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α_{PS2} integrin-mediated muscle attachment in *Drosophila* requires the ECM protein Thrombospondin

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Abstract

During *Drosophila* embryogenesis, the attachment of somatic muscles to epidermal tendon cells requires heterodimeric PS-integrin proteins (α- and β-subunits). The α-subunits are expressed complementarily, either tendon cell- or muscle-specific, whereas the β-integrin subunit is expressed in both tissues. Mutations of β-integrin cause a severe muscle detachment phenotype, whereas α-subunit mutations have weaker or only larval muscle detachment phenotypes. Furthermore, mutations of extracellular matrix (ECM) proteins known to act as integrin binding partners have comparatively weak effects only, suggesting the presence of additional integrin binding ECM proteins required for proper muscle attachment. Here, we report that mutations in the *Drosophila* gene *thrombospondin* (*tsp*) cause embryonic muscle detachment. *tsp* is specifically expressed in both developing and mature epidermal tendon cells. Its initial expression in segment border cells, the tendon precursors, is under the control of *hedgehog*-dependent signaling, whereas *tsp* expression in differentiated tendon cells depends on the transcription factor encoded by *stripe*. In the absence of *tsp* activity, no aspect of muscle pattern formation as well as the initial contact between muscle and tendon cells nor muscle-to-muscle attachments are affected. However, when muscle contractions occur during late embryogenesis, muscles detach from the tendon cells. The Tsp protein is localized to the tendon cell ECM where muscles attach. Genetic interaction studies indicate that Tsp specifically interacts with the α_{PS2} integrin and that this interaction is needed to withstand the forces of muscle contractions at the tendon cells.

Keywords: Integrin; Thrombospondin; Muscle attachment; Tendon cells; Stripe; Hedgehog

1. Introduction

The extracellular matrix (ECM) of cells forms an intercellular layer of secreted glycoproteins, proteoglycans and glycosaminoglycans that is required for the adhesion of neighbouring cells. The interaction of transmembrane proteins with factors of the ECM, rather than direct binding of receptors of the neighbouring cell, facilitates the connection between cells of different layers. A central function of ECM-mediated interactions between cells is to withdraw mechanical forces of contracting muscles to their attachment sites, as in the vertebrate tendons. Similarly, the maintenance of muscle attachment to epidermal tendon cells of *Drosophila* depends on the PS1 and PS2 integrins of type I transmembrane proteins. Muscles and tendon cells express complementary pairs of heterodimeric integrins (PS1 and PS2), composed of a common β-subunit and different α-subunits such as α_{PS1} in tendon cells, and α_{PS2} in muscles (reviewed in Brown et al., 2000; Bökel and Brown, 2002). Mutations of the common β_{PS} subunit, which is encoded by the *myospheroid* (*mys*) gene, cause detachment of most embryonic muscles from the tendon cells once the first muscle contractions occur in the early and still unhatched larva. In contrast, mutations in *multiple edematous wings* (*mew*) and *inflated* (*if*), which encode the α_{PS1} and α_{PS2} subunits, respectively, result in either weaker or no embryonic muscle detachment phenotype (Bogaert et al., 1987; Brower et al., 1995; Leptin et al., 1989).

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Moreover, hypomorphic alleles of integrin mutants result in wing blisters in surviving adult flies, a phenotype likely to be caused by the loss of adhesion between dorsal and ventral wing epidermal blades which express PS1 and PS2, respectively (Bökel and Brown, 2002).

Mutant screens that were based on this easily scoreable wing phenotype were used to identify factors that participate in integrin-mediated adhesion (Prout et al., 1997; Walsh and Brown, 1998). The screen identified both activities required for the expression control of integrins and factors involved in adhesion processes *per se*. A subsequent more detailed genetic and cellular analysis of the cell adhesion system revealed that the transmission of the adhesion force into the cell is enabled by the linkage of the cytoplasmatic tail of integrins with the cytoskeleton, a connection provided by the binding of adaptor proteins encoded by *talin* (*rhea*) (Brown et al., 2002) and *short stop* (*shot*) (Gregory and Brown, 1998; Prokop et al., 1998b; Strumpf and Volk, 1998). *talin* activity was shown to be essential for all integrin-mediated adhesion processes, whereas *shot* participates only in a subset of integrin activities and has integrin-independent functions as well (Brown et al., 2002). At the muscle attachment sites both the activities of *talin* and *shot* are required for the stable anchorage of the muscles to the epidermal tendon cells.

A detailed inspection by electron microscopy showed that two categories of muscle-tendon cell interactions can be distinguished, referred to as direct and indirect attachment, respectively. Direct attachment is observed with a minority of muscles, such as the lateral transverse muscles, and it involves a tight attachment complex between muscles and tendon cells, whereas the indirect attachment is characterised by multiple muscles that attach *inter se* and jointly interact with the tendon cell. Their attachment is mediated by a huge ECM complex that is formed between an epidermal tendon cell, mostly at the segment border, and the multiple muscles attached to them. Therefore, binding of muscles to the ECM and the interaction of the ECM to the epidermal tendon cells are two separable processes (Martin-Bermudo and Brown, 1996; Prokop et al., 1998a).

The observation that adherence is dependent on the expression of two distinct PS integrins on different adjoining tissues raised the question whether the two PS integrins can functionally substitute for each other. Analysis of the larval muscle detachment phenotype proved that the two PS integrins cannot substitute for each other, whereas an exchange of their cytoplasmic tails does not affect their respective activities (Martin-Bermudo et al., 1997), indicating that the specificity of the PS integrins is based on their extracellular ligand binding specificities. Vertebrate α-integrins are grouped into laminin and RGD-motif binding integins based on their interaction specificity. Similarly, the two PS integrins of *Drosophila* can be grouped by their extracellular binding ability, whereby integrin PS1 interacts with laminin and PS2 with ligands that contain RGD motifs (Brown et al., 2000; Bökel and Brown, 2002).

The laminin family of ECM proteins are trimeric proteins formed by one α-, β- and γ-chain, each. *Drosophila* encodes two α chains and one each of the β and γ chains resulting in two different laminin variants being expressed (Gotwals et al., 1994; Martin et al., 1999). The laminin trimer containing the α-chain encoded by *lanA* binds to PS1 but not to PS2 integrin indicating that PS1 is a laminin binding integrin (Gotwals et al., 1994). The laminin α-chain encoded by *wing blisters* (*wb*) contains an RGD motif, which can interact with PS2 (Graner et al., 1998). The RGD dependent interaction with PS2 (Martin et al., 1999), the *wb* expression pattern as well as the mutant phenotypes of *wb* are consistent with a function as a PS2 integrin ligand although an additional function of Wb as a potential ligand for other integrins can not be excluded.

Based on the RGD binding activity of PS2 a second interacting ECM protein was identified. It is encoded by the *tiggrin* (*tig*) gene (Bunch et al., 1998; Fogerty et al., 1994) which is primarily expressed in the fatbody and hemocytes. In addition, the *tig* protein (Tig) was found to be included in the tendon cell matrix at the site where the muscles attach. Consistent with this aspect of Tig localization pattern the *tig* mutants develop a weak integrin-like phenotype, showing that some muscles detach from their attachment sites during the larval stage (Bunch et al., 1998). A detailed analysis of the *tig* mutant phenotype revealed that the initial muscle attachment to tendon cells is not affected, but this connection is less tight and weakened at indirect attachment sites because it cannot withstand the tension of contraction (Bunch et al., 1998; Fogerty et al., 1994). The weak embryonic muscle detachment phenotypes of the three known integrin ligands, in which most muscle/tendon cell connections are unaffected, suggest a functional redundancy and/or the need of an additional integrin interaction partner that is essential for the proper anchoring of muscles to the tendon cells.

