Tweedle proteins form extracellular two-dimensional structures defining body and cell shape in Drosophila melanogaster

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Tissue function and shape rely on the organization of the extracellular matrix (ECM) produced by the respective cells. Our understanding of the underlying molecular mechanisms is limited. Here, we show that extracellular Tweedle (Twdl) proteins in the fruit fly Drosophila melanogaster form two adjacent two-dimensional sheets underneath the cuticle surface and above a distinct layer of dityrosinylated and probably elastic proteins enwrapping the whole body. Dominant mutations in twdl genes cause ectopic spherical aggregation of Twdl proteins that recruit dityrosinylated proteins at their periphery within lower cuticle regions. These aggregates perturb parallel ridges at the surface of epidermal cells that have been demonstrated to be crucial for body shaping. In one scenario, hence, this disorientation of epidermal ridges may explain the squatty phenotype of Twdl mutant larvae. In an alternative scenario, this phenotype may be due to the depletion of the dityrosinylated and elastic layer, and the consequent weakening of cuticle resistance against the internal hydrostatic pressure. According to Barrlow’s formula describing the distribution of internal pressure forces in pipes in dependence of pipe wall material properties, it follows that this reduction in turn causes lateral expansion at the expense of the antero-posterior elongation of the body.

1. Introduction

Extracellular matrices (ECM) contribute to the geometry and consistency of cells and tissues. Generally, the role of an ECM depends on its components and their interactions that ultimately define its organization. Cartilaginous tissues, for instance, consist of a random distribution of the extracellular polysaccharide hyaluronic acid and associated proteins, including collagen and aggrecan produced and secreted by embedded chondrocytes [1–4]. Tension applied on this kind of tissues in concert with swelling forces entail the arrangement of the components and, finally, the shape of the tissue.

A less well-studied ECM is the cuticle of insects that outlines the shape of the organism. In some body regions such as the head capsule of caterpillars or the protective elytra of adult beetles, the hardness of the exoskeleton is sufficient to sustain the required shape. In some other body regions such as the ventral abdomen of many adult insects or the larval body, the respective shape does not only depend on the exoskeleton but involves the inner hydrostatic pressure. Despite these differences the principal organization of the cuticle is well conserved in different body regions and among species. The prototype of the cuticle consists of three composite horizontal layers: the outermost envelope, the middle
epicuticle and the inner procuticle [5,6]. Which factors account for the physical differences of the cuticle in different body regions is a matter of current research.

Like cartilaginous tissues, the procuticle is composed of an extracellular polysaccharide, namely chitin and associated proteins. In the last few years, data accumulated underlining that the proteins binding to chitin and their partners together specify the physical properties of the cuticle. In the elytral procuticle of the red flour beetle Tribolium castaneum, for instance, the chitin-binding proteins Cpr27 and Cpr18 associate with chitin and interact with the cuticle protein CP30 probably over covalent N-β-alamyl-dopamine (NBAD) bridges that are catalysed by the phenoloxidase TmLaccase2 [7,8]. This arrangement correlates with the stiffness of the elytral cuticle. In the fruit fly Drosophila melanogaster, proteins such as the chitin-binding and elastic protein Resilin in the contact region between the procuticle and the epicuticle are cross-linked to each other via dityrosine bonds [9,10]. The formation of this sub-layer involves the C-type lectin Schlaff (Slf) and a yet unknown peroxidase. The dityrosine sub-layer may be important for cuticle elasticity and thereby contributes to its shape.

In order to deepen our understanding of the mechanisms of cuticle ECM organization, we have studied the function of several members of a class of cuticle proteins named Tweedle (Twdl) in the fruit fly D. melanogaster [11]. Twdl proteins are characterized by an N-terminal signal peptide and a domain with four conserved blocks (DUF243), but do not display any homology to any other entry in protein databases. Deletions of stretches of amino acids of these domains provoke body shape changes in larvae and adult flies. In brief, we show that Twdl proteins localize to the epicuticle and the epicuticle are cross-linked to each other via dityrosine bonds [9,10]. The formation of this sub-layer involves the C-type lectin Schlaff (Slf) and a yet unknown peroxidase. The dityrosine sub-layer may be important for cuticle elasticity and thereby contributes to its shape.

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2. Results

2.1. Twdl proteins are expressed at different time points during development

To study the cellular function of Twdl proteins, we first determined the expression pattern of transgenic flies expressing fluorescent-tagged versions of the candidates TwdlA-GFP (Tb-GFP), TwdlD-dsRed, TwdlF-dsRed and TwdlS-GFP under the control of their endogenous promoters (figure 1; see also ‘Material and methods’).

