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Tenectin is a novel αPS2βPS integrin ligand required for wing morphogenesis and male genital looping in *Drosophila*

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ABSTRACT

Morphogenesis of the adult structures of holometabolous insects is regulated by ecdysteroids and juvenile hormones and involves cell–cell interactions mediated in part by the cell surface integrin receptors and their extracellular matrix (ECM) ligands. These adhesion molecules and their regulation by hormones are not well characterized. We describe the gene structure of a newly described ECM molecule, tenectin, and demonstrate that it is a hormonally regulated ECM protein required for proper morphogenesis of the adult wing and male genitalia. Tenectin's function as a new ligand of the PS2 integrins is demonstrated by both genetic interactions in the fly and by cell spreading and cell adhesion assays in cultured cells. Its interaction with the PS2 integrins is dependent on RGD and RGD-like motifs. Tenectin's function in looping morphogenesis in the development of the male genitalia led to experiments that demonstrate a role for PS integrins in the execution of left–right asymmetry.

Introduction

During development, morphogenetic movements are induced and controlled by a variety of molecules such as growth factors or hormones and their receptors. In holometabolous insects like the fruit fly, *Drosophila melanogaster*, and the beetle, *Tenebrio molitor*, ecdysteroids and juvenile hormones control development both during embryogenesis and later during larval and pupal molts. These hormones regulate gene expression patterns required for development. Following changes in gene expression, cells change shape and move by processes that require remodeling of the extracellular matrix (ECM) and alterations in the interactions of cell surface adhesion molecules with their intracellular and extracellular ligands. In both invertebrates and vertebrates, ECM proteins and their receptors are important for the anchorage of cells, cell spreading, migration, proliferation and differentiation and they have been implicated in numerous pathologies (Brown et al., 2000; Hynes 2002; Bökel and Brown, 2002; Brower 2003; Danen and Sonnenberg 2003).

Integrins are a family of heterodimeric transmembrane glycoproteins, consisting of two subunits (α and β) that serve as receptors for ECM molecules and cell surface molecules of neighboring cells. The *Drosophila* genome contains 5 α subunits (αPS1–αPS5) and 2 β subunits (βPS and β_ν) while, in vertebrates, 18 α and 8 β subunits have been identified (Brower, 2003; Takada et al., 2007, for reviews). The best characterized *Drosophila* integrin subunits, αPS1, αPS2 and αPS3, are encoded by the *multiple edematous wings*, *inflated* and *scab* loci, respectively while the βPS locus is encoded by the *myospheroid* locus (Brower and Jaffe, 1989; Wilcox et al., 1989; Wehrli et al., 1993; Brown, 1994; Stark et al., 1997; Grotewiel et al., 1998). A small number of integrin ligands have been identified in *Drosophila*: laminin α1,2 chain (Graner et al., 1998), tiggrin (Fogerty, et al., 1994; Bunch et al., 1998), tenascin-m chain (Graner et al., 1998), thrombospondin (Subramanian et al., 2007) and collagen IV (Borchiellini et al., 1996) but it is not known if tenascin or collagen IV binds integrins in vivo. αPS1 is a typical laminin-binding-type subunit while αPS2 is an RGD (arginine, glycine, aspartic acid)-binding-type subunit. Since αPS1, αPS2 and βPS subunits have clear homologs in vertebrates, the *Drosophila* integrin system is becoming a simple powerful tool in which to characterize integrin functions. Generally, mutants for genes involved in the integrin pathways display clear phenotypes, which facilitate in vivo studies.

Just prior to wing morphogenesis during post-embryonic development, αPS1βPS and αPS2βPS are expressed in a complementary fashion

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in the wing imaginal disc epithelium. αPS1βPS is expressed in the presumptive dorsal surface and αPS2βPS on the ventral surface. At metamorphosis the disc evaginates bringing in apposition αPS1βPS expressing dorsal cells with αPS2βPS expressing ventral cells (Wilcox et al., 1981; Brower et al., 1984; Leptin et al., 1987). Mutations of genes involved in the integrin pathway often cause epithelial detachment and wing-blistering phenotypes. Integrins also function in muscle attachment, short-term memory, olfaction, embryonic midgut migration and axonal pathfinding (Brown et al., 2000; Bökel and Brown, 2002; Brower, 2003, for reviews). Since swapping the cytoplasmic tails between the two subunits does not detectably alter their function, crucial differences between the two subunits are located in their extracellular ligand-binding domains (Martin-Bermudo et al., 1997; Martin-Bermudo and Brown 1999). Thus, the molecular characterization of integrin ligands in *Drosophila* is an important step to understand integrin functions in morphogenesis.

Tenebrin was identified as a potential integrin ligand whose expression is hormonally regulated during morphogenesis in the beetle *Tenebrio molitor*. During the post-embryonic development of holometabolous insects, each molt is induced by a pulse of 20-hydroxyecdysone (20E), while the nature of the molt is controlled by a sesquiterpenoid hormone, the juvenile hormone (JH). 20E directly activates cascades of gene expression by binding to the 20E/receptor complex and inducing expression of early genes encoding transcription factors that coordinate the induction of large sets of secondary-response late genes, leading to the appropriate stage and tissue-specific biological responses (Russell et al., 1996; Thummel, 1996; Richards, 1992; Segraves, 1994; Henrich et al., 1999). Our screen for hormone responsive genes identified *Tenebrin* whose expression is regulated by 20E and JH. *Tenebrin* encodes a putative ECM protein with the RGD integrin-binding motif (Royer et al., 2004).

To analyze the role of *tenebrin* in development, we identified its homolog, *tenectin*, in *Drosophila melanogaster* and described its embryonic expression patterns (Fraichard et al., 2006). In this report we used *tenectin* dsRNA to generate *tenectin* mutants and find phenotypes in the adult wing and male genitalia. *Drosophila* wings originate from small clusters of undifferentiated cells constituting the imaginal discs (Oberlander, 1985), which transform from an essentially flat monolayer of epithelial cells to mature adult structures (Fristrom and Fristrom, 1993). This striking transformation is coordinated by pulses of 20E and requires genes encoding transcription factors, proteases, cytoskeletal proteins, extracellular matrix proteins and their receptors (Fristrom et al., 1993; Brower, 2003; Brabant et al., 1996; Prout et al., 1997; Walsh and Brown, 1998; D'Avino and Thummel, 2000). Ecdysone regulates integrin expression in wing morphogenesis (D'Avino and Thummel, 2000) and in the final stages of wing morphogenesis an epidermal to mesenchymal transition is regulated by the neurohormone bursicon (Kiger et al., 2007).

Looping morphogenesis of the adult male genitalia is also regulated by hormones (Ádám et al., 2003; Wilson et al., 2006). In this process, roles of multiple signaling pathways and an unconventional myosin have been reported but functions of the ECM or its integrin receptors have not (Casanova et al., 1986; Sanchez-Herrero and Crosby, 1988; Macías et al., 2004; Wassarman et al., 2000; Abbott and Lengyel, 1991; Grether et al., 1995; Ádám et al., 2003; Hozumi et al., 2006; Spéder et al., 2006; Coutelis et al., 2008).

Here we report that at metamorphosis both the wing and male genitalia require tenectin and the PS2 integrins for proper morphogenesis. We also show that ecdysone regulates *tenectin* expression in wing morphogenesis. Finally, we directly test the capacity of the PS2 integrins and tenectin to functionally interact in cell spreading and adhesion assays.

Materials and methods

Drosophila stocks

Flies were reared on a standard cornmeal/molasses/yeast medium at 25 °C. w^{1118}, y^1 w^+; *P{Act5C-GAL4}25FO1/CyO*, y^+ (FBst0004414), *P{GawB}elavC155* (*FBst0000458*), w^+; *P{GawB}T80/CyO* (FBst0001878), w^+; *P{GAL4-da.G32}UH1* (FBst0005460), y^1 w^{67c23}; *P{EPgy2}CG31422EY16369* (FBst0021205) respectively named in this paper, WT, *Act*-GAL4, *elav*-GAL4, discs-GAL4, *da*-GAL4, and EY16369 were obtained from the Bloomington stock center (stock ID is indicated in parentheses). mys^{nj42}, mys^{b13}, mys^{b47}, and mys^{b69} have been described (Jannuzi et al., 2004). To obtain mys^{b13} males it was necessary to remove, by recombination, extraneous lethal mutations from the original chromosome.

Rapid amplification of cDNA ends (3′ and 5′RACE)

RNAs were extracted from staged third instar larvae using Izol-RNA reagent (5 Prime). The 3′end of *tenectin* cDNA was obtained using 3′RACE System (Invitrogen). Total RNA was reverse-transcribed using an oligo(dT) anchor primer. Nested PCR used the anchor primer in combination with a specific primer (tnc1-3RACE; 5′-GCAAAC-GAGTCCACGAGCGGTCCC-3′), followed by a second specific primer (tnc2-3RACE; 5′-GGCCGCCGTGGTGTCGGGACG-3′). The 5′end of *tenectin* cDNA was obtained using 5′RACE System (Invitrogen). Reverse transcription used total RNA and an antisense *tnc* internal primer (tnc1-5RACE; 5′-TCATTGGTCATGATGCGG-3′). Homopolymeric oligo-dC tail was added to the 3′end of the purified cDNA using terminal deoxyribonucleotidyl transferase. A supplied sense abridged anchor primer and an antisense *tnc*-specific primer (tnc2-5RACE; 5′-TGTTGG-ATCTCCGTGTACTCC-3′) were used in a subsequent PCR. 3′ and 5′RACE products were cloned in pGEM-T (Promega) and sequenced.

Transgenic tenectin mutants

DNA from coding sequence of the *tenectin* gene was amplified by PCR and cloned into the pUAST vector as an inverted repeat as described (Enerly et al., 2002; Kennerdell and Carthew, 2000). Amplification used sense and antisense primers containing *Eco*RI and *Xba*I sites and *Bgl*II and *Xho*I sites and were 5′-CCGGAATTCTGTT-GAAATCGACACGAAGC-3′, 5′-GAAGATCTTACCTCAGGCTCCTCATGCT-3′, 5′-GCTCTAGATGTTGAAATCGACACGAAG-3′ and 5′-CCGCTCGAGTACCT-CAGGCTCCTCATGCT-3′ respectively. The recombinant vector, pUAST-*tnc*-IR was co-injected into w^{1118} embryos with the helper vector pUChsπΔ2–3. Adult, G0, transformants were identified by outcrossing to w^{1118} and chromosomes bearing *tnc*-IR1a (on chromosome III), *tnc*-IR1b (on chromosome II) were balanced over *TM3*, *Sb* or *CyO* balancer chromosomes.