Expression of the transcription factor encoded by the gene *stripe* in tendon cell precursors is key for both muscle guidance and attachment, and is both necessary and sufficient for the expression of a variety of tendon cell marker genes (Becker et al., 1997; Frommer et al., 1996; Vorbrüggen and Jäckle, 1997). In order to identify novel proteins that function in the integrin-mediated muscle attachment, we searched for genes that are co-expressed with *stripe* in the segment border and in the developing epidermal tendon cells. Here we describe the characterisation of the gene *thrombospondin* (*tsp*). We report that *tsp* is expressed in a pattern identical to *stripe*. *stripe* mutant analysis revealed that the initial expression of *tsp* is independent of *stripe* activity and is initiated in a *stripe*-like fashion in response to Hedgehog signaling at the segment borders (Piepenburg et al., 2000). We also found that ectopic *stripe* expression is sufficient to induce *tsp* expression and that *tsp* expression is reduced in muscle attachment sites of *stripe* mutants during embryonic stages 15–16. We generated a presumptive null-allele of *tsp* and examined the muscle pattern of the mutants. Muscle determination, the fusion of myoblasts

and the outgrowth of myotubes appear unaffected in *tsp* mutants whereas contraction of muscles in the early and unhatched larvae causes muscles to detach, resulting in lethality. During embryogenesis, Tsp protein is initially localized in the ECM of the developing epidermal tendon cells and later included into the tendon cell matrix, suggesting that it functions in tendon cell matrix interactions. In fact, the muscle-muscle attachment at indirect muscle attachment sites is only weakly affected, whereas the tendon cell-muscle interaction in mutants is greatly reduced, resulting in the detachment of individual muscles and groups of muscles from the tendon cells. Genetic interaction studies with *tsp* and α-integrin subunit mutant alleles indicate that Tsp functions in an α_{PS2}-specific manner. Our results suggest that *tiggrin* and *tsp* have complementary functions in the integrin-dependent muscle attachment process.

2. Results

2.1. *stripe*-like thrombospondin expression in epidermal tendon cells

We performed genetic and molecular screens to identify genes that participate in setting up the larval muscle pattern during *Drosophila* embryogenesis. One aspect of the screening involved a gain of function screen in which ectopic gene activities capable of interfering with these processes during *Drosophila* embryogenesis were identified (Staudt et al., 2005). With the identified components, we performed *in situ* hybridization studies using ESTs on whole mounted embryos and in parallel we made use of the *in silico* EST expression pattern databank of the BDGP (http://www.fruitfly.org/cgi-bin/ex/insitu.pl) in order to find ESTs that are expressed in *stripe*-like expression patterns in developing epidermal tendon cells at the segment border. Among the genes that are expressed in the tendon cells in completely developed embryos, only few genes such as *thrombospondin* (*tsp*) were also initially expressed in a *stripe*-like pattern in segment border cells (Adams et al., 2003; Frommer et al., 1996).

The embryonic expression pattern was discerned by whole mount *in situ* hybridization of embryos at various stages of development using antisense RNA probes from the EST GH27479 (Rubin et al., 2000) that reveals all *tsp* splice variants. In early blastoderm embryos small amounts of presumptively maternal derived *tsp* transcripts can be detected that are degraded at cellularisation. After the degradation of maternally distributed *tsp* transcripts the first zygotic *tsp* transcription is detectable in the trunk mesoderm primordium forming a ring like structure around the invaginating midgut (Fig. 1A). In parallel expression

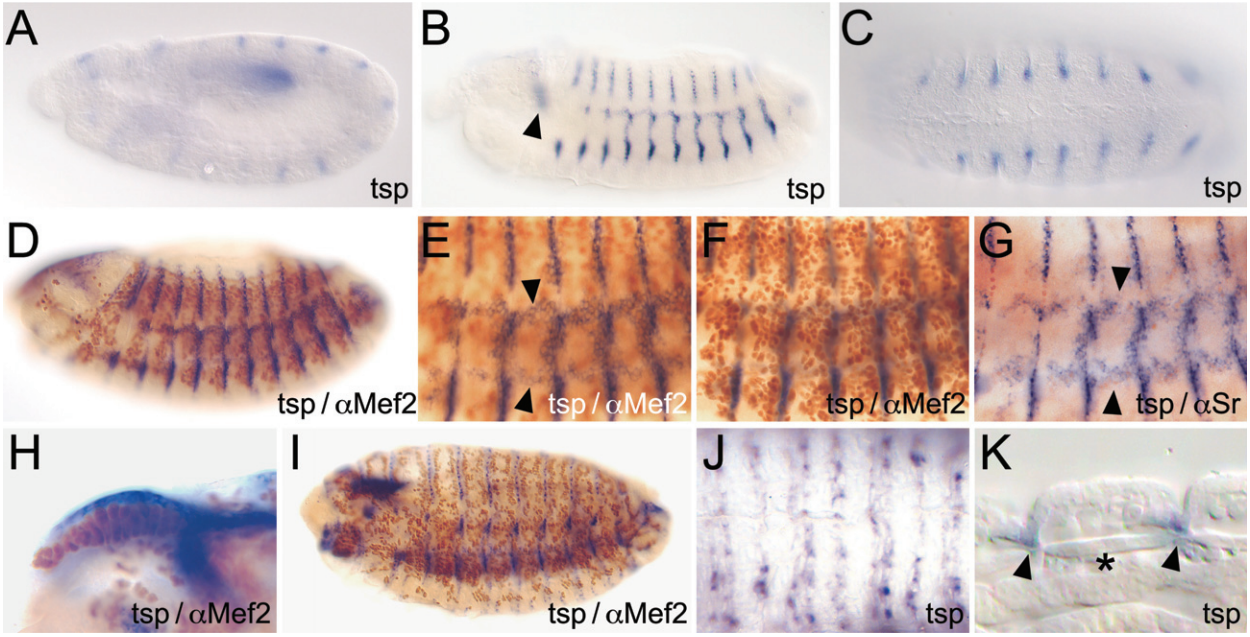

Fig. 1. Spatial expression of *tsp* during embryogenesis. Shown are wild-type embryos stained with a digoxygenin labelled *tsp* antisense probe (blue, A–K), anti-Mef2 (brown, D–F, H, I) and anti-Stripe (G). (A) At stage 11, *tsp* expression in the forming segment borders and in the tip of the trunk mesoderm. (B) Lateral view of a stage 13 embryo, *tsp* expression in the segment borders, weak staining in the developing proventriculus (arrowhead). (C) Ventral view of a stage 13 embryo showing lack of segmental *tsp* expression covering the VNC. (D–G) At stage 14, strong segmental *tsp* expression at the margins of the forming muscles marked by Mef2 expression. Enlarged views with the focal plane at the epidermis (E) and the mesoderm (F) show that *tsp* is expressed in the developing tendon cells of the lateral transverse muscles (LT; arrowheads in E) and not in muscles (different focal plane in F). (G) Double staining with anti-Stripe show co-localization in the segment border cells and partial overlap in the developing tendon cells of the LT muscles at stage 14 (arrowheads). (H) Enlarged view of the pharyngeal muscles marked by Mef2 staining and *tsp* expression in the developing pharyngeal tendon cells at stage 14. (I–K) At late stage 16, *tsp* expression is refined to the tendon cells that contact muscles (I) including the characteristic three rows of tendon cells per segment in the ventral region (ventral view in J). Optical cross section of a dorso-lateral view of late stage 16 embryo (K), showing expression in the epidermal tendon cells (arrowheads) which are already attached to muscles (asterisk).

in the segment borders starting from the ventral region becomes evident. From embryonic stage 13 onward strong expression of *tsp* in the epidermis at segment borders can be detected excluding the region that covers the ventral nerve cord (VNC) (Fig. 1B and C). In addition to the epidermal expression, *tsp* is transiently expressed in a narrow stripe at the intersection between the foregut and the anterior midgut the primordium of the proventriculus (Fig. 1B, arrowhead). At stage 14 strong *tsp* expression in the developing tendon cells at the segment border (Fig. 1D–G) and dynamic expression in the precursors of the tendon cells of the lateral transverse muscles (Fig. 1E, arrowheads) mostly overlapping with Stripe protein expression can be observed (Fig. 1G, arrowheads). In contrast, no *tsp* expression can be detected in the somatic mesoderm that is marked by Mef2 expression (Fig. 1F). Furthermore, *tsp* is strongly expressed in the tendon cells of the pharyngeal muscles from stage 14 onward (Fig. 1H). From early stage 16 onward (Fig. 1I–K), *tsp* expression persists in all tendon cells after muscle attachment including the ventral tendon cells covering the VNC (Fig. 1J). Furthermore, both *tsp* transcript variants, the short and the long version, are expressed in an identical pattern (see Supplementary figure 1). Thus, the initial expression pattern of *tsp* at the segment borders of the developing embryo and later in all epidermal tendon cells continuously overlaps with the expression pattern of the gene *stripe* whose activity determines the fate of epidermal tendon cells (Frommer et al., 1996).