In the early larva (L1), the expression of Tb-GFP was patchy in the epidermis. During later larval stages (L2 and L3), Tb-GFP was found in the whole epidermis. The TwdlS-GFP signal was barely visible in the cuticle of L1 and L2 larvae and became more intense in the whole cuticle of L3 larvae. TwdlF-dsRed was localized in the whole cuticle at all three larval stages. TwdlD-dsRed was expressed in the epidermis during the first two larval stages, but was excluded from the segmental grooves. At the third larval stage, only a faint TwdlD-dsRed signal at the posterior part was detected.

In summary, Twdl proteins are expressed at different time points during development in different, partially overlapping regions of the epidermis.

2.2. Twdl proteins mark the epicuticle

In order to determine the sub-cuticular localization of Twdl proteins, we analysed the distribution of the three fluorescent-tagged Twdl proteins (i.e. TwdlS-GFP, TwdlF-dsRed and Tb-GFP) in the cuticle of live L3 larvae by confocal microscopy (figures 2–4). We used the auto-fluorescence of the cuticle surface excited with a 405 nm laser that represents the envelope [12] and the tagged procuticle markers Cuticle Protein RdR &R 67Fa1 (Cpr67Fa1-dsRed) and Vermiform (Verm-RFP) as landmarks. GFP-conjugated TwdlS was located between the envelope and the broad Cpr67Fa1-dsRed (figure 2a–d”) or Verm-RFP (figure 2d”) region marking the procuticle. When the optical cross-section was perfect, TwdlF-dsRed was localized in a layer above the TwdlS-GFP with partially overlapping signal, but below the envelope (figure 3a). Due to living larvae, a perfect optical cross-section was not always possible (figure 2a). Tb-GFP, by contrast, always overlapped with TwdlF-dsRed (figure 4a). We conclude that these Twdl proteins belong to a region between the envelope and the procuticle (i.e. the epicuticle possibly subdividing it into distinct horizontal domains).

To test this conclusion, we monitored the expression of TwdlF-dsRed in embryos deficient for ecdysone that we had previously shown to be necessary for epicuticle differentiation [13]. In wild-type embryos, TwdlF-dsRed was detected in the entire larval cuticle (figure 5). In embryos mutant for phantom (phm) that codes for a P450 enzyme acting in the ecdysone biosynthesis pathway, TwdlF-dsRed was hardly expressed. Sporadically, dots of an RFP signal were found within epidermal cells. This finding is consistent with our conclusion that Twdl proteins localize to the epicuticle. Moreover, this result also indicates that activation of the twdlF promoter depends on ecdysone signalling.

2.3. Twdl proteins bind selectively to aggregates formed in Tb mutant larvae

To unravel the role of Twdl proteins in epicuticle formation and structure, we analysed the distribution of the tagged Twdl proteins in Tb mutant, i.e. Tb1 and Tb3/3 larvae. In Tb1 larvae, TwdlS-GFP (figure 2b–e”), Tb-GFP (figure 2b–e”) and TwdlF-dsRed (figure 3b–e”) were partially localized correctly in the epicuticle and partially aggregated in lower regions of the cuticle. In the cuticle of Tb3/3 larvae, the signal of aggregated TwdlS-GFP (figures 2c–e”, 3f–e”) and Tb-GFP (figure 4c–e”) was weaker compared with the signal in the Tb1 mutant larvae, while TwdlF-dsRed (figure 3c–e”) did not associate with the aggregates.

In order to find out whether the origin of the aggregates may be a mutated Tb protein, we compared the distributions of non-mutated, GFP-tagged and mutated, RFP-tagged versions of Tb, Tb-GFP and Tb-RFP [14], respectively, in the cuticle of live L3 larvae. Tb-RFP formed aggregates in the cuticle of these larvae (figure 4d,e). Co-expressed
Tb-GFP was recruited to these aggregates (figure 4d–d”). To test whether other Twdl proteins might be part of the Tb1-RFP aggregates, we co-expressed Tb1-RFP with TwdlS-GFP. The TwdlS-GFP signal overlapped with the signal of the Tb1-RFP aggregates (figure 4e–e”). We conclude that mutated Tb forms aggregates within the cuticle, which are able to recruit non-mutated Twdl proteins including Tb itself, TwdlD, TwdlF and TwdlS.