Northern blot analysis

RNA was extracted, fractionated by formaldehyde agarose gel electrophoresis and transferred onto nylon membranes as described (D'Avino et al., 1995). 15 µg of total RNA was loaded per lane. Filters were hybridized, washed, and stripped as described (Karim and Thummel, 1991). To detect *tenectin* transcripts, a 1 kb genomic DNA fragment from exon 5 was PCR-amplified from genomic DNA. All probes were labeled with ^{32}P by random priming (Prime-a-gene, Promega). Each blot was probed with radioactive DNA derived from *E74*, *β-Ftz-F1* or *tenectin*. For reprobing, blots were stripped by boiling for 20–30 min in 10 mM Tris–HCl (pH 7.8), 1 mM EDTA, 1% SDS.

Imaginal discs culture

Staged third instar larvae were dissected in Grace's Insect Medium (Gibco) and the larval organs were cultured in the same at 25 °C for 3 or 6 h. In some cases 20E (5×10^{-6} M) or cycloheximide (7×10^{-5} M) was added to the medium. Cycloheximide treatment reduced incorporation of ^{35}S-methionine into proteins by more than 93% in a parallel experiment (data not shown). After incubation, tissues were collected and total RNA was extracted for Northern blot analysis. The

levels of transcript accumulation were determined with a Molecular Dynamics 300 S computing densitometer.

Quantitative real time PCR

Total RNA (1 µg) from staged third instar larvae was reverse-transcribed using the iScript cDNA Synthesis Kit (Biorad). Q-PCR reactions were carried out on a MyIQ (Biorad) using IQ SYBR green supermix (Biorad) using the following primer pairs; for transcript 1: 5′-AGAAGCCAAATTCCCCAGTT-3′/5′-GCACTTCATGGGTTTGTTCA-3′; for transcript 2: 5′-AGCGGTTGTATCTTGGTGGT-3′/5′-AGAATGGTTTTGGCCAACTG-3′. Each reaction was performed in triplicate and the mean of three independent biological replicates was calculated. All results were normalized to the *RP49* and *Actin 5C* mRNA levels and calculated using the ΔΔCt method (Pfaffl, 2001).

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount *in situ* hybridization was performed using a variation of the protocol described by Tautz and Pfeifle (1989). To prepare the *tenectin in situ* RNA probes, two distinct cDNA encoding regions were PCR-amplified using the primer pairs 5′-GACAATTCCCGAAATCTCCA-3′/5′-CAGCATCCTGAGGAGACACA-3′ and 5′-GATAACCAGGTCTCATTCTC-3′/5′-TCCGGAGAGTGGTAGGGCACG-3′ and cloned in pGEM-T easy vector (Promega). Digoxygenin-labeled sense and antisense riboprobes were synthesized by *in vitro* transcription using T7 and SP6 polymerase, respectively, using the Roche Dig RNA labeling system.

For immunohistochemistry, imaginal discs and larval brains were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 30 min at 0 °C followed by another 30 min incubation with 4% paraformaldehyde and 0.1% Triton X-100 in PBS. After washing in PBS, the imaginal discs and the larval brains were incubated overnight at 4–8 °C with primary antibodies diluted in PBS containing 5% normal goat serum, 0.1% Triton X-100, and 0.02% sodium azide. Primary antibodies were mouse anti-elav (1:1000; Developmental Studies Hybridoma Center, University of Iowa) and anti-tenectin (1:4000; Fraichard et al., 2006). Detection of the different primary antibodies was carried out using alkaline phosphatase anti-rabbit (1:50, Sigma), AlexaFluor594 anti-mouse (1:200, Molecular Probes), and AlexaFluor488 anti-rabbit (1:50, Molecular Probes). Immunolabeled samples were analyzed on a Leica TCS-SP2AOBS spectral confocal microscope.

Tenectin fusion proteins and purification

For the cell spreading assay, a cDNA fragment encoding 68 amino acids (residues 232–299) that includes the RGD cell attachment sequence was cloned into the pQE30 bacterial expression vector (QIA Expressionist, Invitrogen). Soluble tenectin fusion protein was purified by immobilized metal affinity chromatography with Ni-NTA resin and dialyzed into PBS.

For the cell adhesion assays, His-tagged fusion proteins were produced by cloning cDNA fragments encoding VWC#3 or VWC#5, plus 10 amino acids prior to the first cysteine and 8 amino acids following the last cysteine (residues 225–308 and 2819–2731) into pTrcHisC vector (Xpress System, Invitrogen). Mutagenic primers were used to create *Kpn*I and *Eco*RI sites for cloning at these positions. Standard PCR mutagenesis was used to mutate potential integrin-binding motifs. In VWC#3 RGD was mutated to SSL. In VWC#5 RDD, RSD and RYE were mutated to ATA, SSL, and TYI respectively. Urea denatured fusion proteins were purified by affinity chromatography on Ni-NTA agarose (QIAexpresss, QIAGEN). To promote proper folding and formation of the 5 disulphide bonds in the vWFc domains, fusion proteins were dialyzed overnight in refolding buffer (1 mM EDTA, 3.5 M urea, 10 mM beta mercaptoethanol, 20 mM Tris, pH 9.1) followed by dialysis into PBS (Cardamone et al., 1995). Fusion protein concentrations were determined by optical density at 280 nm and then confirmed by polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue.

Cell spreading and adhesion experiments

Drosophila S2/M3 cells and the same stably expressing the αPS2m8 or αPS2c and βPS integrin subunits (under the control of the *Drosophila* HSP70 heat shock promoter) were grown in Shields and Sang M3 medium supplemented with 12.5% heat-inactivated fetal bovine serum. The medium for the transformed cells also contained 2×10^{-7} M methotrexate (Bunch and Brower, 1992; Zavortink et al., 1993).

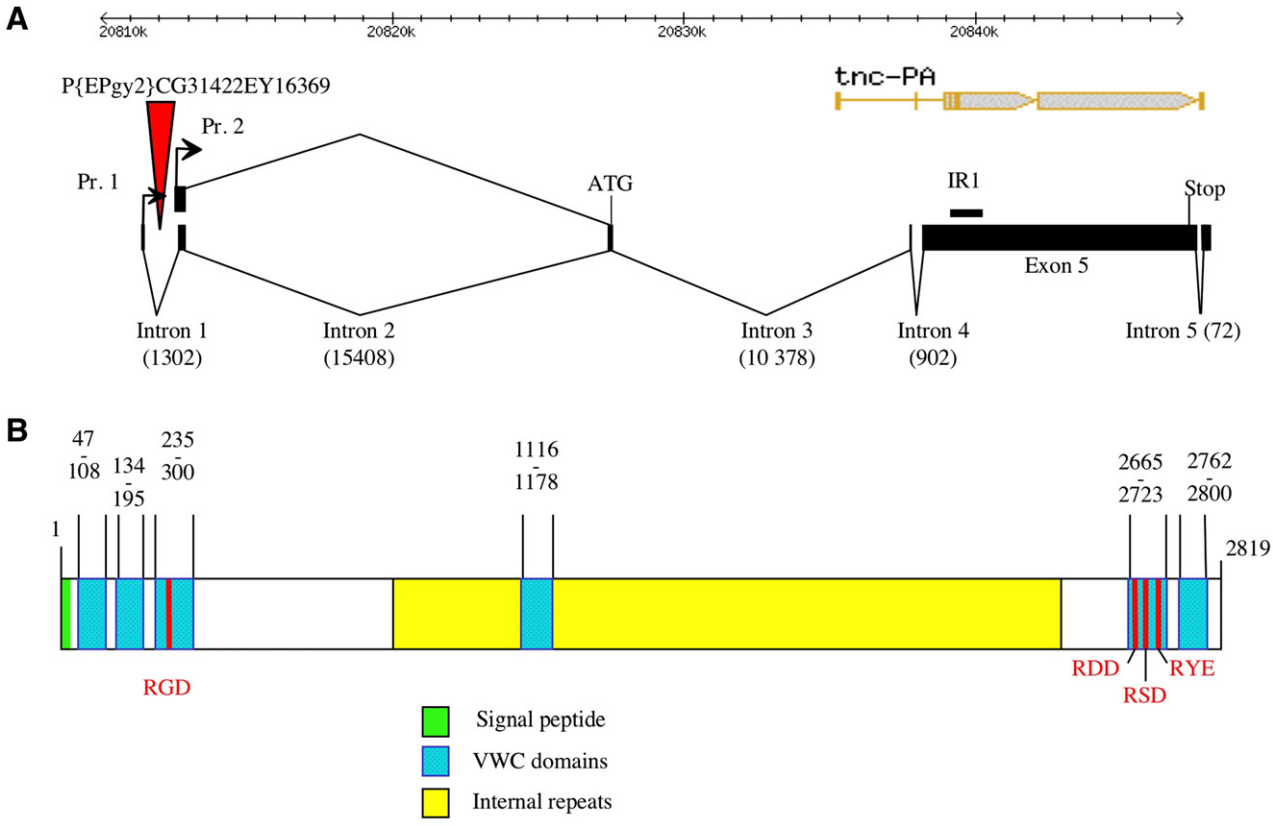
Cell spreading assays were done as described (Jannuzi et al., 2002). Briefly, cells were first cleared of accumulated matrix and other surface proteins by dispase/collagenase treatment at 37 °C. This treatment also heat shocks the cells and induces expression of the integrin transgenes. Cells were allowed to spread in coated 96 well plates for 3–4 h before counting. Each well of a 96 well plate was coated with 50 µl of ligand in PBS overnight at 4 °C. Tenectin VWC#3 fusion protein was used at a concentration of 25 µg/ml. The wells were then washed and non-specific sites on the plate blocked by incubation with 20% dry milk for 1 h at room temperature. Following washing with PBS, 100 µl of cells [at $2–4\times10^5$ cells/ml in M3 medium + 2 mg/ml bovine serum albumin (BSA)] were added. GRGDSP and GRADSP peptides (Calbiochem, San Diego, CA) or purified IgG fractions of the PS integrin function blocking antibody aBG1 (Hirano et al., 1991) and the control PS integrin-binding antibody CF.6G11 (Brower et al., 1984) were added to the cells just prior to their placement onto the coated wells. The number of spread and round cells was determined by phase microscopy using a Nikon phase-contrast microscope (Nikon Diaphot-TMD). For each value, over 100 cells were scored as round or flat from each of 3 different fields and the numbers represent the average of the 3 fields.

