

Mechanical and genetics basis of cellularization and serosal window closure in Tribolium castaneum

Vazquez Faci, T.

Citation

Vazquez Faci, T. (2021, March 9). *Mechanical and genetics basis of cellularization and serosal window closure in Tribolium castaneum*. Retrieved from https://hdl.handle.net/1887/3147347

Version:	Publisher's Version
License:	<u>Licence agreement concerning inclusion of doctoral thesis in the</u> <u>Institutional Repository of the University of Leiden</u>
Downloaded from:	https://hdl.handle.net/1887/3147347

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <u>http://hdl.handle.net/1887/3147347</u> holds various files of this Leiden University dissertation.

Author: Vazquez Faci, T. Title: Mechanical and genetics basis of cellularization and serosal window closure in *Tribolium castaneum* Issue date: 2021-03-09

CHAPTER 4

Tribolium laminin is involved in closure of the serosal window.

Tania Vazquez Faci, Niels Pijnaker and Maurijn van der Zee.

Abstract

The serosa is a crucial extraembryonic epithelium in insects that protects the egg against desiccation and pathogens. As this epithelium is absent in the highly derived main insect model *Drosophila*, knowledge on genes regulating morphogenesis of the serosa is limited. Some transcription factors such as zerknüllt and dorsocross have been identified, but no involved structural molecules are known.

To identify such structural genes, we rescreened 17 potential cell adhesion molecules from the genome-wide *Tribolium* iBeetle screen for involvement in development of the serosa. We find that *Tribolium* laminin α is involved in closure of the serosal window towards the end of gastrulation. Furthermore, we show expression of Laminin protein in the necklace cells around the serosal window during closure. Using live imaging movies, we demonstrate that simultaneous knock down of laminin β and γ results in aberrant and delayed closure of the serosal window towards the end of gastrulation.

Laminin is the first structural molecule now reported to be involved in morphogenesis of the serosa. This study suggests that a basal lamina is involved in closure of the serosal window. As the observed closure defects are similar to the phenotype reported after *dorsocross* knock-down, it is likely that laminins are a target of the Dorsocross transcription factor during serosal window closure in *Tribolium*.

Introduction

Insect eggs possess two extraembryonic epithelia: an inner amnion that covers the embryo at the ventral side, and an outer serosa that completely envelops embryo and yolk (1, 2). These epithelia are an innovation of the insects and are not present in other arthropod groups such as crustaceans or myriapods (3, 4). The serosa secretes a cuticle (5, 6) and has been suggested to play a role in desiccation resistance of the egg (7–9). Furthermore, an immune function has been proposed (10, 11). Both functions have been experimentally demonstrated using *zerknüllt* RNAimediated deletion of the serosa in the beetle *Tribolium castaneum* (12, 13). Serosa-less *Tribolium* eggs desiccate at low relative humidity and do not upregulate an immune response upon infection (12, 13). Thus, the extraembryonic epithelia are of crucial importance in insects.

In the main insect model *Drosophila melanogaster*, the two extraembryonic epithelia have been strongly reduced to a single small amnioserosa that covers the yolk dorsally (14). Its development has been extensively studied. The amnioserosa develops from a small rim of dorsal-most blastodermal cells that express the Hox gene *zerknüllt* (*zen*) (15–17). In *zen* null mutants, the amnioserosa is absent, and germband extension is severely compromised (18). To maintain amnioserosal cell fate, the U-shaped transcription factors are required (19). Loss of the U-shaped gene *hindsight* or all three copies of *dorsocross*, for instance, leads to failure of germ-band retraction (20, 21).Besides these transcription factors, the cell adhesion molecules Laminin and Integrin are required for germband retraction (22).

Finally, the amnioserosa is essential for dorsal closure in *Drosophila* (23). More than 140 genes have been described to be involved in this process (see Kiehart, Crawford, Aristotelous, Venakides, & Edwards, 2017). In the amnioserosa, a contractile actomyosin network (25, 26) regulated by Crumbs (27), Rho GTPases (28) and genes from the PAR complex (29) seems to be a main driving force. In addition, cell adhesion molecules such as DE-cadherin, integrins and Innexin3 have been shown to be required for dorsal closure (30–33).

Despite this massive amount of work on the amnioserosa in Drosophila, surprisingly little attention has been paid to the development of the serosa in other insects (2, 34). The serosa differentiates from the germ anlage at the blastoderm stage (35). In most hemimetabolous insects, the embryo then invaginates into the yolk leaving the serosa to fully cover the yolk (anatrepsis, see Panfilio, 2008 for review). During later katatrepsis, the amnion and serosa fuse, then the serosa retracts, and finally the embryo emerges from the yolk (36). Final dorsal closure follows and is mainly driven by the amnion (37). In many holometabolous insects, such as the beetle Tribolium castaneum, the serosa folds over the germ rudiment during gastrulation. The folds progress to form a serosal window which eventually closes, resulting in an amnion that covers the embryo ventrally and a serosa that completely covers the yolk (38). To achieve dorsal closure, the amnion and serosa attach to each other under the head and rupture. The serosa then retracts together with the amnion to the dorsal side of the egg (39).