2.2. Regulation of tsp expression

In the stages before the muscles contact the tendon cells *tsp* expression is restricted to the developing tendon cell precursors at the segment border in the dorsal and lateral region (Fig. 2A). This clearance of the epidermal region covering the VNC is reminiscent of the expression pattern controlled by a 239 bp minimal promotor element of the *stripe* gene (Piepenburg et al., 2000). We therefore tested, if *tsp* is co-expressed with β-galactosidase under the expression control of the *sr*SB GAL4-driver line (Staudt et al., 2005) which contains this enhancer element. Based on the observed co-expression (Fig. 2B) and the fact that *tsp* expression in response to the *sr*SB GAL4-driver line can be used to rescue *tsp* mutants (see below), we analysed if *tsp* expression is controlled in an analogous way like *stripe.* Initial *stripe* expression is mediated by the 239 bp minimal promotor element and depends on the Hedgehog (Hh) signaling-dependent transcription factor Cubitus interruptus (Ci) at the segment border (Piepenburg et al., 2000). In view of the identical *tsp* transcription pattern, we explored the possibility that *tsp* expression is also dependent on Hh activity using GAL4::UAS-mediated over expression of Hh^{Δ}, which acts over a longer distance than the wild-type Hh protein (Fietz et al., 1995). GAL4::UAS-mediated overexpression of Hh^{Δ} in the *engrailed* (*en*) domain of the embryo caused an induction of *tsp* expression in the *en* domain and an expansion posteriorly to the segment border (Fig. 2C). This result suggests that *tsp* expression at the segment border is controlled by Hh signaling as shown for *stripe* and *rho* (Hatini and DiNardo, 2001; Piepenburg et al., 2000; Sanson et al., 1999). To confirm the inductive function of Hh we analyzed *tsp* expression in *ptc* mutant embryos, in which the loss of *ptc* activity mimics Hh signal reception. The loss of the negative regulator *ptc* induced *tsp* expression strongly (Fig. 2D) similar to the Hh gain of function situation (Fig. 2C). To test for Hh-dependent *tsp* activation directly, we analyzed *tsp* expression in *hh* mutant embryos. Fig. 2E shows that loss of Hh activity results in the complete absence of *tsp* expression in the cells of the segment border, whereas the expression in the pharyngeal tendon cells is not affected. Since Ci mediates Hh signaling via its binding to the *stripe* promotor, we examined whether Hh-dependent *tsp* activation is mediated by Ci. We employed the *sr*SB GAL4 driver line to express the dominant-negative version of the Hh-dependent transcription factor Ci (Suh et al., 2006) in the *tsp* expressing segment border cells and observed suppression of *tsp* expression. Due to the temporal delay of the GAL4::UAS-driven dominant-negative Ci expression, suppression was observed from embryonic stage 15 onwards (Fig. 2F).

Collectively, these results establish that Hh signaling at the segment border activates *tsp* in a Ci-dependent manner. However, the results do not distinguish between the possibility of a direct *tsp* activation in response to Ci or an indirect one that is mediated by the Ci-dependent *stripe* activity. Since *stripe* activity is both necessary and sufficient for the activation of a variety of tendon cell marker genes in ectodermal tissues (Becker et al., 1997; Frommer et al., 1996; Vorbrüggen and Jäckle, 1997), we tested whether the *stripe* activity can also induce ectopic *tsp* expression. Fig. 2G and H show that *stripe* expression in the *en* domain is sufficient to induce *tsp* from embryonic stage 11 onward. In order to establish whether *tsp* expression is initially activated by *stripe* activity in the tendon precursor cells at the segment border and/or *stripe* activity contributes only to the later aspects of *tsp* expression once the tendon cells are already differentiated, we examined the *tsp* expression pattern in *stripe* mutant embryos. Fig. 2I and J show that in embryos lacking *stripe* activity, the early Hh-dependent expression of *tsp* in tendon cell precursors is not affected whereas *tsp* expression in differentiated tendon cells is significantly reduced. These findings establish that the initial *tsp* expression in tendon precursor cells at the segment border depends on Ci-mediated Hh signaling activity and occurs in parallel to *stripe* activation, whereas the second phase of *tsp* expression, in differentiated tendon cells, depends on *stripe* activity.

2.3. Generation and analysis of tsp mutants

The *tsp* gene locus spans about 25 kbp in region 26F7-27A1 of the second chromosome. It is transcribed into four splice variants that are translated into two different protein