Cell adhesion assays were done as described (Jannuzi et al., 2004). Cells were protease treated as for the cell spreading experiments and allowed to recover for 4 h in M3 medium + 2 mg/ml BSA. 100 µl of cells (1.5×10^6 cells/ml) were added to 96 well plate wells coated with ligand and the cells were allowed to attach for 20 min. Nonadherent cells were washed from the wells by pipetting and the remaining cells were stained with crystal violet. Dye levels were quantified using a Synergy2 Microplate Reader (Bio-Tek Instruments) at 562 nm. Plate coating concentrations for tenectin ligands were 20 µg/ml. To determine the tenectin "specific" adhesion we subtracted non-specific values of cells adhering to wells coated with 20 µg/ml BSA. For adhesion assays, wells were coated with ligand for 1 h at room temperature. Adhesion assays were done in three wells for each ligand and cell line displayed. The values given are the mean ± s.e.m. for those 3 wells.

Results

tenectin encodes a putative αPS2βPS integrin ligand

tenectin is the *Drosophila melanogaster* homolog of *tenebrin* from the beetle *Tenebrio molitor*. In the beetle, *tenebrin* was first characterized as a gene whose expression is developmentally regulated by both juvenile hormones and ecdysteroids. Its sequence and subsequent experiments in *Drosophila* indicated that it is a component of the extracellular matrix (ECM) (Royer et al., 2004; Fraichard et al., 2006). To begin the genetic analysis of *tenectin*'s function in development we have characterized its gene structure. Using a combination of 5′ and 3′RACE-PCR and the isolation of multiple cDNAs, we have determined unambiguously the gene structure of *tenectin* (Fig. 1A). Two transcripts, differing by 95



C

```
Tenectin 1 CYYNYAHYQEGDRIMTNEP  CLN CTCHNRMLM CYLRVCPFTKPIGHD   CIVEKREDQCCPIIT  C
           CYYN+ HY EGDRI+TNEP  CLN CTCHNRMLM CYLRVCPFTK IG D   C VEKR DQCCP+IT  C
Tenebrin 1 CYYNFQHYDEGDRIITNEP  CLN CTCHNRMLM CYLRVCPFTKAIGQD   CKVEKRADQCCPVIT  C

Tenectin 2 CSIEEKFYPEGAQVPSNPNKPCEL CYCINNQTK CVMQECTLHVDG      CLPIYNKGSCCPVRYS C
           CSI+  FY +GA+VPS+PNKPCEL CYCI N+T  CVMQECTL V+G      C P+Y +G CCPVRY  C
Tenebrin 2 CSIDGLFYSDGARVPSDPNKPCEL CYCIRNKTA CVMQECTLKVEG      CKPVYQEGVCCPVRYD C

Tenectin 3 CIHDGEIFADGASLKGKNA  CEH CYCMRGDIV CAVQECE-VPMMAANGKSCRAMPAAEGECCPSNYVC
           CIH+ EI+ADGA +       CEH CYCMRGDIV CAVQEC   P+   N   C A+P  EG+CCP NY C
Tenebrin 3 CIHNNEIYADGALVIKDKP  CEH CYCMRGDIV CAVQECSPTPLDKVN---CTALPPKEGQCCPDNYEC

Tenectin 4 CLVEGKTYANNTIVPATAP  CDVSCKCISSLVA CQQMEC-KLPENLEK   CTVAADLLDGCCPTYI C
           CLV+G+TY NN+ VP      C VSCKC+SS++  C+ + C   P NL+    C       D CCPTY  C
Tenebrin 4 CLVDGQTYKNNSNVPPINH  CQVSCKCVSSILQ CESVTCTPPPSNLQH   CMPVYQGPDSCCPTYS C

Tenectin 5 CRYAGKLYVSAQQIPRDDP  CDF CFCFRSDII CLQQSCPPPIAG      CHEEPISGF CCPRYE C
           CRY GK+YVSAQQIPRDDP  CDF CFCFRSDII CLQQSCPPPI        CHEEPI GF CCPRYE C
Tenebrin 5 CRYGGKVYVSAQQIPRDDP  CDF CFCFRSDII CLQQSCPPPIPR      CHEEPIRGF CCPRYE C

Tenectin 6 CLINGRSYRVGEQIESTSGP CIS CTCGGDGKMKCDPQQC
           C I  ++Y VGE+I+S SGP C+  CTCGGDG+MKCDP+ C
Tenebrin 6 CQIRNKAYNVGEEIKSASGP CLH CTCGGDGQMKCDPKAC

vWFc_2     C--------------------C---C-C-------C----C------------C---------CC-----C
```

Fig. 1. *tenectin* gene organization. (A) Schematic of the *tenectin* gene showing the start and stop codons and intron splice sites for the two RNAs transcribed from 2 promoters (Pr. 1 and Pr. 2). The two transcripts contain identical translated sequences. The positions of the P{EPgy2}CG31422EY16369 element and sequence used for making the inverted repeat RNAi construct (IR1) are marked. For comparison, the flybase gene structure, with errors in intron/exon positions, is presented at the top of the figure (tnc-PA). (B) Schematic of the deduced protein showing the signal peptide, the RGD motifs, von Willebrand Factor type-c (VWC) domains and internal repeats. (C) Sequence alignments of VWC domains in tenectin and tenebrin. Consensus cysteines are also shown.

nucleotides in their 5′ untranslated regions have been identified. The two cDNA sequences are 10,376 and 10,281 bp long and contain the same open reading frame of 2819 amino acids followed by 1461 untranslated nucleotides containing a putative polyadenylation signal. This structure for the *tenectin* gene differs markedly from that previously reported in flybase for CG13648 (http://flybase.org/cgi-bin/gbrowse/dmel/?Search=1;name=FBgn0039257) due to CG13648 containing incorrect predictions of transcript start sites, intron/exon positions, and the position of the AUG start codon. Our new description of the gene structure is confirmed by additional ESTs recently deposited in genbank that cover part of the 5′ end of *tenectin* (gb|EY199010.1|) and a region of exon 5 that was previously reported to contain an intron (gb|EC215144.1|, gb|CO309183.1|, gb|CO307535.1|, gb|EC214929.1|, gb|EC077781.1|).

The deduced tenectin protein (Fig. 1B) is very similar to tenebrin (Royer et al., 2004). It has a molecular weight of ~300 kDa, a p*I* of 4, and the putative translational start site is followed by 31 hydrophobic amino acids corresponding to a signal peptide sequence (von Heijne, 1984) with a cleavage site predicted to be located between position 31 and 32 (Nielsen et al., 1997). Royer et al. (2004) described five von

Willebrand type-c (VWC) domains in both tenebrin and tenectin. These motifs are involved in protein–protein interactions (Verweij et al., 1986; Hunt and Barker, 1987) and have been found in ECM proteins like collagens and mucins (Sangiorgi et al., 1985; Wang and Granados, 1997), and also in signaling molecules like chordin (Sasai et al., 1994). Closer examination of the tenectin and tenebrin sequences reveals that they are of the vWFc_2 type (PROSITE, # PS50184) with a consensus sequence of C-X(18–26)-C-X(2,3)-C-X-C-X(6–14)-C-X(3,5)-C-X(1–12)-C-X(8–16)-C-C-X(2–5)-C. In addition to 5 complete VWC domains, there is one partial domain at the end of the protein that contains only the first 6 cysteines. As with tenebrin, the cell attachment sequence Arg-Gly-Asp (RGD; Ruoslahti and Pierschbacher 1987; Yamada, 1991) is found in tenectin within the third VWC domain located in the N-terminus of the protein (Royer et al., 2004). The 5th VWC domain contains 3 variants of the RGD sequence (RDD, RSD and RYE) that may serve as cell attachment motifs as well (Figs. 1B and C). The central region of the protein, excluding the 4th VWC is characterized by the presence of numerous internal repeats and is rich in Glu (19.2%), Thr (11.8%), and Pro (11.9%) as was described for tenebrin (Royer et al., 2004). Though of similar amino acid composition to tenebrin, the central region shows only 36% similarity (21% identities) in a BLASTP 2.2.18 analysis, while the VWC domains display a high degree of similarity (50–92% identities). The high Ser-Thr and Pro content (20.4% and 11.2%, respectively, for the entire protein) and the presence of the central repeats with a high Ser-Thr and Pro content (Fig. S1) suggest that tenectin may be a mucin-related-protein (Syed et al., 2008). Tenectin's presence in the ECM of the CNS, foregut, hindgut, trachea and wing (Royer et al., 2004; Fraichard et al., 2006) and the presence of RGD motifs make it a likely ligand for the RGD-binding αPS2βPS integrins (Bunch and Brower, 1992; Fogerty et al., 1994; Graner et al., 1998; Subramanian et al., 2007).

Temporal profile of tenectin expression during metamorphosis

The *tenectin* homolog in *Tenebrio molitor*, *tenebrin*, is regulated by juvenile hormones and ecdysteroids at each molt (Royer et al., 2004). To ask if the expression of *tenectin* is similarly developmentally regulated during *Drosophila* metamorphosis, Northern blots of total mRNA from staged third instar larvae, prepupae, pupae and unstaged adults were hybridized with a probe derived from the exon 5 shared by the two *tenectin* transcripts. *tenectin* transcripts are not detectable 18 h prior to puparium formation and then increase in the next 8 h and reach a peak level at 2 h after puparium formation (APF). Transcript levels then decline to a low level by 6 h APF. Subsequently, the level of *tenectin* mRNA increases to a second peak during the early pupal period and to a third peak during the mid-pupal period (Fig. 2). These peaks are correlated with peaks in ecdysone titers (Fig. 2) (Richards, 1981; Handler, 1982; Warren et al., 2006). By reprobing the Northern blot we compared the expression of *tenectin* with that of previously described ecdysone responsive genes E74 and β-FTZ-F1 (Andres et al., 1993; Thummel, 1996, 1997). The early expression pattern of *tenectin* is very similar to E74A, a class II early-response gene. The second and third *tenectin* expression phases have some characteristics of the class I early-response gene, E74B. Expression of both rises at 14 h APF and again at 20 h. As β-FTZ-F1 expression is repressed by ecdysone, its expression defines periods of low ecdysone. β-FTZ-F1 expression, and by extension low ecdysone, identifies the stages when *tenectin* is not expressed (hours APF 6–12 and 60–96). Though *tenectin* expression is complicated, the Northern data on whole flies are consistent with it being an ecdysone regulated gene in *Drosophila* just as *tenebrin* is in *Tenebrio*.