A limited number of studies have addressed genes involved in these morphogenetic movements. Zerknüllt (zen) is known to specify serosal cell fate in the flies *Megaselia abdita* and *Episyrphus balteatus* and in the beetle *Tribolium castaneum* (40–42). Furthermore, zen is required to open the amniotic cavity for dorsal closure in *Tribolium* and in the milkweed bug *Oncopeltus fasciatus*. RNAi leads to a completely everted (inside-out) topology of the embryo (42, 43). The U-shaped genes *dorsocross* and *hindsight* are involved in closure of the serosal window in *Tribolium* (44), and depletion of *Folded gastrulation* (Fog) signaling completely blocks all morphogenetic movements (45). Besides these important signaling pathways and transcription factors, no structural molecules have been identified in gastrulation. One exception is Integrin that was recently shown to anchor the anterior-ventral of the germ anlage to the surrounding vitelline membrane to counteract the contractile forces needed for morphogenetic movement of the serosa (46).

Here, we aim to expand our knowledge on structural molecules involved in the morphogenesis of the serosa. From a genome-wide RNAi screen in *Tribolium*, we rescreened 17 genes that were reported to show wing blisters in pupae or adults upon RNAi (47, 48). Wing blisters are indicative of cell adhesion defects, and this phenotype maps to mutations in morphogenetically relevant cell adhesion molecules such as Laminin or

Integrin in *Drosophila* (49–51). We rescreened these 17 wing blisterproducing dsRNAs for defects in the early morphogenesis of the amnion and serosa in *Tribolium*. We find that Laminin is involved in closure of the serosal window towards the end of gastrulation.

Laminins are key components of the extracellular matrix in the basement membrane of all animal epithelia (52, 53). They function as trimeric glycoproteins, consisting of one α , one β and one γ subunit, and form networks that connect to the membrane receptors of epithelial cells (54, 55). Although the human genome contains 5 different α , 4 different β and 3 different γ subunits, practically all invertebrates possess 4 Laminin genes: 2 distinct α subunits, called α 1,2 (orthologous to human laminin α 1 and α 2) and α 3,4 (orthologous to human α 3 and α 4), one β subunit, and one γ subunit (56, 57).

Mutations in *Drosophila* Laminin $\alpha 1, 2$ (called *wing blister, wb*) cause wing blisters and defects in and defects in the dorsal vessel, trachea, muscles and rhabdomeres (50). Null mutations of *Drosophila* Laminin $\alpha 3, 5$ (called *LamininA, LanA*) produce embryonic lethality with defects in somatic muscles, dorsal vessel and endoderm (58, 59). The $\alpha 3, 5$ chain is also required for localization of anterio-posterior markers in the oocyte and for pathfinding of axons in the brain (60, 61). Absence of *Drosophila* laminin β (*called Laminin B1, LanB1*) prevents the normal morphogenesis of most organs and tissues, including the gut, trachea, muscles and nervous system (62). Finally, null mutants of Drosophila Laminin γ (called *Laminin B2, LanB2*) produce a phenotype with reduced midgut regions where gaps appear in the endodermal layer. Laminin γ is essential for the proper organization and arrangement of visceral tissue (63). Importantly, laminins are involved in morphogenetic cell movements, such as germ band retraction (64, 65).

In our screen, we identify *Tribolium* laminin α 1,2 to be involved in proper morphogenesis of the serosa. In addition, we show presence of Laminin γ protein in the necklace cells around the serosal window. Using live imaging movies, we demonstrate that simultaneous knock down of laminin β and γ results in aberrant and delayed closure of the serosal window towards the end of gastrulation.

Materials and methods

Parental RNAi

Seventeen dsRNAs causing wing blisters (Table 1), and dsRNAs against laminin β (iB_08660) and laminin γ (iB_01705) were ordered from the company Eupheria, Germany. pRNAi in 40 female adults per gene was performed as described in M. v. d. Zee, Stockhammer, Fonseca, Levetzow, & Roth, 2006.

Table 1. Genes included in the parental RNAi screen. Their TC-number, the corresponding dsRNA ordered from Eupheria (iB number), and closest Drosophila melanogaster ortholog identified by protein blast are given in the columns, as well as the gene name and possible function as indicated on the NCNI web site <u>https://www.ncbi.nlm.nih.gov/gene/</u>

TC number	iB number	Fly ortholog	Gene Name	Protein function/prediction	
TC012762	iB_02017	CG32138	formin-like protein	actin binding; Rho GTPase binding	
TC012571	iB_05272	CG3403	MOB kinase activator-like 4	protein kinase binding	
TC014797	iB_05697	CG11940	pico-pico (Drosophila)	protein phosphatase 1 binding	
TC003474	iB_00573	CG9984	negative elongation factor D	mRNA binding	
TC007565	iB_01221	CG7392	striatin-3	protein binding	
TC001525	iB_00257	CG7183	protein coding gene	Unknown	
TC001765	iB_00300	CG11081	plexin A	14-3-3 protein binding; GTPase activator activity; heparin binding; protein binding	
TC010914	iB_01762	CG10295	serine/threonine- protein kinase PAK 3	protein serine/threonine kinase activity; Rac GTPase binding; Rho GTPase binding; SH3 domain binding	
TC016062	iB_02548	CG5734	uncharacterized LOC103314950	phosphatidylinositol binding	
TC013912	iB_05522	CG8787	polycomb protein Asx	chromatin binding; deubiquitinase activator activity	
TC014773	iB_05688	CG42677	laminin subunit alpha-1	EGF-like domain; Laminin G domain; Laminin EGF domain;	
TC000185	iB_00037	CG17838	heterogeneous nuclear ribonucleoprotein Q	mRNA binding; nucleotide binding	
TC003007	iB_00499	CG8174	SRSF protein kinase 1	ATP binding; protein kinase activity; protein serine/threonine kinase activity, positive regulation of cell cycle	
TC003342	iB_00557	CG10443	Lar Leukocyte antigen related like (Drosophila)	protein tyrosine phosphatase activity; SAM domain binding, regulation of cell projection organization	
TC004140	iB_00666	CG4931	cytoplasmic FMR1- interacting protein	Rho GTPase binding	
TC005546	iB_00881	CG5092	serine/threonine- protein kinase mTOR	macromolecular complex binding; protein kinase activity; protein serine/threonine kinase activity	
TC013882	iB_02218	CG7266	LIM domain- containing protein jub	peptide methionine sulphoxide reductase MsrA; Peptide methionine sulfoxide reductase	