2.4. Tb93 is a twdlL allele

The differences in the localization of TwdlF-dsRed, TwdlS-GFP and Tb-GFP in both Tb1 and Tb93 alleles suggest that this discrepancy may be a consequence of different mutations in the Tb gene. Indeed, the phenotypes caused by these two alleles differ also between the larval stages. Tb1 larvae start to show the squat phenotype at the L2 larval stage, while Tb93 do so already at L1 (electronic supplementary material, figure S1). The Tb1 allele carries a deletion removing residues 167–190 of the protein including 18 amino acids within the DUF 243 domain [11]. Sequencing of the Tb gene in the Tb93 genome revealed no changes in the Tb protein sequence (data not shown). We reckoned, therefore, that Tb93 is not an allele of Tb. To test this notion, we sought to recombine the Tb1 and Tb93 alleles on one chromosome arguing that recombination would underline that these mutations affect different loci. For this purpose, in a population issued from a cross of Tb1 and Tb93 heterozygous flies segregating both mutations in trans, we screened for larvae that did not show the squat phenotype. We isolated four wild-type larvae in a population of 5860 larvae suggesting that the mutations affect different loci and that these loci are roughly 0.1 cM apart. Based on this result, we sequenced other twdl genes in the twdl cluster on the right arm of chromosome 3 in Tb93 animals in order to identify the mutation responsible for the phenotype. Indeed, we found a missense mutation in the sequence coding for a conserved amino acid of the DUF 243 domain in another Twdl gene, TwdlL (electronic supplementary material, figure S2). In order to confirm that the Tb93 phenotype is caused by the mutation in the TwdlL gene, we expressed a hairpin RNA directed against the twdlL gene in the background of Tb93. The hairpin RNA abolished the squat phenotypes of Tb93 larvae and pupae. By contrast, hairpin RNA against TwdlL did not cause any changes in the phenotype of Tb1 animals. As a control, we also tested several hairpin RNAs against other twdl genes in the Tb93 background (table 1). No phenotype changes were observed in any of these crosses. We conclude that Tb93 is a dominant allele of Tb.
Figure 2. Localization of TwdlS-GFP, Cpr67Fa1-RFP and Verm-RFP in the cuticle of wild-type, Tb1 and Tb93 third instar larvae. (a) In an optical cross-section of the cuticle of wild-type larvae, TwdlS-GFP (green, arrow) is uniformly distributed forming a thin layer under the blue 405 nm-induced auto-fluorescent line that represents the envelope (405AF). dsRed-tagged chitin-binding Cpr67Fa1 (Cpr67Fa1-dsRed, red, triangle), expressed in the epidermis, is plainly distributed in the thick procuticle underneath the TwdlS-GFP layer. (b)–(d') show top views with separated channels. (b) In the cuticle of Tb1 larvae (b–b'), Tb93 larvae (c–c'), TwdlS-GFP partially localizes to the upper epicuticle (arrow) and partially forms aggregates immersed in the procuticle marked by Cpr67Fa1-dsRed (triangle). The upper TwdlS-GFP layer does not overlap with the Cpr67Fa1-dsRed signal (b–b', c–c'). (d) Ubiquitously expressed Vermiform-RFP (Verm-RFP, red, triangle) is plainly distributed in the entire procuticle below the TwdlS-GFP layer (arrow) and forms vesicle-like structures in the cells (*). (d')–(f') show top views with separated channels. In the cuticle of Tb1 larvae (e–e') and Tb93 larvae (f–f'), TwdlS-GFP partially localizes to the upper epicuticle (arrow), and partially forms aggregates immersed in the procuticle (triangle). It does not overlap with the Verm-RFP signal (e–e', f–f').
the twdl gene (henceforth TwdlL93). Taken together, Tb and TwdlL are both needed for correct epicuticle formation.

2.5. Twdl aggregates constitute an ectopic epicuticle immersed in the procuticle

The aggregates visible on optical cross-sections of the cuticle shown in figures 2–4 do not localize to the expected region of the epicuticle just below the surface but scattered along the z-axis of the cuticle. To precisely localize these aggregates, we analysed the ultrastructure of the Tb1 and TwdlL93 larval cuticle by transmission electron microscopy (figure 6). We observed electron-dense aggregates immersed within the procuticle. These aggregates were absent in the cuticle of wild-type larvae.

The interpretation that Twdl aggregates localize to the procuticle is supported by localization experiments with TwdlS-GFP and the tagged chitin-binding, procuticular proteins Cpr6/Fa1-RFP and Verm-RFP co-expressed in Tb1 (figure 7a–d) and TwdlL93 (figure 7e–f) mutant larvae. TwdlS-GFP aggregates were embedded in a region marked by Cpr6/Fa1-RFP and Verm-RFP. Taken together, we conclude that Twdl proteins form an ectopic epicuticle within the procuticle of Tb1 and TwdlL93 larvae recruiting non-mutated Twdl proteins (i.e. TwdlS-GFP) from the epicuticle.