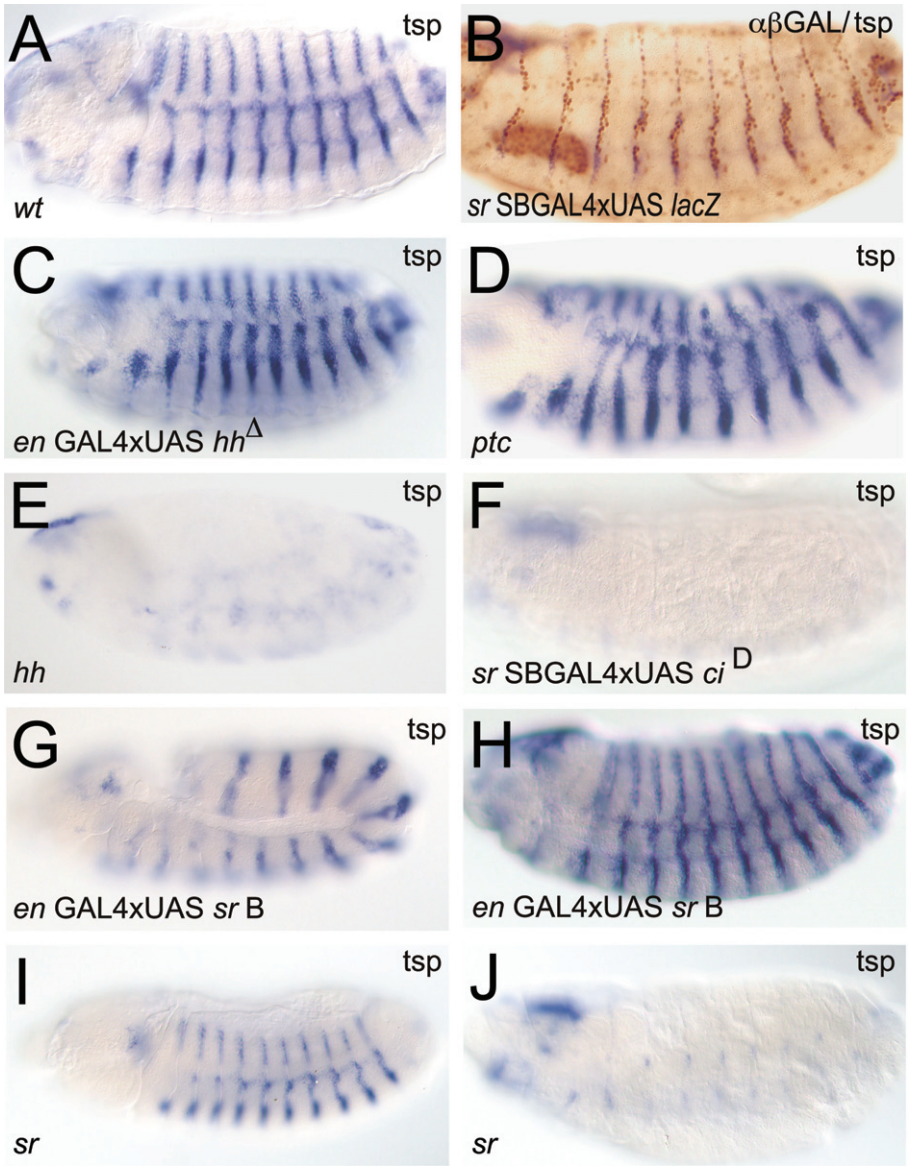

Fig. 2. Regulation of *tsp* expression. Lateral views of embryos at stage 11 (G), stage 13 (I), stage 15 (A–F, H) and stage 16 (J), respectively, which were stained with a digoxygenin labelled *tsp* antisense probe (blue, A–J) and with anti-β-galactosidase antibodies (brown, B). (A) Dorsal and lateral cells but not the ventral region of the segment border show *tsp* expression. (B) *sr*SBGAL4 induces co-expression of UAS *lacZ* in the nuclei of segment border cells with *tsp* excluding the ventral region as shown by the co-localization with *tsp* transcripts. (C) Expression of hh^{Δ} in the *engrailed* (*en*) domain induces ectopic *tsp* in the *en* domain and in a broader stripe posterior. (D) *tsp* expression is ectopically induced in the *en* domain in *ptc* mutant embryos. (E) *tsp* expression is dramatically reduced in the segment border cells but not the tendon cells of the pharyngeal muscles in *hh* deficient embryos. (F) Expression of a dominant negative form of Ci in the segment border using the *sr*SBGAL4 driver line represses *tsp* expression. (G, H) Expression of *sr*B in the *en* domain induces *tsp* expression cell autonomously from stage 11 onwards. (I, J) *tsp* expression in *sr* deficient embryos (*Df(3R)DG4*) is not changed at stage 13 but strongly down regulated at stage 16 in the epidermal segment border cells but not in the pharyngeal tendon cells.

isoforms (Fig. 3A; Grumbling and Strelets, 2006). Three transcripts are identical except for the differential usage of alternate transcriptional start sites, whereas the forth mRNA uses an alternative ninth exon that is translated into a protein with a longer carboxyterminal region (Adams et al., 2003). Based on our results that the shorter and longer transcripts are expressed identically during embryogenesis (Supplementary figure 1), the shorter isoform with 419 amino acids and the longer protein isoform with 1060 amino acids are co-expressed in the developing tendon cells. To generate a mutant for *tsp,* we used the P-element l(2)KG08861 that is integrated 576 bp 5′ of the first, 627 bp 5′ of the second and 698 bp 5′ of the third transcriptional start site. By imprecise remobilization of the P-element, we generated a series of deletions that all retain sequences of the distal P-element border and are directed towards the *tsp* gene locus. Two of the small deletion mutants that were molecularly characterized, *tspΔ6* and *tspΔ79* remove 891 and 1079 bp, respectively. As both deletions remove all alternatively used transcription start

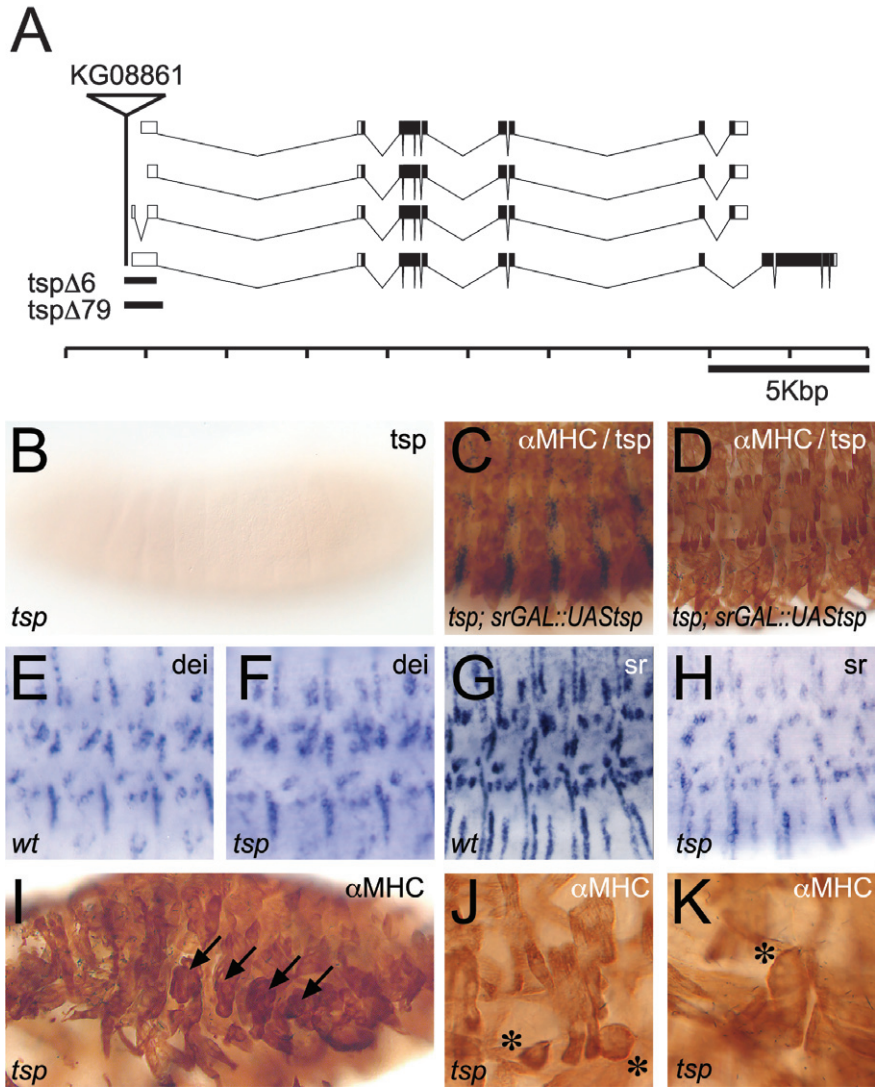

Fig. 3. *tsp* mutants exhibit embryonic muscle detachment phenotypes. Schematic drawing (A), lateral views of early stage 14 (B, C) and late stage 16 embryos (D–K) stained with a digoxygenin labelled *tsp* antisense RNA probe (blue, B–D), a *dei* antisense probe (blue, E, F), a *sr* antisense probe (blue, G, H) and anti-Mhc antibodies to envision the muscle pattern (brown, C, D, I–K). (A) The *tsp* gene locus encodes four different transcripts that are translated into two protein variants with alternative carboxytermini. Position of the *l(2)KG08861* P-element and the extent of the deletions of *tspΔ6* and *tspΔ79* are marked. (B) *In situ* hybridisation of a *tsp* mutant embryo showing the loss of detectable *tsp* expression. (C, D) *sr*SBGAL4 mediated expression of UAS *tsp* in *tsp* mutant embryos induce *tsp* expression in the wild-type pattern (C) and can rescue lethality and muscle detachments (D). (E, F) Comparison of *dei* expression in wild-type (E) and in *tsp* mutant (F) reveals no change in expression. (G, H) No change in *sr* expression between wild-type (G) and *tsp* mutants (H). (I–K) In completely developed *tspΔ79/Df(2)pk78k* mutant embryos muscle detachment occurs mostly in the region of the ventral longitudinal muscles (arrows in I), the detachment phenotypes vary between segments. Note that detached muscles round up but are still connected with other muscles at the segment borders (asterisks in the enlarged views in J, K).

sites (Fig. 3A), we tested whether *tsp* expression is affected in homozygous embryos as well as in embryos that were transheterozygous in combination with the *Df(2L)pk78k* (*tspΔ79/Df(2L)pk78k* and *tspΔ6/Df(2L)pk78k*, respectively). In all mutant combinations, no *tsp* transcript could be observed with a digoxygenin labeled antisense RNA probe that detects all four *tsp* transcripts (see Materials and methods) as shown exemplary in Fig. 3B.