Egg fixation

Eggs were dechorionized in undiluted commercial bleach (4% NaClO) for 4 minutes, and fixed for 20 minutes in 5 ml heptane, 4 ml PBS and 1ml 36% formaldehyde. Eggs were devitellinized using a methanol shock, and stored at -20 °C.

Immunohistochemistry and DAPI staining

The standard buffer used in all steps was PBS with 1% Triton, supplemented with 0.05% acetylated BSA. Blocking was done with BSA and NGS. The primary anti-Laminin γ 1(Abcam, ab47651) antibody was incubated overnight at 4 °C in a dilution of 1:500. The secondary antibody was goat anti-rabbit 488nm Alexa from Boehringer, Mannheim in a dilution of 1:100. DAPI was added 1:1000 to one of the last washes in PBST. DAPI stained embryos of the RNAi screen were imaged using an upright microscope equipped with epifluorescence (Zeiss Axioplan 2 imaging) with a 20X objective. Immunohistochemically stained embryos were imaged on an inverted Zeiss confocal (Zeiss 710). We took 85-200 focal planes with a 40X or 20X objective, and all the focal planes were summed to make one picture.

Western blot

From eight to fifty six hours old eggs that were laid in a period 3-7 days after dsRNA injection into the adults, and 8-72 h old eggs that were laid in a period 7-10 days after injection were homogenized in five times the egg volume of Laemmli buffer (ChemCruz), and were stored at -20 °C. 30 μ l of these samples was loaded on a 30% SDS-PAGE gel for western blotting next to comparable samples of wild type eggs, and 30 μ l of a 1 mg/ml alpha-tubulin solution as control. Primary antibodies used are anti-Laminin γ 1(Abcam, ab47651) in a concentration of 1:500 and anti- α -tubulin (Sigma, T5168) in a concentration of 1:5000. Secondary antibodies were anti-rabbit IgG (Boehringer Mannheim) in a dilution of 1:1000 and anti-mouse (NA931, GE Healthcare) in a concentration of 1:2000. Visualization was done with ECL Western blotting detection reagents (Clarity Western, Biorad).

Live imaging movies

From zero to two hours old eggs from the LAN-GFP transgenic line (chapter 2) were incubated for 6h at 30 °C. A 40 second incubation in bleach(4% NaClO) followed by a 1 minute wash in water was repeated twice to dechorionated the eggs. Eggs were then aligned on a microscope cover slip and covered with Voltalef 10S Halocarbon oil to avoid desiccation. The embryos were imaged on an inverted Zeiss confocal microscope (Zeiss 710) at 30° C. We took eleven focal planes with a 20X or 40X objective in a time interval of 3 minutes. We summed all the focal planes. The total observation time was 14 hours.

Statistical analysis of time for serosal window closure

We measured the time between the frame just before the onset of the differentiated blastoderm stage (i.e. after completion of cellularization) and the completion of serosal window closure in 7 wildtype embryos and in 16 embryos in which laminin β and γ were simultaneously silenced. The results of the two groups of embryos were compared applying a 1-tailed ANOVA.

Results

RNAi against TC014773 disturbs closure of the serosal window

To identify structural genes involved in the morphogenesis, we performed a small-scale parental RNAi screen against 17 genes that showed wing blisters in pupae or adults upon knockdown in the iBeetle screen (47, 48), Table 1. In several cases, RNAi caused a severe reduction in fecundity (<1.0 egg per female per day), indicated with red squares in Figure 1. Analysis of phenotypes in DAPI stained embryos revealed 15 categories of developmental defects, ranging from the undifferentiated blastoderm stage, to dorsal closure (see legend of Figure 1, and illustrated in supplementary Figures 1 - 14). Knockdown of TC007565, TC010914 and TC012571 caused a high percentage of embryos with irregularly spaced nuclei in the syncytial blastoderm (see supplementary Figure 1), possibly indicative of cellularization defects (chapter 3). Notably, knockdown of TC014773 disturbed closure of the serosal window in a considerable

percentage of eggs (Figure 2). Although the beginning of morphogenetic movements seems relatively normal (Figure 2 A, F), the serosal window seems larger during further development (Figure 2 B, G), and sometimes has irregular borders (Fig. 2 C, H). Importantly, closure of the serosal window seems delayed (Figure 2 C, H). Although the serosal window does finally proceed in most eggs (Figure 2 D, I, E, J), the germband is incorrectly positioned (more towards posterior), similar to what has been reported for affected serosal window closure in *dorsocross* RNAi (44). In conclusion, TC014773 RNAi disturbs serosal window closure.



Figure 1. Results of the RNAi screen. TC numbers of the genes against which RNAi was performed are indicated below the bars. Each bar represents the percentage of eggs showing the phenotype of a certain category indicated by the capital letter, see legend on next page. Under every TC-number, the fecundity is indicated as eggs per female per day. The red squares are around TC numbers whose knockdown lead to a fecundity below 1.0 eggs per female per day.