2.6. Distribution of Twdls in hairs differs from their distribution in the naked cuticle

Separation of the pro- and epicuticle in bristles and hairs in the D. melanogaster larvae is less pronounced than in the naked cuticle [15]. For this reason, we had a closer look at the distribution of Twdl proteins in the dorsal hairs in third instar larvae. The organization of the epicuticle in dorsal hairs of wild-type larvae was similar to the naked cuticle: Twdl proteins form adjacent layers under the blue autofluorescent layer (figure 7a–d; electronic supplementary material, figure S3). The procuticle was located in the middle of a hair and reached its tip. In the Tb1 and TwdlL93 mutant background, TwdlS-GFP (figure 7e–f), TwdlF-dsRed (figure 7g–h) and Tb-GFP (figure 7i–j) accumulated at the tips of the hair. The procuticle marked with
Cpr67-dsRed (figure 7a–d') and Verm-RFP (figure 7b–b'') still remained in the middle of the hair, except for the tip, so that these two layers (Twdl and procuticle) did not overlap. Thus, these two layers (Twdl and procuticle) did not overlap. Thus, mutations in Tb$^1$ and TwdlL93 cause a lateral shift of Twdl protein localization from more basal sites of the hair to its tip.

Figure 4. Localization of Tb-GFP, TwdlS-GFP and TwdlF-dsRed in the cuticle of wild-type, Tb$^1$ and Tb$^{93}$ third instar larvae. (a) In the cuticle of wild-type larvae, Tb-GFP (green, arrow) and TwdlF-dsRed (red, triangle) overlap (a: lateral view, d′–d''': top views with separated channels). In the cuticle of Tb$^1$ larvae both proteins are partially aggregated (b–b''). In the cuticle of Tb$^{93}$ larvae Tb-GFP forms aggregates (triangle), while TwdlF-dsRed does not (c–c''). Aggregates are better visible in top views of the same larva (c'-c'''). In the cuticle of the larvae with two additional copies of RFP-tagged, mutated Tb protein (Tb1-RFP, red), the non-mutated Tb-GFP form (green) binds to the aggregates formed by Tb1-RFP (d–d''). TwdlS-GFP protein (green) joins the Tb1-RFP aggregates as well (e–e'').
2.7. Mislocalization of the dityrosine layer in Tb mutant larvae

Recently, we showed that the contact zone between the epicuticle and the procuticle is marked by dityrosinylated proteins in D. melanogaster larvae [10]. In the wild-type third instar one-day-old larvae, Resilin-Venus representing the dityrosinylated layer localized beneath the TwdlF-dsRed layer (figure 8a, b and electronic supplementary material, figure S4). In the Tb1 and TwdlL93 mutant larvae at the same stage, Resilin-Venus was attracted to the aggregates and encased them (figure 8a0, a00, b0, b00). In the dorsal hairs of the young wild-type third instar larvae just after moulting Resilin-Venus occupied the space below the TwdlF-dsRed layer in the dorsal hair (figure 8d), while in the hairs of the Tb1 and TwdlL93 mutant larvae, it accumulated also at the hair tips, where the aggregated Tweedle proteins occurred (figure 8d0, d00). In one-day-old third instar wild-type larvae localization of Resilin-Venus was unchanged compared with young third instar larvae (figure 8c), while in one-day-old Tb1 and TwdlL93 mutant larvae Resilin-Venus was not localized at the hair tips anymore but encased the Twdl aggregates at the hair tip (figure 8c0, c00). Thus, Twdl proteins dictate the localization and the shape of the dityrosinylated layer at the epicuticle–procuticle interface.

2.8. Non-cell autonomous localization of the aggregated Twdls in the cuticle

In our live localization experiments, we noted that, as reported, TwdlD-RFP was expressed in a striped pattern in wild-type larvae. Dorsal hairs were lacking TwdlD-RFP. In the cuticle of Tb1 and TwdlL93 larvae (electronic supplementary material, figure S5), the Twdl aggregates were visible in the areas of dorsal hairs including the tips of hairs. Two alternative scenarios may explain this observation. Either the expression pattern of twdlD was changed in Tb1 and TwdlL93 larvae, or, in addition to the vertical, the lateral mobility of the TwdlD within the cuticle was enhanced in these larvae. In order to distinguish between these two possibilities, we monitored the expression of a nuclear-binding RFP under the control of the TwdlD promoter (TwdlD > RFP-NLS) in wild-type, Tb1 and TwdlL93 larvae. In all three cases RFP-NLS was detected in a striped pattern in the epidermis of developing embryos and larvae. This finding indicates that there were no changes in the expression pattern of twdlD. Thus, Twdl proteins dictate the localization and the shape of the dityrosinylated layer at the epicuticle–procuticle interface.