tenectin transcription is inducible by ecdysone

To demonstrate directly that *tenectin* expression is regulated by ecdysone, we analyzed the transcription of *tenectin* in mass-isolated third instar larval organs cultured for 0, 3 and 6 h in the absence or presence of physiological levels of 20-hydroxyecdysone (20E) (Fig. 3A). In the absence of added 20E, *tenectin* mRNA levels rise dramatically at 3 and 6 h following dissection (black bars). This increase in *tenectin* mRNA is likely due to the larval organs responding to the pulse of ecdysone that took place prior to dissection (at 16 h prior to puparium formation (see Fig. 2). The addition of 20E further increases *tenectin* mRNA levels at both 3 and 6 h following dissection (grey bars Fig. 3A) suggesting that the *tenectin* promoter is activated directly by the high titer 20E pulse that triggers puparium formation. To further address this possibility, we assessed the effect of cycloheximide on the stimulation of *tenectin* expression by 20E. Mass-isolated late third instar larval organs were cultured for 6 h with 20E alone, 20E and cycloheximide, or cycloheximide alone (Fig. 3B). In the presence of physiological levels of 20E, *tenectin* mRNA was increased twofold by 6 h. *tenectin* transcription was also induced by 20E in the presence of cycloheximide, although to reduced levels. These results indicate that *tenectin* expression is directly inducible by 20E but that protein synthesis is required to obtain the full induction. This would suggest that *tenectin* is an early-late, or a type II early, ecdysone responsive gene (Stone and Thummel, 1993).

tenectin is widely expressed in imaginal discs and brain

If *tenectin* is important to the hormonally regulated developmental processes of metamorphosis, it should be expressed in imaginal discs at this time. To determine the spatial expression of *tenectin* at the

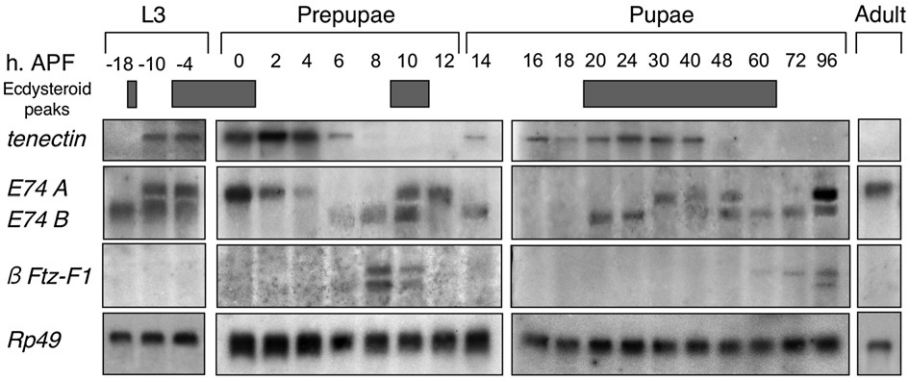


Fig. 2. Developmental profile of *tenectin* expression. Northern blot hybridization of RNA isolated from staged late third instar larvae, prepupae, pupae and unstaged adults. The probe used for hybridization was prepared from the common fifth exon. The blot was reprobed to detect an early gene (*E74*) and a prepupal gene (*β-Ftz-F1*). Previously identified peaks in ecdysone titer are shown (Richards, 1981; Handler, 1982; Warren et al., 2006). Developmental times are shown on top, in hours after puparium formation (APF). Hybridization to detect *rp49* mRNA (O'Connell and Rosbash, 1984) was used as a control for loading and transfer. This experiment was performed twice with very similar results (data not shown).

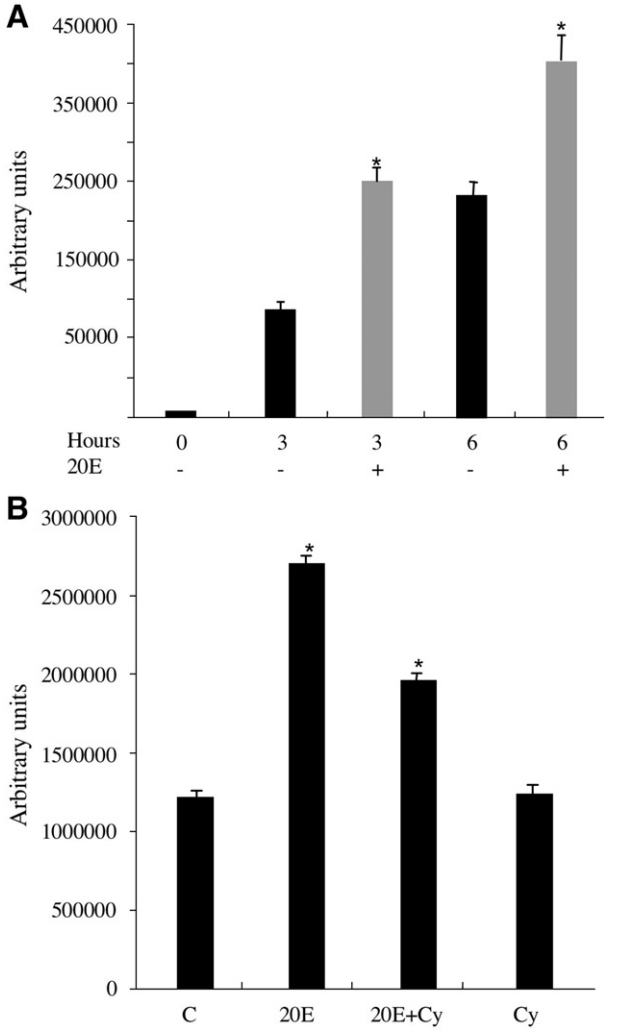


Fig. 3. Stimulation of *tenectin* transcription by 20E. (A) *tenectin* RNA levels are shown for mass-isolated third instar larval imaginal discs maintained in culture without added hormone (black bars) or treated with 5×10^{-6} M 20E (grey bars) for 0, 3 or 6 h. (B) *tenectin* mRNA levels in larval organs cultured for 6 h in the absence (C) or presence of 20E alone, 20E and cycloheximide (20E + Cy), or cycloheximide (Cy) alone. Total RNA was analyzed by Northern blot hybridization and *tenectin* mRNA was quantified by volume integration densitometry. Hybridization to detect *rp49* mRNA (O'Connell and Rosbash, 1984) was used as a control for loading and transfer. Error bars are s.e.m. and asterisks indicate significant differences from control using Student's *t* test ($P<0.01$).

beginning of metamorphosis, *tenectin* mRNA and protein distributions were determined in a variety of imaginal discs and brains dissected from late third instar larvae. *tenectin* mRNA is detected in all of these structures. In the brain, *tenectin* transcript is expressed in voluminous cells of thoracic and abdominal ganglia and in small cells of the optic lobes corresponding to the lamina region (Fig. 4A; arrowhead). *tenectin* mRNA is detected in the eye-antennal (Fig. 4B), leg (Fig. 4C), male genital (Fig. 4D) and wing (Fig. 4E) discs. In the eye disc, *tenectin* mRNA is expressed in undifferentiated cells, anterior to the morphogenetic furrow as shown by the result of a double labeling for *tenectin* mRNA and Elav antibodies (Fig. 4B). In the male genital disc, tenectin mRNA is localized to regions derived from A8 and A9 segments (Fig. 4D). In the wing disc, *tenectin* mRNA is strongly expressed in the wing hinge and in the notum (Fig. 4E). Tenectin protein is localized to the entire neuropil of the CNS (Fig. 4A) and later, it is found at the surface of the two prepupal wing layers (Fig. 4J). As expected for an ECM protein, tenectin's distribution is wider than its mRNA expression (Figs. 4F–I). These results indicate that just prior to, or during, metamorphosis *tenectin* is widely expressed on most discs.

RNAi tenectin knockdown mutants

To begin a genetic analysis of *tenectin*'s in vivo functions, in the absence of mutations in the *tenectin* gene, we used RNA interference (RNAi) to reduce *tenectin* expression. Transgenic lines were produced, expressing a 1 kb hairpin loop RNA under the control of the UAS/GAL4 system. An inverted repeat sequence was constructed from the large fifth exon of *tenectin* (Fig. 1A) in the region encoding the N-terminus of the protein. Two transgenic lines, *tnc*-IR1a, *tnc*-IR1b, were chosen that gave adult escapers with phenotypes when the *tenectin*-hairpin loop genes were driven by the expression of the ubiquitous *Actin 5c*-GAL4 (*Act*-GAL4) and *daughterless*-GAL4 (*da*-GAL4) drivers. We confirmed by Northern blot analysis (data not shown) and by Q-PCR that expression of IR1 in *tnc*-IR1a and *tnc*-IR1b leads to a significant decrease of *tenectin* transcript. *tnc*-IR1a in combination with *Act*-GAL4 results in a 90% reduction in both *tenectin* transcripts while *tnc*-IR1b results in only a 50% reduction (Fig. 5).

tnc-IR1 expression results in lethality, and the level of lethality correlates with the effectiveness of the hairpin loop genes. *Tnc*-IR1a driven by *Act*-GAL4 is 90% ($n=205$) lethal while *tnc*-IR1b is only 35% ($n=145$) lethal (Table 1). Comparable values are seen when these hairpin loop genes are driven by the *da*-GAL4 driver (Table 1). The reduced viability is due to death during several phases of development with a large proportion of embryonic lethality (~75% for *tnc*-IR1a and ~20% for *tnc*-IR1b). Adult escapers are lethargic and unable to jump or fly, and a large number die within one or two days after eclosion. Morphological defects of male genitalia and wings are common.

tenectin and integrin mutants display male genitalia anomalies

When the *tnc*-IR1b line was crossed to *Act*-GAL4, 72% of the adult male progeny showed a characteristic malrotation of genitalia (Table 2; Fig. 6B) by up to 180°. Similar experiments with *tnc*-IR1b crossed to *da*-GAL4 (Table 2), or *tnc*-IR1a crossed to *Act*-GAL4 (not shown), also resulted in adult male progeny displaying malrotation of genitalia. Correct positioning of the male genitalia takes place during metamorphosis. At this stage, the distal part of the male reproductive apparatus, the genital plate, undergoes a stereotyped 360° clockwise rotation, inducing the spermiduct to loop around the gut in a clockwise direction (Fig. 6C) (Gleichauf, 1936). Because external malrotation does not allow discrimination between under-, hyper- or counter-rotation of the genitalia, mutant males were dissected and the looping of their spermiduct analyzed. All dissected males expressing *tnc*-IR1 showed a clear under-rotation phenotype (Fig. 6F). Our results demonstrate that *tenectin* is required for the genital plate and spermiduct to undergo complete looping, but has no role in directionality.