-nuclei irregularly spaced A -holes in the blastoderm B Differentiated blastoderm: C -condensation of the germ rudiment C disturbed C -irregularly positioned germ rudiment C Gastrulation: C	A B C D
-holes in the blastoderm B <u>Differentiated blastoderm</u> : -condensation of the germ rudiment disturbed -irregularly positioned germ rudiment D <u>Gastrulation</u> :	B C D
Differentiated blastoderm: -condensation of the germ rudiment disturbed -irregularly positioned germ rudiment distrulation:	C D
-condensation of the germ rudiment disturbed -irregularly positioned germ rudiment Gastrulation:	C D
disturbed -irregularly positioned germ rudiment Gastrulation:	D
-irregularly positioned germ rudiment D Gastrulation:	D
Gastrulation:	
Gastrulation:	
restriction distant of	
-gastrulation disturbed	E ,
-delayed serosal window formation F	F
-irregularly positioned germ rudiment G	G
Germ band stage	
-(irregularly) curved germband H	н
-irregularly positioned germ band	
-head lobe defects J	J
-border of the embryo irregular K	K
-flat/broad germ band	
<i>, , , ,</i>	
Late	
-germ band retraction defects	М
-dorsal closure defects	N
-Serosa nuclei irregular spaced	D



Figure 2. RNAi against TC014773 disturbs closure of the serosal window. (A-E) DAPI stainings after RNAi against TC014773. (F-J) Stills from a live imaging movie using LAN-GFP (chapter 2) showing the comparable wild type. (B) The serosal window seems larger than in wildtype. (C) Serosal window closure seems delayed, and the borders of the window are irregular. (D, E) Serosal window closure proceeds, but the position of the germ band is more toward posterior than in the wild type.

TC014773 is the laminin α 1,2 subunit

In NCBI and iBeetle databases, TC014773 is annotated as *laminin subunit* α *1,2*. Analysis of the protein domains using Prosite (67, 68) confirmed this annotation and unambiguously identified TC014773 as the *Tribolium* Laminin α 1,2 ortholog (Figure 3). Like in practically all invertebrates (57), we identified three other Laminin subunits in the *Tribolium* genome. All of them showed the highly conserved protein domains typical of the respective subunits. LOC661583, a fusion of the former gene models TC003460 and TC032383, is Laminin α 3,5; TC005184 is Laminin β , and TC010540 is Laminin γ (Figure 3).



Figure 7. Comparison of domains in Laminin subunits of Tribolium and Drosophila. Domains were identified using Prosite (67, 68). High conservation in protein architecture shows that TC014773 is Laminin α 1,2; LOC661583, a fusion of the former gene models TC003460 and TC032383, is Laminin α 3,5; TC005184 is Laminin β , and TC010540 is Laminin γ .

As laminin $\alpha 1,2$ is a subunit of the Laminin trimer, we wondered if knockdowns the other members of the trimer give the same phenotype. We therefore performed RNAi against laminin β , laminin γ , and a combination of the two, and analyzed the phenotypic effect during closure of the serosal window. Similar to laminin $\alpha 1,2$ RNAi, these knockdowns all generated high percentages of defects in closure of the serosal window (Table 2). This suggests that the phenotype we observe is indeed caused by the absence of functional Laminin trimers. Simultaneous RNAi against the β and γ subunits gives the highest percentage of defects in serosal window closure (Table 2).

TC NUMBER	Laminin subunit	% Serosal window closure defect
TC14773	Laminin α 1,2	58%
TC05184	Laminin β	44%
TC10540	Laminin y	30%
TC05184, TC10540	Laminin β and γ	60%

Table 2. percentage of embryos showing defects in serosal window closure upon RNAi against Laminin subunits

Laminin $\boldsymbol{\gamma}$ is enriched around necklace cells during closure of the serosal window

Although several antibodies are present against vertebrate Laminins, the only currently available antibody recognizing a *Drosophila* Laminin subunit is anti-Laminin $\gamma 1$ (Abcam, ab47651). To obtain an indication whether this antibody also recognizes the very similar *Tribolium* Laminin γ , we performed a Western blot (Figure 4). In wildtype samples, we observe a band around 180 kDa, the expected size of Laminin γ (Figure 4, 3rd and 6th lane). Importantly, this band was not present after laminin γ RNAi (4th and 5th lane). These data indicate that ab7651 recognizes *Tribolium* laminin γ , silencing the protein.



Figure 4. The ab7651 Drosophila laminin \gamma antibody recognizes Tribolium Laminin γ . The first lane shows a 10-180 kDa ladder; the second lane a blotting control of alpha-Tubulin. In the wildtype lanes (lane 3 and 6), a 180 kDa band is visible, whereas this band is absent after the RNAi samples in lane 4 and 5. Alpha tubulin was used as loading control.

We used this antibody to investigate expression pattern of laminin γ during closure of the serosal window. In wildtype embryos, we observe an enrichment of Laminin γ in the so-called "necklace cells", the cells at the border of the amnion and the serosa (69). A possibly extracellular enrichment of the protein is visible at the inner rim of the serosal window (Figure 5 A, arrowhead). This suggests that a basal lamina is present during closure of the serosal window. This expression pattern is absent after laminin γ knockdown (Figure 5 B), confirming that RNAi against laminin γ effectively prevents expression of the protein.