2.9. Epidermal cell shape is altered in Twdl mutant larvae

Compared with wild-type larvae, Tb1 and TwdlL93 mutant larvae are wider, but shorter (electronic supplementary material, figure 5a, b).
Figure 6. In Tb mutant larvae, Twdl proteins aggregate in the procuticle. (a) The wild-type first instar larval cuticle consists of three composite horizontal layers: the envelope (env), the epicuticle (epi) and the procuticle (pro). (b) The cuticle of Tb1 mutant first instar larvae is stratified as the wild-type cuticle. Occasionally, electron-dense material is found in the procuticle (arrow). (c,d) At later stages, large electron-dense material accumulates within the procuticle in Tb1 and Tb93 mutant larvae. Electron micrographs. Scale bar, 500 nm.

2.10. Movement capabilities of the Tb1 and Twdl93 larvae are normal

An intact exoskeleton is a prerequisite for insect locomotion. To find out whether the altered body shape of Tb1 and Twdl93 larvae affects crawling efficiency, we measured the ratio of the body length in the most stretched to the most contracted state of young third instar wild-type and Tb1 and Twdl93 larvae (electronic supplementary material, figure S6). We observed there was no significant difference between the ratios of the wild-type and Tb1 and Twdl93 larvae. We also investigated whether the barrel-like larval shape limits the crawling capabilities of the Tb1 and Twdl93 larvae. For this purpose, we measured the height on which larvae formed pupae on the vial wall. We found that in all cases, the wild-type, Tb1 and Twdl93 homozygous larvae, the average pupariation height was comparable (electronic supplementary material, figure S8). Taken together, we conclude that the stiffness of the cuticle of the Tb1 and Twdl93 larvae and their movement efficiency are unchanged.

2.11. Basal cuticular ridges are disorganized in Tb1 and Twdl93 mutant larvae

The cuticular protein Obstructor-E (Obst-E) is needed for ridge formation at the interface between the procuticle and the apical plasma membrane of epidermal cells [16]. These ridges are missing in obst-E mutant larvae that by consequence do not contract during pupariation. The question is whether these ridges are altered in Tb1 and Twdl93 mutant larvae. We analysed the basal site of the procuticle in wild-type, Tb1 and Twdl93 late third instar larvae by atomic force microscopy (AFM). The procuticle of wild-type larvae formed long convexities along the antero-posterior axis of the larva (figure 9a). The inner cuticular surface of Tb1 and Twdl93 larvae formed convexities that were comparably flat with random orientation (figure 9d,e). Hence, Twdl proteins are needed for correct orientation of the basal procuticular ridges.

In confocal imaging experiments, we observed that the cuticular aggregates in Tb1 and Twdl93 larvae were in contact with the apical site of the epidermal cells (electronic supplementary material, figure S9). Thus, the effect of Twdl proteins on apical ridge formation is either direct or indirect.

2.12. Localization of the Obst-E does not depend on Twdl function

Both, Twdl and Obst-E proteins influence the formation of basal cuticular ridges. To test whether Twdl proteins may influence Obst-E localization and function, we monitored Obst-E-GFP localization in wild-type, Tb1 and Twdl93 late third instar larvae. In non-squat L3 larvae Obst-E-GFP was uniformly localized in the procuticle (figure 9b–d). In Tb1 and Twdl93 larvae Obst-E-GFP localization was unchanged and did not bind to the Twdl aggregates (figure 9e,f). We conclude that the localization of Obst-E does not depend on the function of Twdl proteins.