Under-rotation of the genitalia can be due to an abnormal neuroendocrine function, due to mutation of the adhesion molecule fasciclin2, leading to an elevated level of juvenile hormone. The function of fasciclin2 is required in the nervous system and mutants are rescued by ectopic expression of wild type fasciclin2 protein promoted by the neuronal-specific *elav*-GAL4 driver (Ádám et al., 2003). As *tenectin* is also expressed in the CNS (Fig. 4A), we asked whether *tenectin* function is required in the nervous system or in the genital disc for correct genital rotation. When expression of the *tnc*-IR1 transgene is restricted to the imaginal discs using the discs-GAL4 driver, the genitalia undergo incomplete rotation (Table 2). In contrast, and when the *tnc*-IR1 transgene is expressed in neuronal cells, using the *elav*-GAL4 driver, the male genitalia were unaffected (Table 2). Taken together, these results suggest that the phenotype observed is due to abnormal *tenectin* expression in the genital disc and that *tenectin* expression is not required in the neuroendocrine system for correct genital rotation.

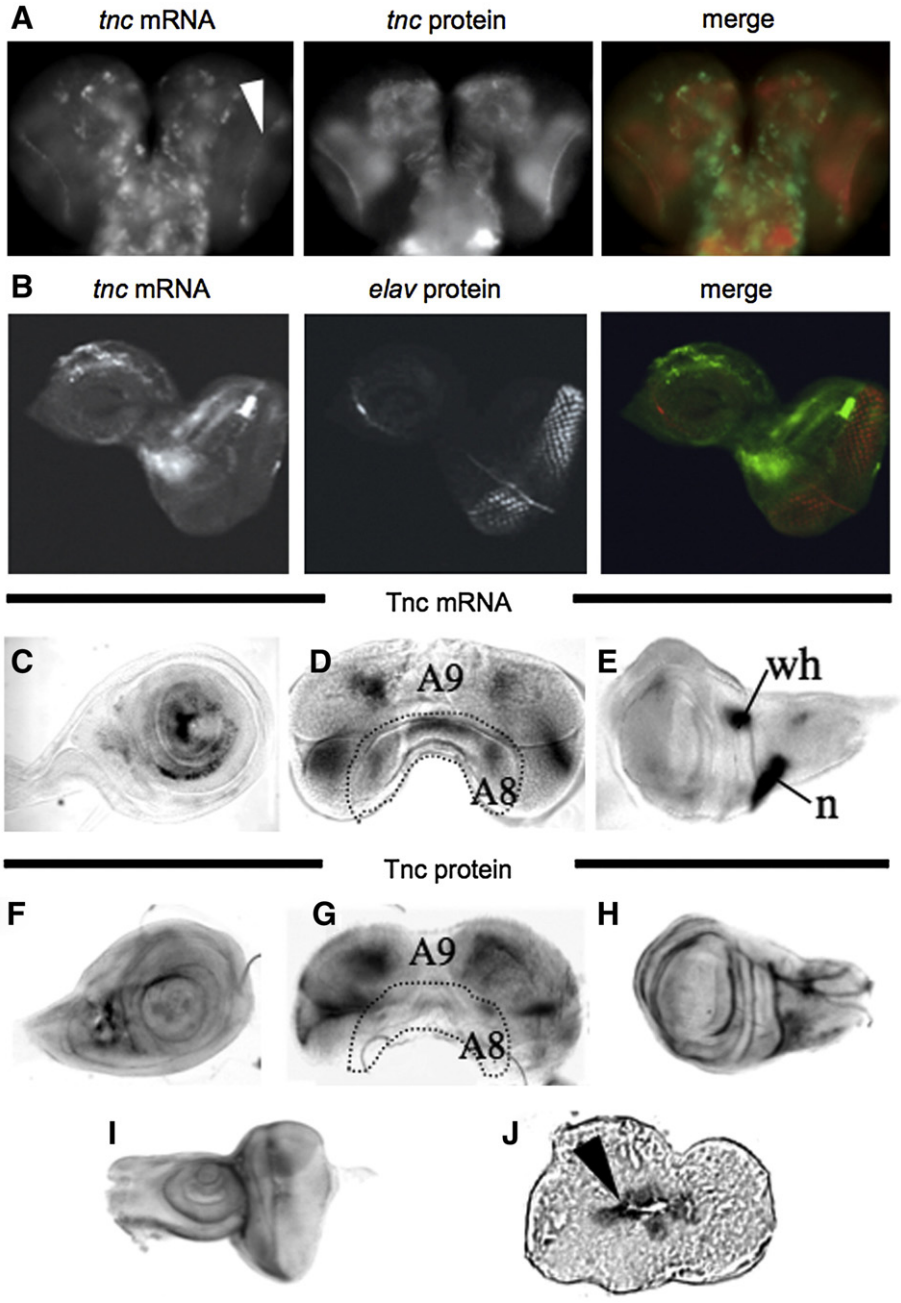


Fig. 4. *tenectin* is expressed in brain and imaginal discs during post-embryonic development. *tenectin* nucleic acid or antibody probes were hybridized to brain and discs dissected from late third instar larvae (A–I). Brain (A) and eye-antennal disc (B) stained for *tenectin* transcript (green) and tenectin protein (red: A) or elav protein (red: B). Cells of the optic lobes corresponding to the lamina region expressing tenectin transcripts are indicated (arrowhead in A). Leg (C), male genital (D) and wing (E) discs probed for *tenectin* transcript. Leg (F), male genital (G) wing (H) and eye-antennal discs (I) probed for *tenectin* protein. Prepupal wing disc, transversal cut, stained for tenectin protein (J) showing its localization to the apposed surfaces of the dorsal and ventral wing layers (arrowhead). Regions of the male genital discs corresponding to different abdominal segment cells (Casares et al., 1997) are indicated (D and G). n: notum; wh: wing hinge.

We are proposing that tenectin is a new PS2 integrin ligand and one potential integrin mutant previously displayed malrotation of male genitalia (Deak et al., 1982). Unfortunately, that allele was never molecularly characterized and no longer exists. Therefore, we assayed our collection of *myospheroid* (*mys*; βPS integrin subunit) function-altering mutations (Jannuzi et al., 2004) for their effects on male genitalia. Three alleles mys^{b47}, mys^{b69} and mys^{b13} gave males with under-rotated genitalia by up to 180° (Fig. 6C, Table 2). The frequency of rotation defects is dependent on temperature as it is reduced when flies are reared at 22 °C. To ask specifically if PS2 integrins are required for this process we combined the *inflated* (*if*; αPS2 integrin subunit) hypomorphic allele if^3 (Lindsley and Grell, 1968; Brower and Jaffe, 1989; Wilcox et al., 1989), with mys^{b13}. if^3 males display no under-rotated genitalia and mys^{b13} males display slightly under-rotated genitalia at a frequency of just 2% at 22 °C. mys^{b13}, if^3 males have under-rotated genitalia at a frequency of 100% (Table 2) and these are typically very extreme with 61% ($n=41$) of them being greater than 180° under-rotated. Thus, in addition to the extracellular matrix molecule tenectin, its proposed cell surface integrin receptor is required for proper looping morphogenesis of male genitalia.

tenectin mutants display wing defects and interact with integrin mutants

tenectin also has a role in wing morphogenesis. *tnc*-IR; *Act*-GAL4 transheterozygous adults exhibit multiple wing defects. Depending

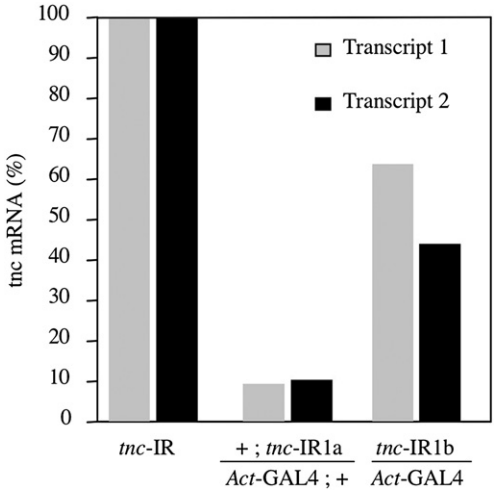


Fig. 5. *tenectin* mRNA is reduced in flies expressing *tnc*-IRs. *tenectin* transcript levels were quantified by real time PCR in staged third instar larvae heterozygous for *tnc*-IR1a or *tnc*-IR1b and these levels were set as 100% for each line (*tnc*-IR). Levels of both transcripts produced from the *tenectin* gene were measured and compared with the levels in flies carrying one copy of *tnc*-IR1a (or *tnc*-IR1b) and one copy of the *Act*-GAL4 driver.

on the specific transgene used to inhibit *tenectin* expression the range of defects observed included wing blisters, failure of wing expansion, wing margin nicking, and malformed wings (Fig. 7). *tenectin* wing phenotypes resemble the phenotypes associated with mutations in integrins or their ligands (Brower and Jaffe, 1989; Brabant and Brower, 1993; Wilcox et al., 1989; Wehrli et al., 1993; Zusman et al., 1993; Brower et al., 1995; Bloor and Brown, 1998; Henchcliffe et al., 1993; Martin et al., 1999; Bunch et al., 1998). To assess whether tenectin and integrins function together to ensure proper wing morphogenesis, we tested whether *tenectin* mutations could enhance the wing blister phenotypes associated with a viable hypomorphic βPS integrin mutation, mys^{nj42} (Costello and Thomas, 1981). This allele has been used for several genetic interaction studies of cell adhesion (Wilcox et al., 1989; Prout et al., 1997; Schöck and Perrimon, 2003). *tnc*-IR1b/*Act*-GAL4 flies do not produce wing blisters and hemizygous mys^{nj42} display a low frequency of wing blisters (10%). In mys^{nj42} flies, *tnc*-IR1b/*act*-Gal increased the frequency of blistering by approximately fourfold and the overall frequency of wing defects by sevenfold (Table 3). Taken together, these results suggest that tenectin and integrins function in a common pathway during wing morphogenesis.

tenectin RNAi and mys^{nj42} rescue ectopic tenectin expression

Overexpression of *tenectin* results in defects that can be rescued by *tenectin* RNAi or mys^{nj42}. An Epgy2 transposable element, EY16369, has been found inserted between the two *tenectin* transcription initiation sites (Fig. 1A; Bellen et al., 2004). This transposable element contains GAL4-UAS sequences allowing *tenectin* to be overexpressed by GAL4 drivers. Combining the ubiquitous GAL4 driver, *Act*-GAL4, and EY16369 resulted in 92% lethality (Table 4). Of the adult escapers, 66% ($n=33$) showed wing defects characterized by an absence of wing expansion and a large number die within one or two days after hatching. No male genitalia defects were observed.

tnc-IR1b, which reduces *tenectin* expression, shows partial rescue of the *tenectin* overexpression by EY16369. *Tnc*-IR1b/*Act*-GAL4; EY16369/+ are only 23% lethal (Table 4). Also, the overexpression by EY16369 reduced the genitalia and wing defects of *Tnc*-IR1b (Tables 2 and 4). To ask if the lethality of *tenectin* overexpression was due to integrin-mediated processes we combined EY16369 and mys^{nj42}. The 92% lethality of males expressing EY16369 is reduced to only 25% by the presence of mys^{nj42} (Table 4).