Figure 5. Laminin γ is enriched in necklace cells around the serosal window. Embryos of around 10 hours after cellularization at 30oC. In green is Laminin stained with 488nm of Alexa and blue are the nuclei stained with DAPI. A) Wild type embryo; Laminin is expressed mainly at the edge of the serosal window (arrowhead) B) Laminin γ knock down embryo; Laminin is only detected at low levels all over the egg. Scale bar 50 μ m.

Absence of Laminin delays closure of the serosal window

To investigate the laminin knockdown phenotype in more detail, we analyzed development in live imaging movies of the LAN-GFP transgenic *Tribolium* line (chapter 2). We applied double RNAi against laminin β and γ , as this simultaneous knockdown generated the highest percentage of defects in closure of the serosal window (Table). Strikingly, these movies reveal an irregularly formed serosal window, with some necklace cells staying behind (red arrows in Figure 6 A, B and C), instead of a regularly formed serosal window. Importantly, serosal closure is delayed (Figure 6 C, D), and the germband irregularly curved and positioned more towards the posterior (Figure 6 D), as reported for dorsocross knockdown (44).



Figure 6. Serosal window closure is delayed in laminin 6 and γ double knock down embryos. Stills of temporary aligned time lapse movies of a wild type embryo (Top) and an embryo with Laminin beta-gamma silenced (Bottom) of the LAN-GFP transgenic Tribolium line. Red arrows indicate necklace cells that stay behind at the irregular borders of the serosal window. Serosal window closure is delayed (C, D), and the germband is folded and positioned more towards posterior after RNAi (D). Scale bar 100 μ m.

To quantify the delay in closure of the serosal window upon simultaneous laminin β and γ RNAi, we measured the time between the onset of the differentiated blastoderm stage (i.e. the end of cellularization) and the completion of the serosal window closure in 16 knockdown embryos and 7 wildtype embryos. This revealed a conspicuous difference in closing time of the serosal window between wild type embryos (3.67 hours) and Laminin β and γ knock down embryos (3.92 hours) (Figure 7). Given the small number of movies in which this time could be measured, the p-value of this difference (0.07 in an ANOVA) might well drop when a higher number of embryos will be analyzed.



Figure 7. Comparison of the average of the time of serosal window closure between wild type and Laminin β and γ silenced. Using a t-test, we found that the difference of the serosal window closure time is different with a p-value of 0.07.

Taken together, we have identified laminin α 1,2 to be involved in closure of the serosal window in *Tribolium*. We have shown enrichment of Laminin γ in necklace cells of the serosa, and reveal that serosal window closure is delayed in laminin β and γ knockdown embryos. This demonstrates that the Laminin trimer is a key structural molecule for proper closure of the serosal window.

Discussion

We identify Laminin as key structural molecule involved in closure of the serosal window in Tribolium. Furthermore, we show the enrichment and potentially extracellular localization of Laminin γ protein around the necklace cells of the serosal window. As laminin is a component of the basal lamina in all animals (52, 53), it is likely that a basal lamina is present around the serosal window, and required for proper closure. It would be interesting to investigate this hypothesis by electron microscopy. In addition, disturbance of the basement membrane upon laminin RNAi could be studied using antibody stainings for other components of the basal lamina, such as Collagen IV or Perlecan (62).

We have identified four laminin subunits in the *Tribolium* genome, like in all invertebrates. These subunits all show high conservation in protein domains with their respective orthologs. However, in a frequency below 10%, we observed a splice variant of laminin γ that lacks the N-terminal domain. Interestingly, one of te three human laminin γ genes (laminin γ 2) also lacks the N-terminal domain, suggesting that this *Tribolium* splice variant might well have a biological function (70). Knock down of subunit α 1,2, subunit β or subunit γ all give the same phenotype in serosal window closure. This strongly suggests that it is the laminin trimer composed of these three subunits that is required for proper closure of the window.

Finally, we show that closure of the serosal window is delayed in laminin deficient embryos. As the duration of serosal window closure can only be measured in particularly oriented embryos, and only during this particular period in development, we could only analyze 16 movies of the RNAi treatment. In addition, RNAi effects vary in strength per embryo. It is therefore not surprising that the p-value we found for the difference in serosal window closure time between wildtypes and knockdowns is slightly higher than 0.05 (p=0.07). Considering same striking movies like the one in Figure 6, and conspicuous pictures of fixed material, we feel confident that analysis of a larger number of eggs will decrease this p-value.

The phenotype we observe after laminin RNAi is comparable to the one reported for dorsocross RNAi (44). Not only the delay of closure of the serosal window is similar, but also the curved germ band that is positioned more towards the posterior (44). In absence of proper separation of the amnion and the serosa, the amnion connects the germ band to the serosa which is anchored to the outer vitelline membrane, preventing anterior movement of the germ band (44, 46). This probably happens I both *dorsocross* and laminin RNAi. In addition, the expression pattern of laminin γ resembles the reported expression pattern of *dorsocross* (44). This makes it highly likely that laminins are important target genes of the Dorsocross transcription factor during closure of the serosal window in *Tribolium*.

In conclusion, we have shown that Laminin is required for closure of the serosal window, a crucial step in the development of the serosa. As more genetic tools such as RNAi and CRISPR-Cas are now available for nonmodel organisms, more genes will probably soon be described to be involved in morphogenesis of this crucial extraembryonic epithelium in insects.

Acknowledgments

We thank Rianne Grond from the group of Catherine Rabouille at the Hubrecht Institute for help with the Western blot.