3. Discussion

ECMs and the ECM-producing cells adopt a concerted shape that is potentially important for tissue function.
Figure 7. (Caption overleaf.)
Figure 7. Localization of Twdl fluorescent proteins in the hairs of wild-type and Tb mutant larvae. In the dorsal hairs of wild-type larvae TwdlS-GFP (green) forms a layer under the auto-fluorescent line (blue) at the bases of the hairs but does not reach their tip (a,b). Cpr67Fa1-dsRed (a) and Verm-RFP (b) are localized in the centre of hairs (both red). In the hairs of Tb1 and Tb93 larvae, TwdlS-GFP accumulates at the tips and forms smaller aggregates in the middle of the hair, while Cpr67Fa1-dsRed and Verm-RFP are localized in the centre, excluding the TwdlS areas (d–d'; b–b'). TwdlF-dsRed (red) forms a layer between the auto-fluorescent layer and TwdlS-GFP layer (405AF) in the hairs of wild-type larvae (c). In the hairs of Pb1 larvae stratification under the envelope seems to be retained, while at the hair tip and in the aggregate proteins overlap (c'). In Pb93 larvae stratification is retained and only TwdlS-GFP is mislocalized (c''). Localization of Tb-GFP (green) in the hairs of wild-type, Tb1 and Tb93 larvae is similar to TwdlS-GFP (d–d'''); these larvae also expressed TwdlF-dsRed. Mutated Tb1-RFP (red) form attracts unmutated Tb-GFP (e, green) and TwdlS-GFP (f, green).

Figure 8. Mislocalization of the dityrosine layer in Tb mutant larvae. In the wild-type third instar one-day-old larvae Resilin-Venus (green) is plainly distributed in the whole cuticle (a, top view on the larval segment), while in the Pb1 (a') and Pb93 (a'') mutant larvae it is attracted to the aggregates and encircles them (red: TwdlF-dsRed, blue: 405 nm-induced auto-fluorescent envelope signal 405AF). In the optical cuticle cross-section of the wild-type one-day old larvae Resilin-Venus localizes just below the TwdlF-dsRed layer (b), while in Pb1 mutant larvae it predominantly surrounds the aggregates visualized by TwdlF-dsRed (b'). In the dorsal hairs of the young wild-type third instar larvae just after moulting Resilin-Venus forms a uniform layer just after the TwdlF-dsRed layer below the hair, while the hair core contains less signal (c). In the hairs of the respective Pb1 (c') and Pb93 (c'') mutants, Resilin-Venus appears to be normal. TwdlF-dsRed localizes in the presumptive hair procuticle. In one-day-old third instar wild-type larvae localization of Resilin-Venus is unchanged in comparison with the third instar larvae just after hatching (d), while in one-day-old Pb1 (d') and Pb93 (d'') larvae Resilin-Venus is not localized at the hair tip anymore. TwdlF-dsRed localization is unchanged compared with younger larvae.
The insect integument consisting of the epidermis and the apical cuticle, for instance, conceivably plays a key role in body shape determination. The two Twdl-class cuticle proteins Tb and TwdlD have been shown to be involved in this process in *D. melanogaster* [11].

### 3.1. Twdl proteins form two-dimensional sheets within the epicuticle

Live imaging experiments with fluorescent-tagged proteins using CLSM reveal that Tb, TwdlF and TwdlS proteins...

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**Figure 9.** The structure of procuticular ridges but not the localization of Obstructor-GFP is changed in Tb mutant larvae. The internal cuticular surface of the wild-type L3 larvae scanned by the atomic force microscope shows longitudinal ridges running parallel to the anterior–posterior axis (a). In Tb1 (a') and TwdlL93 mutants (a''), the structure of the ridges is disrupted, they run in different directions and seem to be flatter. GFP-tagged chitin-binding protein Obstructor-E (Obst-E-GFP, green) is plainly distributed in the whole procuticle of the naked cuticle (b: the top view of the dorsal cuticle; d: the lateral view; blue: auto-fluorescence of the external cuticular envelope, 405AF) and in the centre of the dorsal hairs of wild-type larvae (c). On the top view the cuticular ridges are discernible (b, marked with arrow). In the Tb1 (b'-d') and the Tb93 larvae (b''-d'') Obst-E-GFP is still plainly distributed in the procuticle, excluding the epicuticular aggregates.
form two two-dimensional adjacent horizontal sheets (i.e. the TwdlF and the Tb/TwdlS sheets underneath the surface and above the procuticle that is characterized by the presence of dityrosinylated proteins). This localization indicates that Twdl proteins possibly belong to the epicuticle. In agreement with this interpretation, in ecdysone biosynthesis mutants where the epicuticle is absent [13], twdl gene expression is strongly reduced. We also conclude that the uniform appearance of the epicuticle in electron micrographs probably does not reflect the stratified organization revealed by fluorescence CLSM. Thus, the epicuticle may be a more complex structure than supposed by mere ultrastructure analysis.