All mutant alleles cause embryonic lethality in different allelic combinations. To confirm that embryonic lethality as well as the below described muscle detachment phenotypes are caused by the loss of *tsp* activity, we used *sr*SBGAL4::UAS-mediated expression of a *tsp* cDNA in *tsp* mutants. *sr*SBGAL4 mediated expression of the shorter version of Tsp in the developing tendon cells at the segment border (Fig. 3C) rescued the lethality and resulted in the complete rescue of the muscle pattern defects (Fig. 3D) in four independent *tsp* jump out alleles tested of which two were not molecularly characterized. Furthermore, tendon cell-specific expression of the rescuing *tsp* transcripts showed no difference to wild-type *tsp* expression in the segment borders (Fig. 3C). These results establish that the loss

of *tsp* RNA expression in the tendon cell precursors at the segment border is responsible for the cause of the muscle detachment phenotype described below and embryonic lethality associated with the mutants.

The somatic muscle pattern of *Drosophila* is fully developed at stage 16 of embryogenesis. Shortly thereafter, the muscles firmly attach to the tendons cells. The analysis of the muscle pattern in homo- or transheterozygous *tsp* mutant embryos revealed no detectable defect in early muscle developmental processes such as the determination, differentiation, and migration of the individual muscles and their attachment to the tendon cells. Furthermore, the differentiation of the tendon cells is also not affected as indicated by the normal expression of various marker genes such as *delilah* (compare Fig. 3E with F) *stripe* (compare Fig. 3G and H) and *odd skipped* (data not shown) in late stage 16 embryos. Though, after the first muscle contractions occurred, the muscles were found to be detached (Fig. 3I). Detachment results in the rounding up of the muscles which, however, remain loosely connected to neighboring muscles on one side of the segment, a phenomenon that results in gaps in the stereotypical muscle pattern (Fig. 3J and K). Although the detachment can be noted in each of the *tsp* mutant alleles analyzed, the strength and patterns of detached muscles varies from embryo to embryo and between individual segments. However, all embryos have in common that detachment is only seen in those muscles that (i) are spanned between tendon cells at the segment border and (ii) are linked to the tendon cells via indirect attachment complexes. This includes the lateral longitudinal as well as the ventral longitudinal muscles (Fig. 3I–K).

Muscle detachment can only be observed in early, not yet hatched larvae, which unfortunately are barely accessible to antibody staining due to the synthesis of the cuticula that occurs from late stage 16 onward. We therefore stained the actin filaments of the muscle using phalloidin, a specific marker that can penetrate the embryo even in the presence of a partial cuticula, and thereby allows visualization of the muscle pattern at a stage, when muscle contractions occur. In contrast to *mys* mutant embryos, even at that stage not all muscles detach in *tsp* mutants, although many ventral longitudinal muscles and the lateral longitudinal muscles are detached in the majority of segments. In contrast, the dorsal oblique and acute muscles are only partially disorganized but not detached and the lateral transverse muscle appeared normal in most segments, indicating that other activities can at least partially substitute for the lack of *tsp* activity (Fig. 4A and B).

Next we examined somatic muscle detachment by confocal microscope analysis, which allowed a more detailed analysis of muscles, which are positioned in different layers of the musculature (Bate, 1993). The detailed analysis revealed the detachment of all ventral longitudinal muscles in one segment, their rounded up appearance and their contacts to ventral longitudinal muscles of the neighboring segments (Fig. 4C and G). Furthermore, optical sectioning of the musculature revealed that in addition to the rounded up muscles, groups of interconnected muscles lose contact with the tendon cells at segment borders and become mispositioned by up to 2 μm into the interior of the segment.

Muscle attachment is known to depend on integrins and their ECM-interaction partner Tiggrin (Bunch et al., 1998; Fogerty et al., 1994). Thus, we next asked whether expression of Mys, which is the common β-subunit of the PS integrins, α_{PS1}, which is the integrin α-subunit that is expressed in the epidermal tendon cells, and Tig are altered in *tsp* mutant embryos by staining them with the respective antibodies. The results summarized in Fig. 4D–J show that Tig, Mys and α_{PS1} are normally expressed and properly localized in *tsp* mutant embryos. However, detached muscles are not stained with anti-Mys and anti-α_{PS1} antibodies, indicating the loss of polarization after the detachment of muscles which is confirmed by the loss of polarization of the actin cyto-skeleton in the detached muscles as revealed by staining with fluorescently marked phalloidin (Fig. 4G). These findings establish that the muscle detachment in *tsp* mutants is the result of loss of Tsp protein in the tendon cell matrix and not due to an indirect effect such as the reduction in integrin expression.

2.4. Thrombospondin is localized to the tendon cell matrix

The expression of the ECM protein Tsp in the tendon cells and the muscle detachment phenotype of the *tsp* mutants are consistent with a role of *tsp* in integrin-mediated muscle attachment. In order to visualize the cellular localization of the *Drosophila* Tsp protein, both in fixed and live embryos, we generated a Tsp–GFP fusion protein using the shorter Tsp protein that was used for the rescue experiments of the mutant (see above). *sr*SBGAL4-mediated expression of Tsp–GFP resulted in the secretion of the fusion protein into the tendon cell matrix at the segment border of embryos that were co-stained with fluorescently marked phalloidin to visualize the developing muscle pattern (Fig. 5A and B). Comparison of Tsp and Mys localization, in optical cross-sections of embryos, revealed that up to embryonic stage 16, the Tsp protein is enriched at the ECM located between the epidermal tendon cells (green in Fig. 5C; white in C′) and the polarized ends of the attached muscles which were marked by Mys staining (red in Fig. 5C; white in C′′). Similar to the Tsp protein (green in Fig. 5D; white in D′) the integrin α-subunit αPS1, which is expressed in tendon cells, is localized to the ECM between tendon cells and muscles (red in Fig. 5D; white in D′′) at late stage 15. Subsequently, when the larvae were differentiated, Tsp becomes highly enriched in the tendon cell matrix, which was marked by Tiggrin (red in Fig. 5E; white in E′′). Furthermore, the muscles, which were marked by fluorescently marked phalloidin staining were attached to this matrix (red in Fig. 5F; white in F′′). Thus, the process of muscle attachment during stage 16 parallels the concentration of Tsp at the tendon