References

- 1. Panfilio, K.A. 2008. Extraembryonic development in insects and the acrobatics of blastokinesis. Dev. Biol. .
- Schmidt-Ott, U., and C.W. Kwan. 2016. Morphogenetic functions of extraembryonic membranes in insects. Curr. Opin. Insect Sci. 13: 86– 92.
- 3. Jacobs, C.G.C., and M. Van Der Zee. 2013. Immune competence in insect eggs depends on the extraembryonic serosa. Dev. Comp. Immunol. .
- Zeh, D.W., J.A. Zeh, and R.L. Smith. 1989. Ovipositors, amnions and eggshell architecture in the diversification of terrestrial arthropods. Q. Rev. Biol. .
- 5. Hinton, H.E. 2013. Biology of Insect Eggs. .
- 6. Lamer, A., and A. Dorn. 2001. The serosa of Manduca sexta (insecta, lepidoptera): Ontogeny, secretory activity, structural changes, and functional considerations. Tissue Cell. .
- Goltsev, Y., G.L. Rezende, K. Vranizan, G. Lanzaro, D. Valle, and M. Levine. 2009. Developmental and evolutionary basis for drought tolerance of the Anopheles gambiae embryo. Dev. Biol. .
- 8. Rezende, G.L., A.J. Martins, C. Gentile, L.C. Farnesi, M. Pelajo-Machado, A.A. Peixoto, and D. Valle. 2008. Embryonic desiccation resistance in Aedes aegypti: Presumptive role of the chitinized Serosal Cuticle. BMC Dev. Biol. .
- Vargas, H.C.M., L.C. Farnesi, A.J. Martins, D. Valle, and G.L. Rezende.
 2014. Serosal cuticle formation and distinct degrees of desiccation resistance in embryos of the mosquito vectors Aedes aegypti, Anopheles aquasalis and Culex quinquefasciatus. J. Insect Physiol. .
- 10. Chen, G., K. Handel, and S. Roth. 2000. The maternal NF-κB/Dorsal gradient of Tribolium castaneum: Dynamics of early dorsoventral patterning in a short-germ beetle. Development.
- 11. Gorman, M.J., P. Kankanala, and M.R. Kanost. 2004. Bacterial challenge stimulates innate immune responses in extra-embryonic tissues of tobacco hornworm eggs. Insect Mol. Biol. .
- Jacobs, C.G.C., G.L. Rezende, G.E.M. Lamers, and M. van der Zee.
 2013. The extraembryonic serosa protects the insect egg against desiccation. Proc. R. Soc. B Biol. Sci. 280.

- 13. Jacobs, C.G.C., H.P. Spaink, and M. van der Zee. 2014. The extraembryonic serosa is a frontier epithelium providing the insect egg with a full-range innate immune response. Elife. 3: 1–21.
- 14. Schmidt-Ott, U. 2000. The amnioserosa is an apomorphic character of cyclorrhaphan flies. Dev. Genes Evol. .
- 15. Doyle, H.J., K. Harding, T. Hoey, and M. Levine. 1986. Transcripts encoded by a homoeo box gene are restricted to dorsal tissues of Drosophila embryos. Nature. .
- 16. Rushlow, C., H. Doyle, T. Hoey, and M. Levine. 1987. Molecular characterization of the zerknüllt region of the Antennapedia gene complex in Drosophila. Genes Dev. .
- 17. Rushlow, C., and M. Levine. 1990. Role Of The zerknüllt Gene In Dorsal-Ventral Pattern Formation In Drosophila. Adv. Genet. .
- 18. Wakimoto, B.T., F.R. Turner, and T.C. Kaufman. 1984. Defects in embryogenesis in mutants associated with the antennapedia gene complex of Drosophila melanogaster. Dev. Biol. .
- Frank, L.H., and C. Rushlow. 1996. A group of genes required for maintenance of the amnioserosa tissue in Drosophila. Development. 122: 1343–52.
- 20. Reim, I., H.H. Lee, and M. Frasch. 2003. The T-box-encoding Dorsocross genes function in amnioserosa development and the patterning of the dorsolateral germ band downstream of Dpp. Development.
- Yip, M.L., M.L. Lamka, and H.D. Lipshitz. 1997. Control of germ-band retraction in Drosophila by the zinc-finger protein HINDSIGHT. Development. 124: 2129–2141.
- 22. Schöck, F., and N. Perrimon. 2003. Retraction of the Drosophila germ band requires cell-matrix interaction. Genes Dev. 17: 597–602.
- 23. Scuderi, A., and A. Letsou. 2005. Amnioserosa is required for dorsal closure in Drosophila. Dev. Dyn. 232: 791–800.
- Kiehart, D.P., J.M. Crawford, A. Aristotelous, S. Venakides, and G.S. Edwards. 2017. Cell sheet morphogenesis: Dorsal closure in drosophila melanogaster as a model system. Annu. Rev. Cell Dev. Biol. .
- Pasakarnis, L., E. Frei, E. Caussinus, M. Affolter, and D. Brunner. 2016. Amnioserosa cell constriction but not epidermal actin cable tension autonomously drives dorsal closure. Nat. Cell Biol. .