Table 1
tnc-IR induced lethality.

Genotype	Lethality
tnc-IR1a/+; *Act*-GAL4/+	90% ($n=205$)
tnc-IR1a/+; *da*-GAL4/+	80% ($n=180$)
tnc-IR1b/*Act*-GAL4	35% ($n=145$)
tnc-IR1b/Da-GAL4	36% ($n=175$)

Table 2
Malrotated genitalia in *tnc*-IR and *mys* males.

Genotype	Rotated genitalia
tnc-IR1b/*Act*-GAL4	72% (240)
tnc-IR1b/*Da*-GAL4	54% (218)
tnc-IR1b/discs-Gal4	48% (100)
tnc-IR1b/*elav*-GAL4	0% (95)
tnc-IR1b/*Act*-GAL4; EY16369/+	20% (140)
mys^{b13} 28 °C[a]	10% (231)
mys^{b47} 28 °C[a]	16% (219)
mys^{b69} 28 °C[a]	20% (129)
mys^{b13} 22 °C	2% (179)
mys^{b47} 22 °C	0% (360)
mys^{b69} 22 °C	1% (114)
if^{3} 22 °C	0% (262)
mys^{b13}, if^{3} 22 °C	100% (41)

Number of flies examined (n) is given in parentheses.
[a] To avoid lethality at 28 °C, flies were raised at 18–22 °C through first or second instar larvae and then shifted to 28 °C prior to pupal development.

Tenectin mediates cell spreading and adhesion via αPS2βPS integrins

Tenectin's RGD integrin-binding motif coupled with the phenotypic and genetic interaction data strongly suggest that tenectin is an extracellular αPS2βPS integrin ligand. To confirm this, we tested the ability of αPS2βPS to mediate spreading of S2 cells on plates coated with recombinant tenectin. As reported for other RGD-containing ligands (Graner et al., 1998), tenectin supports the spreading of these cells efficiently (Fig. 8A). Cells that are not transformed with integrins displayed no spreading (not shown). To further demonstrate the integrin requirement for tenectin-mediated spreading we blocked cell spreading with a monoclonal antibody, aBG1, that is known to block the function of βPS integrins (Fig. 8C). That tenectin interacts with the RGD-binding domain of integrin is supported by the ability of a soluble competing peptide, GRGDSP, to inhibit cell spreading on tenectin (Fig. 8C).

The recombinant tenectin VWC#3 used in the cell spreading studies contains the integrin-binding RGD motif. VWC#5 repeat has an RSD sequence in the same location as the RGD of VWC#3. VWC#5 also contains two other potential integrin-binding motifs, RDD and RYE (Fig. 1C). Additional complexity in PS2 integrin–ligand interactions arises due to the presence of two isoforms of the αPS2 integrin, αPS2m8 and αPS2c, that are present in flies and display differences in their interactions with ligands (Graner et al., 1998). To examine the importance of VWC#3 and VWC#5 and their individual binding motifs to both isoforms of PS2 integrins, we performed cell adhesion assays. Untransformed cells (S2) not expressing PS2 integrins displayed low levels of adhesion to fusion proteins containing either VWC#3 or VWC#5 and this was increased by the expression of either PS2 integrin isoform (Figs. 9A and B, black bars). Thus, two widely separated VWC domains have the ability to promote cell adhesion via both forms of PS2 integrins.

To confirm the importance of the RGD motif in VWC#3 and to explore potential binding motifs in VWC#5, fusion proteins with mutated motifs were tested. The RGD sequence of VWC#3 was mutated to SSL. VWC#5 motifs were mutated as follows; RDD>ATA,

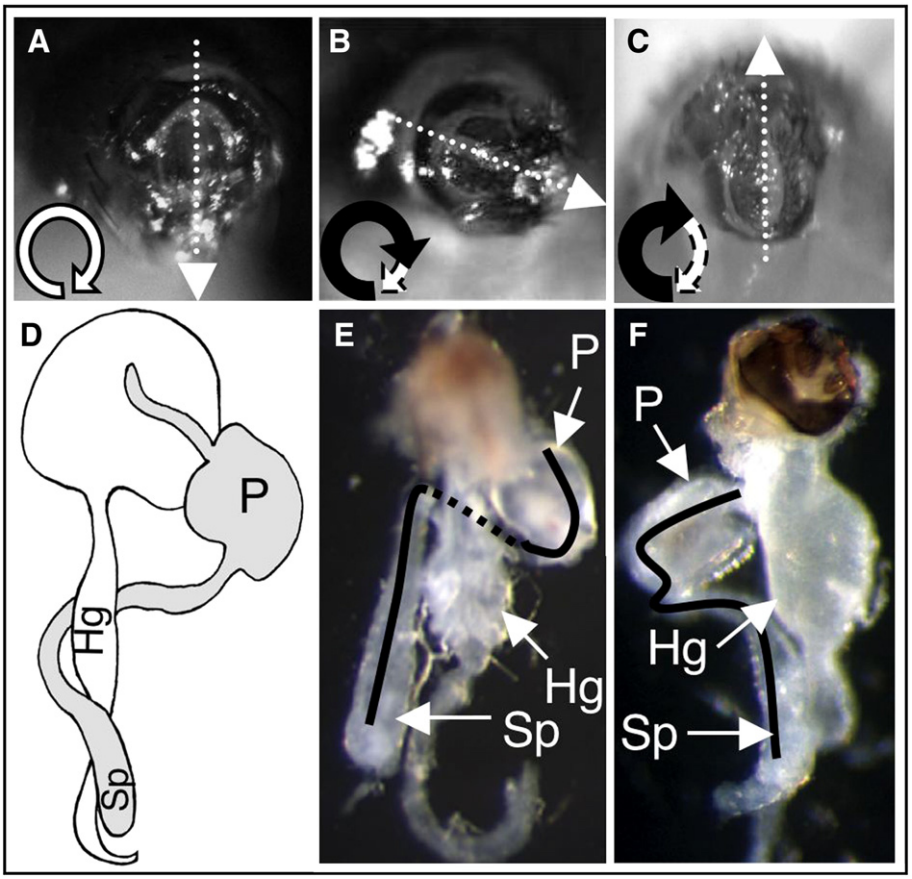


Fig. 6. Rotation of genitalia and spermiduct looping in *tnc*-IR and *mys*^{b13} males. (A) Image of a wild type male external genitalia (posterior view with dorsal upwards), showing the position of the anus and penis. The direction and extent of genitalia rotation is schematized by a looping arrow (bottom left). (B and C) Images of a representative *tnc*-IR and *mys*^{b13} males showing genitalia malrotation. (D) Schematic representation (Ádám et al., 2003) and (E) dissected wild type male abdomen showing the rightward (when viewed from the posterior) looping of the spermiduct. (F) Dissected *tnc*-IR male abdomen with under-rotation phenotype. sp, spermiduct; g, gut; p, penis.

RSD>SSL, and RYE>TYI. ATA, SSL and TYI were chosen as substitutions for the potential integrin-binding motifs due to their presence at identical locations in VWC#4 (Fig. 1C). This makes it unlikely that the substitutions will cause structural changes to the VWC domains. Significantly reduced adhesiveness was found when VWC#3 RGD motif was mutated to SSL confirming a role for the RGD motif in cell adhesion (Fig. 9A). Mutation of the 3 potential motifs in VWC#5 gave varied results. Two of the potential motifs, RSD and RYE, showed no evidence for promoting adhesion, as mutating them to SSL and TYI did not decrease adhesion. For the PS2c integrin-expressing cells, mutations of the RSD and RYE sequences increased adhesion (Fig. 9B) suggesting that RSD and RYE might interfere with adhesion to tenectin by PS2c integrins. In contrast, RDD>ATA reduced adhesion by both PS2 integrin-expressing cell lines. Thus, RDD can serve as an integrin-binding motif in VWC#5. PS2c integrin-mediated adhesion is reduced to levels similar to untransformed S2 cells by mutations of either the RGD or the RDD motif. For cells expressing PS2m8 the effects of these mutations are significant but not as dramatic.

Discussion

tenectin mutants

Tenectin is a protein localized to the ECM during *Drosophila* embryonic development (Fraichard et al., 2006). The presence of an integrin-binding RGD motif led us to speculate that tenectin could be a new integrin ligand. To study the function of tenectin during *Drosophila* development, we generated *tenectin* knockdowns by RNA interference (Fortier and Belote, 2000; Kennerdell and Carthew, 2000; Lam and Thummel, 2000). Two strains of *tenectin* knockdown flies were selected that gave visible hypomorphic phenotypes. We also characterized flies that give phenotypes due to overexpression of the endogenous *tenectin* gene. Lowering mRNA level by RNAi partially rescued the effects of *tenectin* overexpression and overexpression of *tenectin* partially rescues *tenectin* knockdown phenotypes. Thus, we are confident that our *tenectin* knockdown phenotypes result specifically from reduced *tenectin* expression.