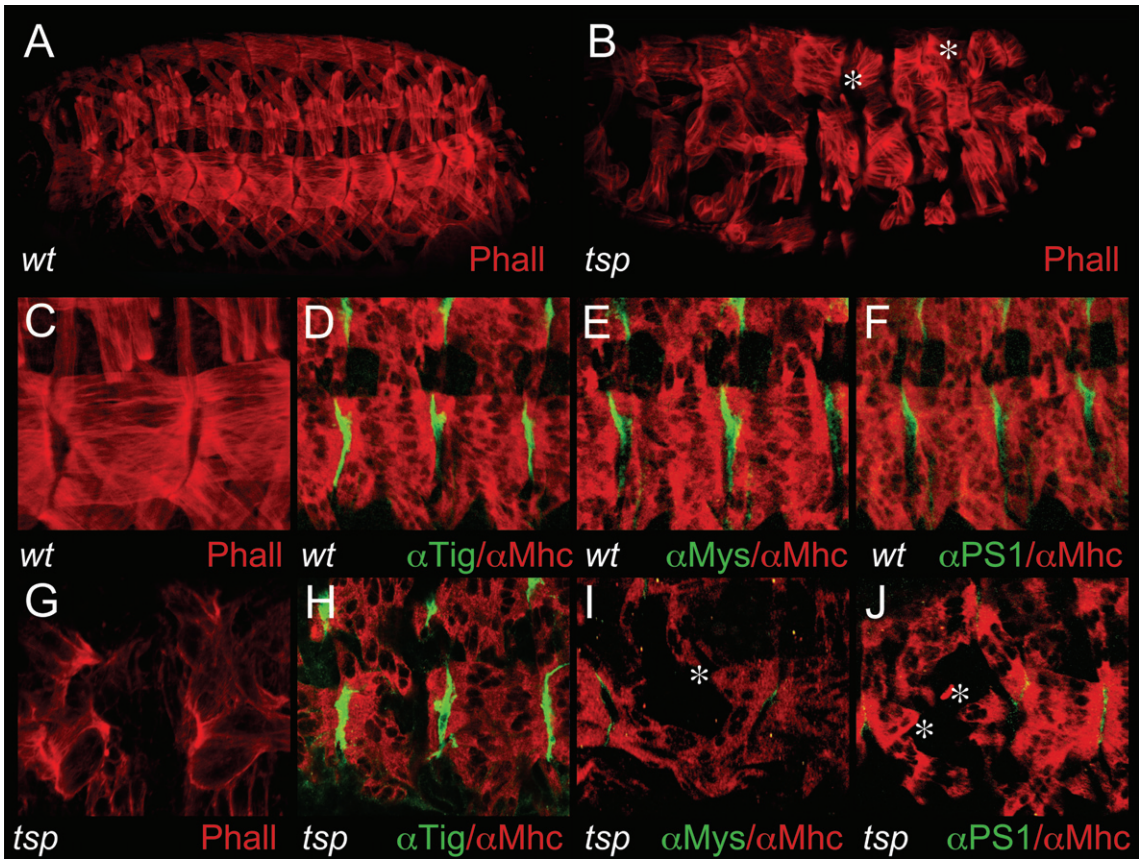

Fig. 4. *tsp* mutants have no consequence on integrin expression. Lateral views of late stage 16 wild-type (A, C–F) and *tspΔ79/Df(2)pk78k* embryos (B, G–J) stained with fluorescently marked phalloidin (A–C, G), and anti-Mhc (D–F, H–J) together with anti-Tig (D, H), anti-Mys (E, I) and anti-PS1 (F, J). (C–J) Enlarged views of two segments with the focal plane on the ventral longitudinal muscles (VL). (A) Phalloidin staining of a fully developed wild-type embryo with a partially secreted cuticula reveal a regular muscle pattern, whereas (B) in *tsp* mutant embryos the musculature is strongly disturbed. Most VL muscles are detached (see enlarged view in G) and the dorsal acute and oblique muscles are abnormally shaped in some segments (asterisks in B). In contrast, the lateral transverse and ventral acute muscles are only weakly affected. (C–F) Wild-type embryos with VL muscles attached to the tendon cells at the segment border (C) where the ECM protein Tig (D), the integrin β-subunit βPS/Mys (E) and the integrin α-subunit αPS1 (F) are strongly enriched in the tendon cell matrix. (G) In *tsp* mutants VL muscles are detached resulting in a rounded appearance with actin enrichment at their surface. (H) *tsp* mutant embryos express the ECM protein Tig normally. Note that detached VL muscles are connected with the tendon cell matrix at the segment border marked by Tig. (I, J) PS integrin subunits (βPS/Mys in I; αPS1 in J) are expressed in *tsp* mutant embryos. Note the absence of staining in detached, rounded muscles that have lost their polarisation (asterisks in I, J).

cell matrix, a position where integrins and Tig were shown to concentrate as well (Brown, 2000; Prokop et al., 1998a).

2.5. Genetic interactions reveal a Tsp function as an α_{PS2} integrin ECM interactor

Attachment of muscles to tendon cells critically depends on integrin activity (Brown, 2000; Bökel and Brown, 2002). The integrin-like muscle detachment phenotype of the *tsp* mutants as well as the co-localization of Tsp and integrin proteins strongly suggest that Tsp plays a decisive role in the integrin-mediated cell adhesion process of muscles and tendon cells. In fact, Tsp was shown to encode a pentameric glycosylated protein that is part of the ECM (Adams et al., 2003). Thus, it could indeed function as a direct binding partner of the PS-integrins.

In order to establish such a functional link between *tsp* and PS-integrins by genetic means, we generated double mutant embryos which carry either a strong loss of function allele for the α_{PS1} subunit of integrin due to the mew^{M6} mutation (Martin-Bermudo et al., 1997) or a hypomorphic allele for the α_{PS2} subunit due to the if^{B2} mutation (Bloor and Brown, 1998) in combination with only one wild-type copy of *tsp* (*tspΔ6* and *tspΔ79*).

In comparison with wild-type embryos (Fig. 6A) mew^{M6} mutants bearing two copies of *tsp* develop a normal muscle pattern with muscle detachment in only few segments (Fig. 6C). mew^{M6} mutant embryos that have only one wild-type copy of *tsp* develop a weak muscle detachment phenotype affecting a small number of longitudinal muscles (Fig. 6D). In contrast, if^{B2} mutants bearing two copies of *tsp* developed a mild detachment phenotype in several segments (Fig. 6E and F), which was strongly enhanced, both with respect to the extent of detachment and penetrance in embryos with only one remaining wild-type copy of *tsp* (Fig. 6G and H). In these embryos, longitudinal, ventral as well as dorsal muscles were found to be detached (Fig. 6H), a phenomenon not observed in *tsp* mutant

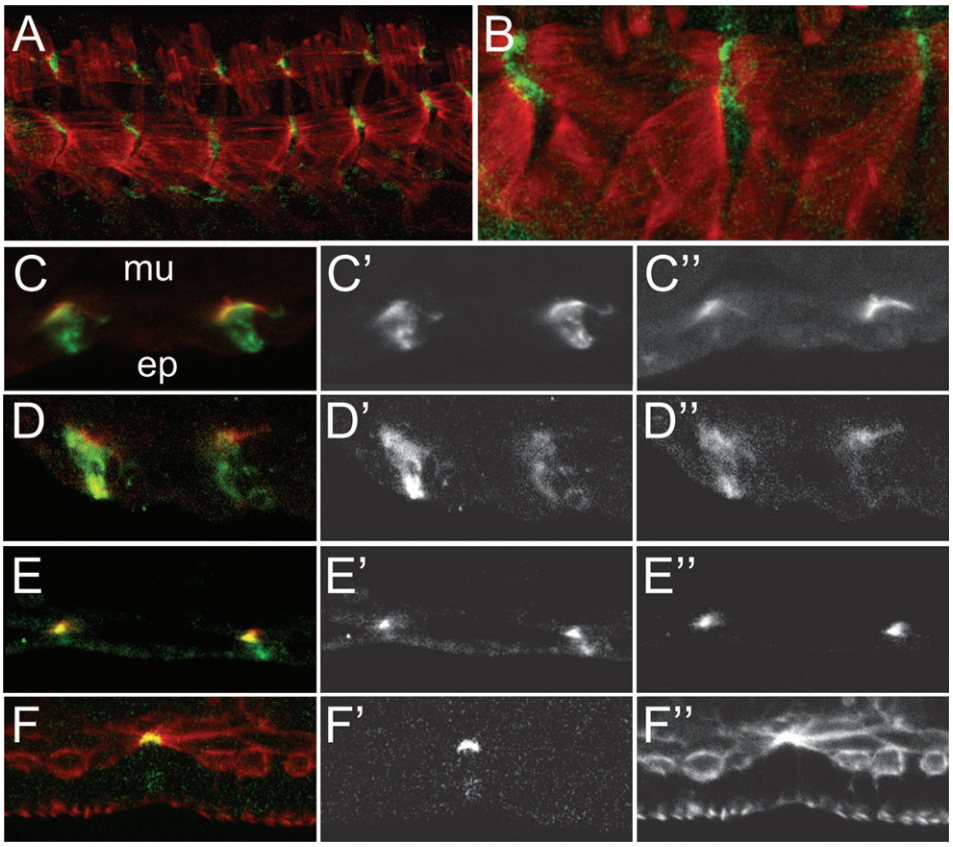

Fig. 5. Tsp becomes localized to the tendon cell matrix during embryogenesis. Lateral views (A, B), and optical cross-sections of dorso-lateral views (C–F) of *sr*SBGAL4::UAS*tspGFP* embryos (A–F) with Tsp-GFP auto-fluorescence (green in A, B) and stained with fluorescently marked phalloidin (red in A, B, F), anti-Mys (red in C), anti-αPS1 (red in D), anti-Tig (red in E) and anti-GFP (green, C–F). (A, B) *sr*SBGAL4 mediated Tsp-GFP expression result in the specific localization of Tsp-GFP to the tendon cell matrix at the segment borders in fully developed embryos (see enlargement in B). (C) At late stage 15, Tsp-GFP (green in C, C′) is enriched to the ECM between tendon cell and muscles (muscles: mu; epidermis: ep in C). Note that only a fraction of the protein is localized to the tendon cell matrix marked by Mys (red in C, C′′). (D) Similar to Tsp-GFP (green in D, D′) αPS1 (red in D, D′′) is enriched to the ECM between tendon cell and muscles at late stage 15. (E) At early stage 16 Tsp-GFP (green in E, E′) becomes more restricted to the tendon cell matrix, marked by Tig (red in E, E′′). (F) In fully developed embryos, TSP–GFP is exclusively localized to the tendon cell matrix (green in F, F′) resulting in an overlay with actin bundles that are enriched in the muscle termini (red in F, F′′).