- 26. Young, P.E., A.M. Richman, A.S. Ketchum, and D.P. Kiehart. 1993. Morphogenesis in Drosophila requires nonmuscle myosin heavy chain function. Genes Dev. .
- 27. Flores-Benitez, D., and E. Knust. 2015. Crumbs is an essential regulator of cytoskeletal dynamics and cell-cell adhesion during dorsal closure in Drosophila. Elife. .
- Harden, N., M. Ricos, Y.M. Ong, W. Chia, and L. Lim. 1999. Participation of small GTPases in dorsal closure of the Drosophila embryo: Distinct roles for Rho subfamily proteins in epithelial morphogenesis. J. Cell Sci.
- 29. David, D.J.V., A. Tishkina, and T.J.C. Harris. 2010. The PAR complex regulates pulsed actomyosin contractions during amnioserosa apical constriction in Drosophila. Development.
- 30. Brown, N.H. 1994. Null mutations in the α (PS2) and β (PS) integrin subunit genes have distinct phenotypes. Development.
- Giuliani, F., G. Giuliani, R. Bauer, and C. Rabouille. 2013. Innexin 3, a New Gene Required for Dorsal Closure in Drosophila Embryo. PLoS One. 8: 1–16.
- Tepass, U., E. Gruszynski-DeFeo, T.A. Haag, L. Omatyar, T. Török, and V. Hartenstein. 1996. shotgun encodes Drosophila E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. Genes Dev. .
- Uemura, T., H. Oda, R. Kraut, S. Hayashi, Y. Kataoka, and M. Takeichi. 1996. Zygotic Drosophilia E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the Drosophila embryo. Genes Dev. .
- Horn, T., M. Hilbrant, and K.A. Panfilio. 2015. Evolution of epithelial morphogenesis: Phenotypic integration across multiple levels of biological organization. Front. Genet. 6: 1–7.
- 35. Roth, S. 2004. Gastrulation in other insects. In: Gastrulations: from cells to embryo. .
- 36. Panfilio, K.A. 2009. Late extraembryonic morphogenesis and its zenRNAi-induced failure in the milkweed bug Oncopeltus fasciatus. Dev. Biol. .
- 37. Panfilio, K.A., and S. Roth. 2010. Epithelial reorganization events during late extraembryonic development in a hemimetabolous

insect. Dev. Biol. .

- Handel, K., C.G. Grünfelder, S. Roth, and K. Sander. 2000. Tribolium embryogenesis: a SEM study of cell shapes and movements from blastoderm to serosal closure. Dev. Genes Evol. 210: 167–179.
- Hilbrant, M., T. Horn, S. Koelzer, and K.A. Panfilio. 2016. The beetle amnion and serosa functionally interact as apposed epithelia. Elife. 5: 1–17.
- Rafiqi, A.M., S. Lemke, S. Ferguson, M. Stauber, and U. Schmidt-Ott.
 2008. Evolutionary origin of the amnioserosa in cyclorrhaphan flies correlates with spatial and temporal expression changes of zen.
 Proc. Natl. Acad. Sci. U. S. A. .
- 41. Schmidt-Ott, U., Rafiqi, A. M. and Lemke, S. 2010. Hox3/zen and the Evolution of Extraembryonic Epithelia in Insects. In Hox Genes: Studies from the 20th to the 21st Century. J. S. Deut. New York, NY: Springer New York.
- 42. Van Der Zee, M., N. Berns, and S. Roth. 2005. Distinct functions of the Tribolium zerkn??Ilt genes in serosa specification and dorsal closure. Curr. Biol. 15: 624–636.
- 43. Panfilio, K.A., P.Z. Liu, M. Akam, and T.C. Kaufman. 2006. Oncopeltus fasciatus zen is essential for serosal tissue function in katatrepsis. Dev. Biol. .
- 44. Horn, T., and K.A. Panfilio. 2016. Novel functions for Dorsocross in epithelial morphogenesis in the beetle Tribolium castaneum. Dev. .
- Benton, M.A., N. Frey, R.N. da Fonseca, C. von Levetzow, D. Stappert, M.S. Hakeemi, K.H. Conrads, M. Pechmann, K.A. Panfilio, J.A. Lynch, and S. Roth. 2019. Fog signaling has diverse roles in epithelial morphogenesis in insects. Elife. .
- 46. Münster, S., A. Jain, A. Mietke, A. Pavlopoulos, S.W. Grill, and P. Tomancak. 2019. Attachment of the blastoderm to the vitelline envelope affects gastrulation of insects. Nature.
- Dönitz, J., C. Schmitt-Engel, D. Grossmann, L. Gerischer, M. Tech, M. Schoppmeier, M. Klingler, and G. Bucher. 2015. iBeetle-Base: A database for RNAi phenotypes in the red flour beetle Tribolium castaneum. Nucleic Acids Res. .
- Schmitt-Engel, C., D. Schultheis, J. Schwirz, N. Ströhlein, N. Troelenberg, U. Majumdar, V.A. Dao, D. Grossmann, T. Richter, M. Tech, J. Dönitz, L. Gerischer, M. Theis, I. Schild, J. Trauner, N.D.B.

Koniszewski, E. Küster, S. Kittelmann, Y. Hu, S. Lehmann, J. Siemanowski, J. Ulrich, K.A. Panfilio, R. Schröder, B. Morgenstern, M. Stanke, F. Buchhholz, M. Frasch, S. Roth, E.A. Wimmer, M. Schoppmeier, M. Klingler, and G. Bucher. 2015. The iBeetle large-scale RNAi screen reveals gene functions for insect development and physiology. Nat. Commun. 6.