Dominant mutations in twdl genes provoke mislocalization of the mutated proteins and their accumulation as three-dimensional aggregates within the procuticle. Non-mutated Twdl proteins are incorporated into these structures. Based on these data we assume that Twdl proteins interact with each other. These observations argue that the mutated Twdl protein sequence loses its ability to form a flat two-dimensional ECM but recruits normal and mutated protein sequences to form ectopic three-dimensional aggregates without losing the ability to self-assemble. The recruitment, however, is selective (i.e. not all Twdl proteins tested are attracted to these aggregates). In addition, mutant Twdl proteins attract dityrosinylated proteins from the epicuticle–procuticle interface that had previously been shown to have an important role in cuticle organization and function [10,17]. This supports the notion that Twdl protein polymers are responsible for the formation and orientation of the adjacent dityrosine sub-layer including Reslin that is assumed to confer elasticity.

In summary, the epicuticle consists of polymers of Twdl proteins that partition this layer into two two-dimensional horizontal sheets by self-assembly.

3.2. Squat body shape as a consequence of body wall tension changes

Our data underline that Twdl proteins play a key role in body shape determination or maintenance. This function might rely on their localization and function within the cuticle. Alternatively, the dominant phenotype caused by Twdl mutations, however, suggests that the defects may be neomorphic (i.e. they might be unrelated to the normal function of these proteins). Together, three possible scenarios can explain the mechanism of Twdl protein function in body shape implementation: (1) the ‘cytoplasmic’, (2) ‘epidermal–cuticular interface’ and (3) ‘cuticle’ scenarios.

The ‘cytoplasmic’ theory relies on mutated Twdl proteins that fail to be transported to the cuticle but accumulate in cytoplasmic structures, probably vesicles, during cuticle formation when massive secretion and vesicle sorting occur [5,18]. Accumulation of Twdl-vesicles may perturb plasma membrane dynamics, and thereby cause loss of correct cell shape along the antero-posterior axis. The altered cell shape would, in turn, influence the body shape.

The alternative ‘epidermal–cuticular’ theory relies on the extracellular Twdl aggregates that are in close contact with the apical surface of the epidermal cells. According to this theory, these aggregates in the procuticle are responsible for the dis-organization of the regular ridges that run along the antero-posterior axis in the epidermal–cuticular interface [16]. These ridges have recently been shown to depend on the presence of the procuticular protein Obst-E that controls longitudinal contraction and lateral expansion of the L3 larvae during pupariation. The deletion of obst-E causes flattening of the ridges and the formation of longer and thinner pupae. In twdl mutant larvae, Obst-E localization appears to be normal. We, therefore, reckon that body shape changes in twdl mutant animals are independent of Obst-E function. As a consequence of ridge mis-orientation in twdl mutant larvae, however, the epidermal cells lose their longitudinal antero-posterior direction and adopt a shape with random orientation. Accordingly, the change of orientation preference of epidermal cells may be responsible for the overall shorter but thicker body shape.

The third, ‘cuticle’ theory considers that the depletion of either the epicuticle itself or the epicuticle–procuticle interface might be the reason for the aberrant body shape in twdl mutant animals. In this view, the Twdl polymers and/or the dityrosinylated proteins of the epicuticle–procuticle interface confer the elastic forces resisting the internal hydrostatic pressure. A thin epicuticle and/or procuticle–procuticle sub-layer may be insufficient to withstand these forces. According to the formula of Barlow, a weakened wall of a closed pipe or cylinder would allow radial rather than longitudinal expansion of the object (figure 10). In analogy, due to a weakened cuticle and assuming a normal hydrostatic pressure, twdl mutant larvae become thick and short. Epidermal cells would, in this scenario, passively follow cuticle stretching in the lateral direction.

In any case, body shape change in twdl mutant larvae does not affect locomotion efficiency. Importantly, the twdl mutant phenotype enables us to distinguish body shape and locomotion as distinct functions of the cuticle that are not necessarily linked.

3.3. Twdl evolution

The D. melanogaster genome encodes 27 twdl genes. Different combinations of the respective proteins in different body regions allow establishment of different types of epicuticles, thereby, conforming with our ‘cuticle’ theory; probably influencing the physical properties of the cuticle. So far so good. By contrast to D. melanogaster some insects such as the bedbug Cimex lectularius or the honeybee Apis mellifera do only have two or three copies of twdl in their genome [19]. How is epicuticle complexity that we encounter in D. melanogaster achieved in these species? We can only speculate that, testified by the varying number of twdl genes, the epicuticle is a fast-evolving structure and therefore reckon that other types of epicuticular proteins may contribute to its construction to accommodate its different functions in species with only a few twdl genes.