embryos (Fig. 6B). Furthermore, in mew^{M6} mutants lacking both wild-type copies of *tsp,* only a mild enhancement of the *tsp* mutant phenotype was observed (Fig. 6I). In contrast, if^{B2} lacking both wild-type copies of *tsp* develop dramatic muscle pattern defects which is beyond an additive effect of the two individual mutant phenotypes (Fig. 6J). The enhancement of the muscle detachment phenotype of *if* mutants by the removal of one copy of *tsp* and the dramatic enhancement that affects even the set of muscles that are not affected in each of the single *if* or *tsp* mutants establishes an essential role of Tsp in the α_{PS2}-dependent muscle attachment process. In contrast, the weak effects of the reduction of the *tsp* dose in hemizygous mew^{M6} mutants makes a prominent role of Tsp in α_{PS1}-dependent muscle attachment rather unlikely.

3. Discussion

We present evidence that Tsp, an evolutionarily conserved ECM protein that has been shown to participate in intercellular adhesion processes of vertebrates, participates preferentially or even specifically in α_{PS2} integrin-mediated muscle attachment to tendon cells. Vertebrate Tsp is a glycosylated protein that forms oligomers and is capable of interacting with both calcium and heparin (Adams, 2001). Furthermore, it has been shown to directly interact with the extracellular part of integrin proteins (Lawler et al., 1988). This interaction depends on a highly conserved RGB motif, which is characteristic of integrin binding proteins of the ECM (Lawler et al., 1988; Ruoslahti, 1996).

Vertebrate genomes code for up to five Tsps (Adams, 2001), whereas the *Drosophila* genome contains only a single *tsp*-coding sequence (Adams et al., 2003), which, however, encodes two Tsp variants which differ in their carboxyterminal regions. Previous biochemical studies on *Drosophila* Tsp showed that the protein is secreted and able to form a pentameric structure as suggested by the molecular weight of the secreted native complex (Adams et al., 2003). At the sequence level, the conserved *Drosophila* Tsp contains all functionally characterized domains including the critical RGD motif required for integrin binding. In

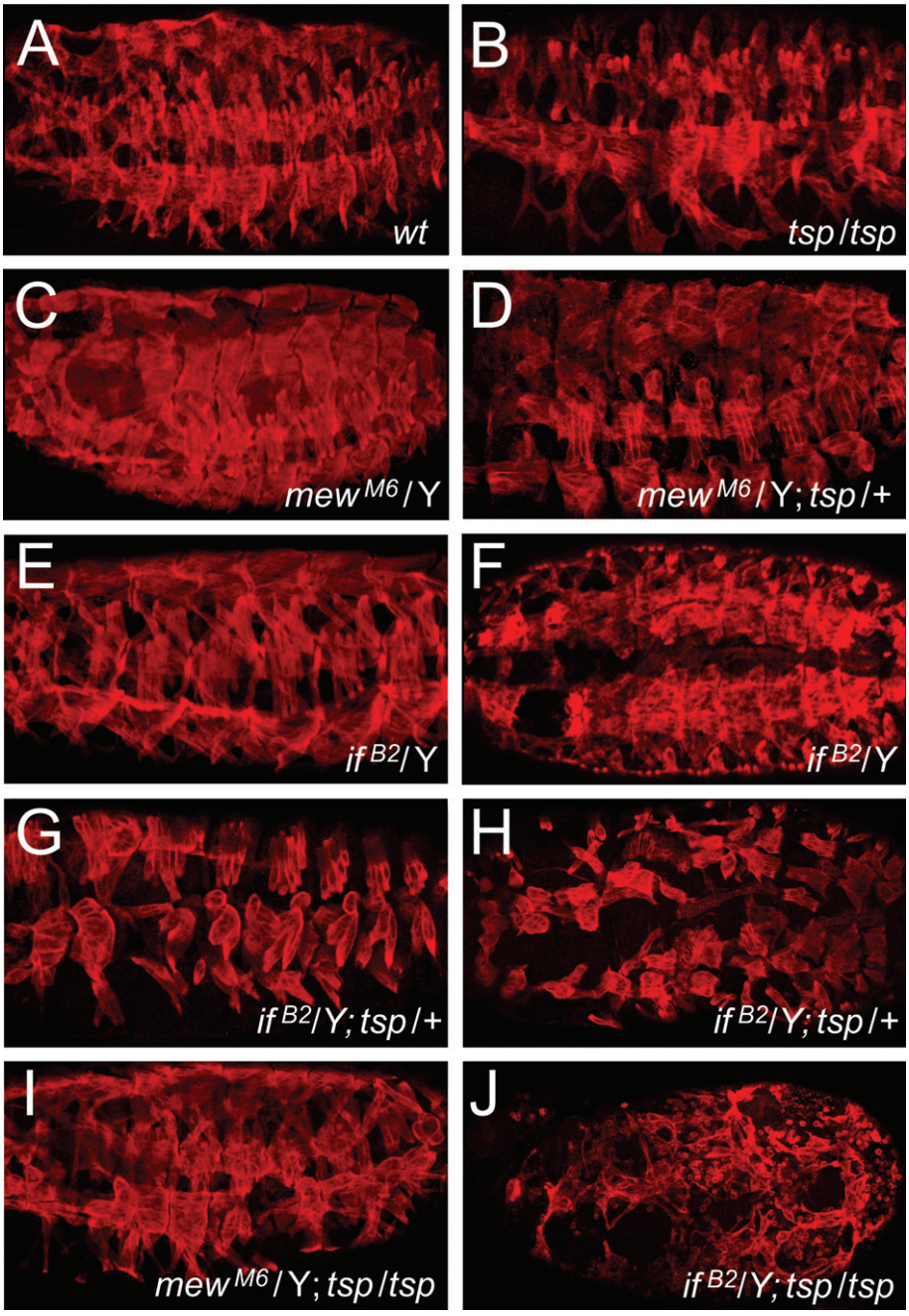

Fig. 6. Genetic interaction of *tsp* with *if* encoding α_{PS2}. Lateral views (A–E, G, I, J) and dorsal view (F, H) of late stage 16 embryos stained with anti-Tropomyosin antibodies. (A) Wild-type with stereotype muscle pattern. (B) Homozygous *tsp* mutant embryo with ventral lateral muscles detaching from tendon cells at segment borders. (C) Hemizygous mew^{M6}/Y mutant embryos show mild detachment phenotype in few segments. (D) Hemizygous mew^{M6}/Y mutant embryos, that have one *tsp* copy show mild detachment phenotypes. (E, F) Hemizygous if^{B2}/Y embryos show muscle detachment mostly in the region of the ventral longitudinal (VL) muscles (E) and in few dorsal muscles (F). (G, H) Hemizygous if^{B2}/Y embryos which carry only one wild-type copy of *tsp* show dramatic detachment phenotype of most VL muscles (G) and disordered dorsal muscles with detached muscles (H). (I) Double mutant embryos of mew^{M6} and *tspΔ79* show weakly enhanced muscle pattern defects as compared to *tspΔ79* mutants. (J) Double mutant if^{B2}, *tspΔ79* embryos exhibit a strongly enhanced phenotype as compared to the single mutants, respectively.

contrast to vertebrate Tsp, the RGD motif in *Drosophila* Tsp is positioned in the aminoterminal region, close to two BBXB sequence motifs known to bind to heparin, instead of the third Tsp/COMP domain (Adams et al., 2003). In addition, we observed a KGD motif in the third Tsp/COMP domain of *Drosophila* Tsp which was shown to serve also as an interaction motif for integrins (Ruoslahti, 1996). *Drosophila* Tsp contains therefore two RGD/KGD motifs that would allow direct binding of PS2, the integrin heterodimer that was previously found to associate with Tig, an interaction that was shown to be dependent on the presence of the RGD motif (Bunch et al., 1998; Fogerty

et al., 1994). Furthermore, an RGD motif is also required to mediate the interaction of PS2 with the laminin α-chain Wb (Graner et al., 1998; Martin et al., 1999).