Tenectin is a new ligand of αPS2βPS in wing epithelia

Lethality is the most prevalent phenotype displayed by ubiquitous reduction in tenectin expression but in this study we have focused on adult phenotypes to ascertain tenectin's function in morphogenetic processes of metamorphosis. The most striking adult phenotype observed in adult flies with reduced tenectin expression is deformed wings including blisters, nicks, lack of expansion and malformation. These phenotypes resemble those associated with mutations in integrin subunits (Brower and Jaffe; 1989, Wilcox et al. 1989; Zusman et al., 1990; Brabant and Brower, 1993), their extracellular ligands (Henchcliffe et al., 1993; Bunch et al., 1998; Martin et al., 1999), and genes encoding intracellular proteins that interact with integrins (Bökel and Brown, 2002; Brower, 2003 for reviews). Three lines of evidence support tenectin functioning as a PS integrin ligand to facilitate wing morphogenesis. First, we find tenectin protein localized between the dorsal and ventral epithelial cell layers in prepupal wings. Integrins function at this location to promote adhesion of these cell layers (see Brower, 2003 for a review). Second, a mutation of *mys*, encoding the βPS subunit, interacts genetically to increase the

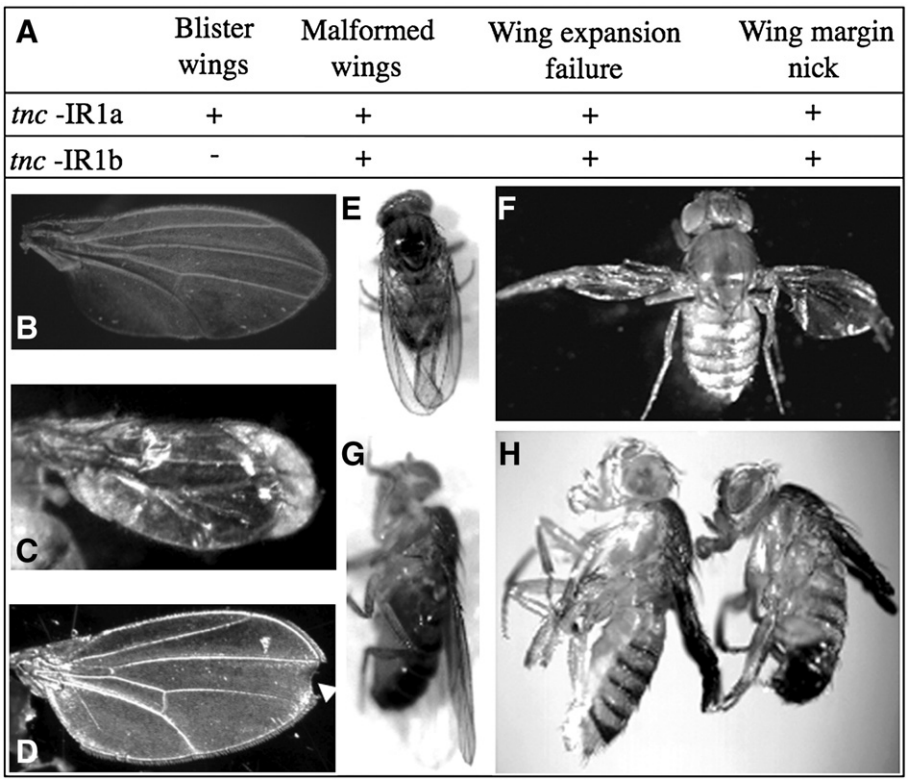


Fig. 7. *tnc*-IRs knockdowns exhibit wing defects. Table (A) shows the different classes of wing defects exhibited by *tenectin* RNAi lines. Dorsal views of adult wings (B–F) either wild type (B and E) or double heterozygous for *tnc*-IR and *Act*-GAL4 (C, D and F). Lateral views of wild type (G) or transheterozygous for *tnc*-IR and *Act*-GAL4 (H). *tenectin* mutants exhibit wing defects: blistered wings (C), malformed wings (F), wing expansion failure (H), and wing margin nicking (D).

frequency of blisters in flies with reduced tenectin expression. Finally, *in vitro* experiments demonstrate that tenectin, through multiple RGD motifs, can function to promote αPS2βPS-mediated cell spreading and adhesion. Taken together, these genetic and biochemical data provide strong evidence that tenectin is a new ligand of αPS2βPS integrin in the wing.

Perhaps relevant to tenectin's function in the wing, Syed et al. (2008), using a bioinformatics approach, identified tenectin as being a mucin-related-protein. In our analysis of the tenectin protein we also notice mucin like repeats. Mucins are highly hydrated O-glycosylated macromolecules that are important to the mucosal lining of mammalian organs. In addition to serving a protective function, various mucins interact with growth factors and cell surface receptors to modulate signaling. It has been shown in vertebrates that mucins also modulate cell adhesion. For example, MUC4 was found to sterically reduce the accessibility of integrins to extracellular matrix ligands and thereby interfere with adhesion (Hollingsworth and Swanson, 2004; Chaturvedi et al., 2007, 2008). Interestingly, Zhang et al. (2008) have recently shown in *Drosophila* that a mucin-type glycosyltransferase, PGANT3, glycosylates another PS2 integrin ligand, tiggrin. Moreover, mutation of the *pgant3* gene results in a wing-blistering phenotype. In the developing wing disc PGANT3 glycosylates tiggrin and other matrix molecules, thus potentially modulating cell adhesion through integrin–ECM interactions. Future biochemical experiments will be needed to determine if tenectin is a bona fide mucin, glycosylated by PGANT3, and whether glycosylation down- or up-regulates its adhesive function.

Table 3
Wing defects in *tnc*-IR and *mys* males.

Genotype	BW	WD	*n* =
mys^{nj42}/Y	10%	10%	104
tnc-IR1b/*Act*-GAL4	0%	15%	240
mys^{nj42}/Y; *tnc*-IR1b/*Act*-GAL4	45%	68%	29

BW; blistered wings, WD; wing defects.

tenectin expression is regulated by 20E during metamorphosis

The formation of the flat bi-layered wing from a folded imaginal disc involves several steps of apposition and separation of the ventral and dorsal epidermal sheets followed ultimately by an epithelial to mesenchymal transition and migration of the cells out of the wing (Waddington, 1940; Johnson and Milner, 1987; Fristrom et al., 1993; Brabant et al., 1996; Kiger et al., 2007). The resulting wing is predominantly two layers of cuticle cemented together by ECM. These studies point out the importance of regulating the adhesive properties of the wing epidermal cells by modulating the activity of integrins and their intracellular and extracellular binding partners. One mode of regulation is at the transcriptional level and several studies have demonstrated that the hormone 20E plays an important role in regulating at least some of these morphogenetic events including integrin expression levels (Fristrom et al., 1993; D'Avino and Thummel, 2000). Consistent with *tenectin*'s role in wing morphogenesis we find that during metamorphosis *tenectin* mRNA expression correlates with the ecdysone titer profile. *In vitro*, imaginal disc

Table 4
Rescue of *tenectin* overexpression.

Genotype	Lethality	WD
+/*Act*-GAL4; EY16369/+	92% (437)	66% (33)
tnc-IR1b/*Act*-GAL4	35% (145)	10% (462)
tnc-IR1b/*Act*-GAL4; EY16369/+	23% (368)	6% (283)
mys^{nj42}/Y; *Act*-GAL4/+	24% (156)	ND
mys^{nj42}/Y; *Act*-GAL4/+; EY16369/+	25% (201)	ND

WD; wing defects, ND; not determined.
Number of flies examined (*n* =) is given in parentheses.

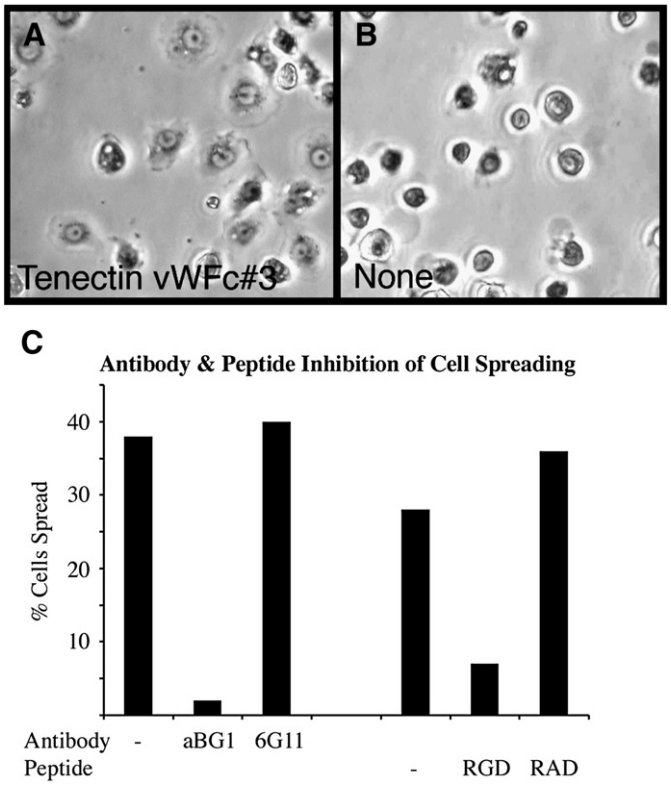


Fig. 8. Tenectin supports PS2 integrin-mediated cell spreading. (A) Phase-contrast microscopy of cells transformed to express PS2m8 integrins cells spreading on tenectin VWC#3. (B) In the absence of ligand the cells remain round and unspread. The parental S2/M3 cells, which do not express PS2 integrins, show no spreading on tenectin VWC#3 (not shown). (C) The anti-βPS integrin function blocking antibody aBG1 inhibits cells spreading on tenectin VWC#3 while the control anti-βPS integrin CF.6G11 has no effect. Both antibodies were purified and used at a concentration of 15 μg/ml. 1.5 mg/ml of an integrin inhibitory peptide GRGDSP (RGD) inhibits cell spreading on tenectin VWC#3 while the same concentration of a control peptide GRADSP (RAD) has no effect.

cultures demonstrate that *tenectin* is a 20E target gene. The comparison of the developmental *tenectin* expression profile with those of early (*E74A*, *E74B*) and prepupal (*β-Ftz-F1*) genes defined more precisely the temporal expression pattern of *tenectin*. *E74B* is a class I transcript, induced in mid-third instar larvae in response to a low concentration of 20E and repressed at higher ecdysone concentrations. In contrast, the class II transcripts, including *E74A*, are induced by high 20E concentration and their expressions are unaffected by higher 20E concentrations (Karim and Thummel, 1992). The temporal profile of *tenectin* is similar to those of *E74A*, with a slight delay in the peak levels of *tenectin* mRNA accumulation. This temporal delay in *tenectin* is similar to the delay observed in the early-late gene profiles. The early-late genes appear to share properties with both the early genes and late genes (Stone and Thummel, 1993). Early-late genes respond directly to ecdysone even in the presence of protein synthesis inhibitors like cycloheximide but unlike early genes their full induction requires protein synthesis due to a requirement for other ecdysone induced gene products. We propose that *tenectin* is an early-late gene as its expression in cultured larval organs was induced by 20E in the presence of cycloheximide but maximal induction required protein synthesis. In the wing, we propose that 20E also regulates morphogenesis by regulation of *tenectin* mRNA levels, suggesting that ecdysone controls wing morphogenesis and cell adhesion not only by regulating integrin expression but also their ECM ligand expression. Just as E74A and E75B do not display identical expression profiles, the tenectin expression pattern is complicated and likely involves additional modes of regulation that will need to be elucidated.