4. Material and methods

4.1. Fly husbandry

Flies were kept in cages with apple juice agar plates with yeast, from which embryos and larvae were collected. Embryonic stages were recognized according to the gut morphology described by Hartenstein & Campos-Ortega [20]. Homozygous mutants non-carrying any GFP- or YFP-constructs were identified from the rest of the embryos that were heterozygous or homozygous for the balancer chromosome expressing YFP or GFP (Dfd::YFP or Kr::GFP). Collected embryos were dechorionated in chlorine bleach diluted 1:1 in tap water, manually freed from the vitelline membrane or left
in the vitelline membrane, subsequently mounted in Voltalef 105 oil (VWR Chemicals) and observed by microscopy.

Transgenic flies harbouring constructs were generated by the BestGene Inc (USA) company. The constructs used are: knkpcpr6/Fa-rfp with the promoter of the knk gene [13] upstream of the coding region of cpr6/Fa fused to the open reading frame of rfp in pW8; TwdlD-RFP-NLS with the coding regions of twdlD and rfp-nls fused together and downstream of the twdlD promoter [11] in pW8, Tb1-GFP and TwdlS-GFP from the Flyfos library (electronic supplementary material, figure S10) [21], Tb1-RFP [14], TwdlF-dsRed and TwdlD-dsRed [11], UAS: Verm-RFP [22], UAS-Resilin-Venus [10] and Obst-E-GFP [16]. The Gal4 line to drive UAS:Verm-RFP and UAS-Resilin-Venus expression was daughterless:Gal4.

4.2. Molecular biology

In order to identify a mutation in TwdlL, gene standard PCR reaction and sequencing were performed. The primers used for the amplification and sequencing of the twdl gene are listed in electronic supplementary material, table S1.

4.3. Genetics

In order to downregulate transcript levels of twdl genes in a Tb101/Twdl103 background, the RNAi technique combined with the UAS:Gal4 system was applied. Flies carrying a UAS: RNAi construct coding for a hairpin RNA directed against a corresponding twdl transcript were crossed to flies harbouring ubiquitously or epidermally expressed Gal4 (daughterless:Gal4 or e22c:Gal4, respectively). The larval and pupal phenotypes of the progeny were observed. The RNAi lines used in this work are listed in table 1 and were purchased from the VDRC (Vienna, Austria).

For confirmation that Tb1 and Tbw103 mutations are not alleles of the same gene we performed a complementation test by crossing Tb1 and Tbw103 flies with each other, subsequently crossing daughters (F1 females) with wild-type males and counting the number of the wild-type and tubby looking pupae.

4.4. Microscopy and image preparation

For live imaging, larvae were anaesthetised with ether, mounted in halocarbon 700 or 50% glycerol and observed either by confocal laser scanning microscopy (CLSM, using Zeiss LSM 710, 780 and 880) or fluorescent binocular Leica M205 FA.

For imaging of the cuticle preparations, the larvae were mounted in Hoyer’s medium (30 g gum arabic, 50 ml distilled water, 200 g chloral hydrate, 20 g glycerol, mixed 1:1 with lactic acid), kept overnight at 65°C and observed on a binocular Leica M205 FA. Transmission electron microscopy was performed following our extensive protocol published in 2010 [23]. For examination of the cuticular ridges, third instar larvae were digested in Hoyer’s medium and their cuticles washed several times in distilled water. Afterwards they were stacked to the metal plate and their inner side was scanned by the atomic force microscope (Innova AFM, Bruker).

For figures’ preparation Adobe Photoshop CS3/CS6 and Adobe Illustrator CS4/CS6 software were used without changing initial microscope settings. For cell measurements, AxioVision Rel. 4.7 was used.

4.5. Body length measurements

Third instar larvae were placed on an agar plate and the movie of 10 steps (1 step = contraction and subsequent stretching) was made. Afterwards in every step the body in the most contracted and stretched state was measured and the difference between the shortest and the longest measurement of all 10 steps of 5 wild-type, homozygous Tb1 and Tbw103 larvae was counted and compared.

4.6. Movement measurements

In total, 100 third instar wild-type, homozygous Tb1 and Tbw103 larvae were placed into vials with agar food, 20 larvae of each kind in one vial. After pupariation of all larvae, the distance between the pupae on the vial wall and the food level was...
measured and the average of measurements of 20 larvae was determined. To compare the crawling efficiency between wild-type and \texttt{twdl} mutant larvae, five larvae of each genotype were filmed. The maximally stretched and contracted states of the body were determined and measured on images extracted from the movies.

Data accessibility. This article does not have additional data.

Authors’ contributions. R.Z., Y.W. and B.M. designed and performed experiments. N.G. performed experiments. R.Z., S.B. and B.M. analysed the data. B.M. supervised and designed the project. R.Z. and B.M. wrote the manuscript. All authors have approved the final version of the manuscript.

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