Drosophila tsp is expressed in all ectodermal tendon precursor cells, strongly enriched in those positioned at the segment border of the embryo. Furthermore, *tsp* is expressed in all differentiated tendon cells after muscle contact. Therefore, *tsp* is expressed in all cells that have previously been identified by the expression of *stripe*. *stripe* encodes an EGR-type Zn-finger transcription factor that is required for tendon cell differentiation (Becker et al., 1997; Frommer et al., 1996; Vorbrüggen and Jäckle, 1997). Like *stripe*, the initial expression of *tsp* is controlled by Hedgehog signaling at the segment borders (Piepenburg et al., 2000) and requires *stripe* activity only during the later stages when the tendon cells are already differentiated. Our results suggest that the genes *stripe* and *tsp* are activated in parallel by Hh-dependent Ci activity, and that *stripe* activity maintains the expression of *tsp* during the later stages when Ci activity has ceased.

Tsp is secreted from epidermal tendon cells and accumulates at the tendon cell matrix, a specific ECM where the muscles attach to. The functional characterisation of the newly generated *tsp* alleles, which fail to express detectable amounts of *tsp* transcript, showed that Tsp is necessary for the proper anchoring of muscles at the tendons cells. As observed with mutants affecting the β subunit and the α_{PS2} subunit of integrin, *mys* and *if*, respectively, the muscles were found to detach from their epidermal attachment sites once muscle contraction occurs. Thus, *tsp* activity is not required for any aspect of muscle pattern formation and/or muscle guidance as well as proper adherence to tendon cells but plays an essential role in maintaining the interconnection between muscles and tendons cells once contraction occurs. Although the muscle detachments are less pronounced than in *mys* or *if* mutants, the detachment phenotype of *tsp* mutants is by far stronger than the corresponding phenotypes that are caused by the loss of other ECM proteins, such as Tig (Bunch et al., 1998; Fogerty et al., 1994), Wb (Graner et al., 1998; Martin et al., 1999) and LanA (Gotwals et al., 1994), known to be integrin interaction partners. The strong and specific enhancement of the detachment phenotype of mutants that carry a weak *if* allele, in response to the loss of one or both *tsp* wild-type alleles strongly suggests that Tsp functions as an essential ECM binding partner of α_{PS2} encoded by *if*. The specificity of the genetic interaction shown here is consistent with the finding that binding to α_{PS2} requires an RGD motif as has been found in Tig and Wb as well (Bunch et al., 1998; Fogerty et al., 1994; Martin et al., 1999). Mutations of either *tig* or *wb* cause weak muscle detachment phenotypes as observed with *tsp* mutants, suggesting a redundant α_{PS2} integrin interaction system in which ECM binding is provided by different partners and that each of them is required for the proper anchoring of the muscles. This conclusion is consistent with the finding that *tig, wb* and *tsp* mutants (Bunch et al., 1998; Martin et al., 1999) display a weaker phenotype than the *if* loss of function mutants (Bogaert et al., 1987; Brown, 1994). Based on the specific expression of *tsp* in both tendon cell precursors and differentiated tendon cells, which differs from the multiple expression sites of *tig* and *wb*, together with the strong enhancement of the muscle detachment phenotype in *if* and *tsp* double mutants, it appears likely that *tsp* is the crucial interaction partner of the α_{PS2} integrin subunit to provide proper anchoring of muscles to tendon cells. This proposal, and the relative contribution of each of the by now three different α_{PS2} integrin subunit binding proteins, can be tested once double and triple mutant combinations for all the genes involved and biochemical test systems become available.

4. Materials and methods

4.1. Fly strains

Wild-type Oregon R, if^{B2}, mew^{M6}, ptc^{9}, hh^{2}, *Df(3R)DG4*, *Df(2)pk78k* and *l(2)KG08861* were obtained from the Bloomington stock center. UAS Ci^{D} were kindly provided by J.M. Graff, *en* GAL4 by A. Brand and the *sr*SBGAL4 driver is described (Staudt et al., 2005). Generation of *tsp* mutants by mobilising the P-element KG08861 and subsequent analysis was performed as described earlier (Steigemann et al., 2004). We obtained 8 independent alleles, that all contain sequences from the distal P-border. Two of them were molecularly characterized und used in the study, *tspΔ6* and *tspΔ79*. To avoid effects by second site mutations, the alleles were analyzed in *trans* or using a deficiency in transheterozygous conditions.

4.2. Molecular biology

EST GH27479 (Rubin et al., 2000) (encoding the shorter Tsp protein variant) was used to generate specific *tsp* Digoxygenin labelled antisense RNA probes according to the protocol of the manufacturer (Roche, Basel, Schwitzerland). The pUAST *tsp* construct was cloned using an EcoRI and XhoI fragment that includes the full *tsp* ORF and the *tsp* 5′- and 3′-UTR sequences. The pUAST *tsp* ORF GFP fusion construct was generated by PCR using the primers CAGAATTCACCAGCAACAACGAACACGAT GAAT to introduce an *Eco*RI site 5′ of the ORF removing 5′-untranslated sequences and CGCCTCGAGGTTCGTAACGACCGAGTAT replacing the STOP codon by a *Xho*I site that was used to clone GFP in frame. To detect specifically the short *tsp* transcript a 500 bp fragment of the unique sequence of its 3′UTR were cloned using the primers CAACATT CTAGTGCCCTCGCAT and GTGGCCAATGATGGAGTTTATT. In parallel, a 820 bp fragment specific for the long version was amplified and cloned using the primers TTCGCACTCTTCAAACCATTCCAC and GTCCTGCAACTCCACCTTCGTCTT.

4.3. Fly techniques and staining of embryos

Whole mount *in situ* hybridisation and antibody staining were performed as described (Steigemann et al., 2004; Vorbrüggen and Jäckle, 1997). In order to perform phalloidin stainings, embryos were fixed normally but the viteline membrane was removed by shaking the embryos with equal volumes of heptane and 80% ethanol instead of methanol or the vitelin membrane was removed by hand. Rabbit anti-Mhc (1:2000; kindly provided by D. Kiehart), mouse anti-Mys (1:5; kindly provided by N. Brown), rat anti-PS1 (1:5; kindly provided by N. Brown), rabbit anti-GFP (1:1000, Synaptic Systems, Goettingen, Germany), mouse anti-Tiggrin (1:400, kindly provided by L. Fessler), rabbit anti-Mef2 (1:700, kindly provided by H. Nguyen), rabbit anti-Stripe (1:250, Frommer et al., 1996) and rat anti-Tropomyosin (1:400, Babraham Tetronix,

Cambridge, U.K.) were used as primary antibodies, anti-mouse, anti-rat and anti-rabbit (coupled to biotin, 1:500; Vectastain or with Alexa 488 or 567 1:500; Invitrogen) were used as secondary antibodies. 2 units phalloidin coupled to Alexa 488 (Invitrogen) was added. Images were taken with a Leica TCS SP2 laser scanning microscope (Bensheim, Germany).

All stocks were kept with balancer chromosomes with a *hb*lacZ or a *ftz*lacZ transgene integration to enable the identification of homozygous embryos. As the if^{B2}; *tsp* and mew^{M6}; *tsp* double mutants were only marked by an actin GFP transgene on the FM7 balancer, *tsp* mutant embryos were identified by *in situ* hybridisation before the antibody staining. Based on problems to distinguish heterozygous *if*/FM7 mutant from homozygous FM7/FM7 embryos it was impossible to analyze an enhancement of the *tsp* mutant phenotype by the reduction of the *if* dose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2007.03.005.

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