Tenectin and PS2 integrins are required for male genital disc rotation

Tenectin knockdown resulted in reduced rotation of male genitalia. Looping morphogenesis of the male genitalia occurs during the pupal stage as the genital disc undergoes a 360° dextral (clockwise) looping around the hindgut (Gleichauf, 1936). A variety of genes expressed in larval posterior abdominal segments A8, A9 and A10 have been identified that affect male genital rotation. These include genes encoding a signaling protein (*Pvf1*), a transcription factor (*Taf1*, formerly *TAF250*), and a pro-apoptosis gene (*hid*) (Casanova et al., 1986; Sanchez-Herrero and Crosby, 1988; Macías et al., 2004; Wassarman et al., 2000; Abbott and Lengyel, 1991; Grether et al., 1995). One adhesion molecule, fasciclin-2, was genetically demonstrated to be involved in genital rotation. However, the effect was indirect as *Fas2^{spin}* mutant alters the synapses connecting neurosecretory cells to the organ that produces juvenile hormone (the corpora

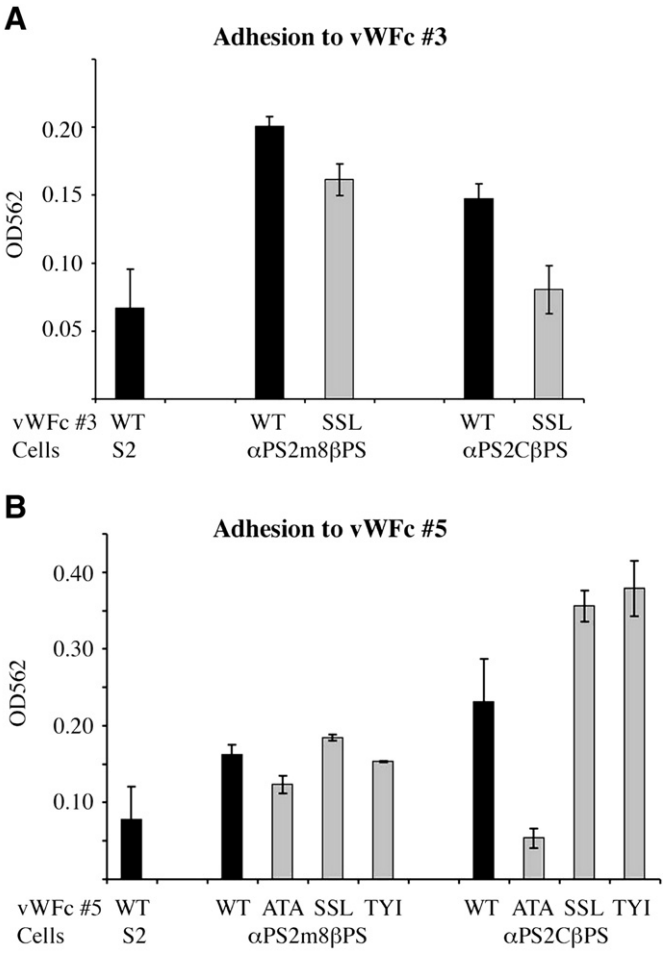


Fig. 9. Tenectin VFC#3 andVFC#5 support PS2-mediated cell adhesion. Untransformed (S2) cells or the same expressing PS2m8 (αPS2m8βPS) and PS2c (αPS2cβPS) integrins were allowed to adhere to tenectin VWC#3 (A) or VFC#5 (B). The tenectin fusion proteins used were either wild type (WT) or the same whose potential integrin-binding motifs had been mutated; RGD or RSD>SSL (SSL in A and B respectively); RDD>ATA (ATA); RYE>TYI (TYI). Adhesion was defined by the number of cells remaining attached after washing, 20 min after settling on the plate. The number of cells was determined by staining the cells with crystal violet and dye levels were determined using a microplate reader. To obtain tenectin dependent adhesion values, background adhesion observed in wells coated with BSA was subtracted. Three wells were scored for each ligand and the values are the mean ± s.e.m. of these three values. Differences between S2 cells and PS2m8 cells on VWF#3, between both PS2m8 cells and PS2c cells on VWF#3 wild type versus RGD>SSL, and PS2c cells on wild type VWF#5 versus RDD>ATA were significant ($P<0.05$). Consistent, but less dramatic, were the differences between PS2c cells and S2 cells on VWF#3 ($P=0.06$), PS2c cells and S2 cells on VWF#5 ($P=0.10$), PS2m8 cells on wild type VWF#5 versus RDD>ATA ($P=0.09$), PS2m8 cells and S2 cells on VWF#5 ($P=0.13$).

allata), and genitalia under-rotation is due to an excess of juvenile hormone (Ádám et al., 2003). These authors have demonstrated that the effects on genitalia rotation are mediated by an excess of juvenile hormone, a retinoic-like molecule, establishing a parallel between vertebrate and invertebrate left right asymmetry, since the retinoic acid is involved in the control of asymmetry in vertebrates (Spéder et al., 2007 for review). In *Drosophila*, excessive juvenile hormone may result in the attenuation of ecdysone regulated processes required for male genital rotation as mutations in *Broad-Complex*, an ecdysone early-response gene, also result in malrotation of male genitalia (Wilson et al., 2006). Mutations of the unconventional *myosin* 31DF gene (*Myo*31DF) have been shown to uniquely reverse the looping direction of genitalia (Hozumi et al., 2006; Spéder et al., 2006). Knockdown of tenectin in imaginal discs, but not in neuronal cells, resulted in incomplete rotation of the genitalia but not in direction of looping. Thus, we have for the first time identified a *Drosophila* ECM component required for genital looping morphogenesis.

The *tenectin* mutant phenotype in male genitalia prompted us to re-examine integrin hypomorphic mutations for a similar phenotype. Males bearing 3 different hypomorphic mutations in the gene encoding the βPS integrin subunit, mys^{b13}, mys^{b47}, and mys^{b69} (Jannuzi et al., 2004) displayed under-rotated male genitalia when raised at elevated temperatures. Deak et al. (1982) also described a mutation that was likely in *myospheroid* that produced under-rotated male genitalia when larvae and pupae were raised at elevated temperatures. Combining mys^{b13} with the if^{3} mutation in the gene encoding the αPS2 integrin subunit caused a dramatic increase in the expressivity of the rotated genitalia phenotype. Therefore, tenectin's proposed cell surface adhesion receptor is also required for the execution of looping morphogenesis. In addition to adhesion, the PS integrins function in the regulation of intracellular signaling pathways and specifically the JNK pathway (Lee et al., 2003; James et al., 2007). JNK signaling pathway has also been suggested to function in apoptosis required for rotation of male genitalia (Macías et al., 2004). Thus, tenectin and PS integrin function in looping morphogenesis could be at the level of adhesion and/or signaling. Additional experiments are required to distinguish between these two models.

Tenectin has multiple integrin-binding motifs

Tenectin's RGD sequence in the 3rd VWC domain is conserved in the beetle homolog, tenebrin, and supported PS2 integrin-mediated cell spreading. This result is expected given that RGD is a well known integrin-binding motif of the PS2 integrins. More novel is the presence in the identical location in the 5th VWC of the sequence RSD and elsewhere in this 5th repeat the occurrence of RDD and RYE sequences. The biological importance of the 5th VWC domain is supported by the extraordinary high degree of conservation in this domain between *Drosophila* tenectin and *Tenebrio* tenebrin. The two proteins share 92% (62/67) sequence identity in the 5th VWC repeat and this includes the RDD, RSD, and RYE sequences. To date, this domain is found conserved, with greater than 84% sequence identity, in mosquitoes, honey bees, crickets, wasps, the beetle, and aphids (not shown). While RGD is the best studied integrin-binding motif, experimental evidence is accumulating that variants of this sequence are also important. These variants include KQAGD, KGD, RSD, WGD, MVD and RYD found in fibrinogen, thrombospondin, tenascin-W, CD40, snake venom disintegrins, viral coat proteins, and ligand mimetic monoclonal antibodies (Springer et al., 2008; Subramanian et al., 2007; Meloty-Kapella et al., 2008; Prasad et al., 2003; Juárez et al., 2008; Van de Walle et al, 2008; Taub et al., 1989; Tomiyama et al., 1992; Hamidpour et al., 2006). Our cell adhesion assays demonstrate that VWC#5 as well as VWC#3 promotes cell adhesion mediated by PS2 integrins. Mutations of the individual RGD-variant motifs in VWF#5 suggest that they have differing effects on different integrins. The RDD is required for strong adhesion by both the PS2m8 and PS2c integrin isoforms as mutation of this sequence reduced adhesion of cells expressing either integrin. To our knowledge, this is the first time the RDD tripeptide in an ECM protein has been found to function in integrin-mediated adhesion. It also appears that the RSD and RYE motifs may be inhibitory for adhesion mediated by the PS2c isoform as their mutations increased cell adhesion. With multiple integrin-binding domains, both positive and inhibitory, tenectin potentially functions in multiple processes in development and specifically in metamorphosis.

Future experiments will be required to address the many unanswered issues regarding tenectin–PS integrin interactions including: which PS integrin(s) interact with tenectin in vivo; how the function of the motifs may be affected by the context of other ECM proteins; and how other regions of tenectin and modifications, such as glycosylation or cleavage, influence the functionality of the putative integrin-binding motifs. The presence of multiple motifs also raises the possibility that tenectin can bridge integrins on neighboring cells, or on the surface of the same cell. Finally, the different motifs may be needed to bind different integrins at different times in development and this binding of different motifs may have different adhesive and/or signaling consequences.

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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.ydbio.2010.02.008.

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