- 49. Brower, D.L., and S.M. Jaffe. 1989. Requirement for integrins during Drosophila wing development. Nature. .
- Martin, D., S. Zusman, X. Li, E.L. Williams, N. Khare, S. DaRocha, R. Chiquet-Ehrismann, and S. Baumgartner. 1999. wing blister, A new Drosophila laminin chain required for cell adhesion and migration during embryonic and imaginal development. J. Cell Biol. 145: 191– 201.
- 51. Wilcox, M., A. DiAntonio, and M. Leptin. 1989. The function of PS integrins in Drosophila wing morphogenesis. Development. .
- 52. Hohenester, E., and P.D. Yurchenco. 2013. Laminins in basement membrane assembly. Cell Adhes. Migr. .
- 53. Pozzi, A., P.D. Yurchenco, and R. V. Iozzo. 2017. The nature and biology of basement membranes. Matrix Biol. .
- 54. Aumailley, M. 2013. The laminin family. Cell Adhes. Migr. .
- 55. Hohenester, E. 2019. Structural biology of laminins. Essays Biochem.
- Aumailley, M., L. Bruckner-Tuderman, W.G. Carter, R. Deutzmann, D. Edgar, P. Ekblom, J. Engel, E. Engvall, E. Hohenester, J.C.R. Jones, H.K. Kleinman, M.P. Marinkovich, G.R. Martin, U. Mayer, G. Meneguzzi, J.H. Miner, K. Miyazaki, M. Patarroyo, M. Paulsson, V. Quaranta, J.R. Sanes, T. Sasaki, K. Sekiguchi, L.M. Sorokin, J.F. Talts, K. Tryggvason, J. Uitto, I. Virtanen, K. Von Der Mark, U.M. Wewer, Y. Yamada, and P.D. Yurchenco. 2005. A simplified laminin nomenclature. Matrix Biol. 24: 326–332.
- 57. Fahey, B., and B.M. Degnan. 2012. Origin and evolution of laminin gene family diversity. Mol. Biol. Evol. 29: 1823–1836.
- Henchcliffe, C., L. Garcia-Alonso, J. Tang, and C.S. Goodman. 1993.
 Genetic analysis of laminin A reveals diverse functions during morphogenesis in Drosophila. Development.
- 59. Yarnitzky, T., and T. Volk. 1995. Laminin is required for heart, somatic muscles, and gut development in the Drosophila embryo. Dev. Biol.

- 60. Deng, W.M., and H. Ruohola-Baker. 2000. Laminin A is required for follicle cell-oocyte signaling that leads to establishment of the anterior-posterior axis in Drosophila. Curr. Biol. .
- 61. García-Alonso, L., R.D. Fetter, and C.S. Goodman. 1996. Genetic analysis of Laminin A in Drosophila: Extracellular matrix containing laminin A is required for ocellar axon pathfinding. Development.
- Urbano, J.M., C.N. Torgler, C. Molnar, U. Tepass, A. López-Varea, N.H. Brown, J.F. de Celis, and M.D. Martín-Bermudo. 2009. Drosophila laminins act as key regulators of basement membrane assembly and morphogenesis. Development.
- 63. Wolfstetter, G., and A. Holz. 2012. The role of LamininB2 (LanB2) during mesoderm differentiation in Drosophila. 2: 267–282.
- 64. Schöck, F., and N. Perrimon. 2003. Retraction of the Drosophila germ band requires cell-matrix interaction. Genes Dev. 17: 597–602.
- 65. Urbano, J.M., P. Domínguez-Giménez, B. Estrada, and M.D. Martín-Bermudo. 2011. PS integrins and laminins: Key regulators of cell migration during drosophila embryogenesis. PLoS One. .
- Zee, M. v. d., O. Stockhammer, R.N. d. Fonseca, C. v. Levetzow, and S. Roth. 2006. Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. Proc. Natl. Acad. Sci. 103: 16307–16312.
- 67. Sigrist, C.J.A., L. Cerutti, N. Hulo, A. Gattiker, L. Falquet, M. Pagni, A. Bairoch, and P. Bucher. 2002. PROSITE: a documented database using patterns and profiles as motif descriptors. Brief. Bioinform.
- Sigrist, C.J.A., E. De Castro, L. Cerutti, B.A. Cuche, N. Hulo, A. Bridge,
 L. Bougueleret, and I. Xenarios. 2013. New and continuing developments at PROSITE. Nucleic Acids Res. .
- 69. Dearden, P., M. Grbic, F. Falciani, and M. Akam. 2000. Maternal expression and early zygotic regulation of the Hox3/zen gene in the grasshopper Schistocerca gregaria. Evol. Dev. 2: 261–270.
- Tzu, J., and M.P. Marinkovich. 2008. Bridging structure with function: Structural, regulatory, and developmental role of laminins. Int. J. Biochem. Cell Biol. 40: 199–214.

Supplementary

Examples of the defects found in the embryos

All the embryos are stained with DAPI as mentioned in the method.



Figure 1. Embryo during the nuclei division with TC10914 gene silenced showing nuclei irregular spaced.



Figure 2. Embryo during the nuclei division with TC3474 gene silenced showing a hole in the blastoderm.



Figure 3. Embryo after cellularization with TC7565 gene silenced showing a wrong condensation of the germ rudiment.



Figure 4. Embryo after gastrulation with TC1525 gene silenced showing a gastrulation disturbed.



Figure 5. Embryo after cellularization with TC14773 (Laminin α) gene silenced showing a delay serosa closure.



Figure 6. Embryo after gastrulation with TC3007 gene silenced showing irregular positioned germ rudiment.



Figure 7. Embryo in the germband stage with TC10914 gene silenced showing irregular curved germband.



Figure 8. Embryo in the germband stage with TC14773 gene silenced showing irregular positioned germband.



Figure 9. Embryo in the germband stage with TC5546 gene silenced showing head lobes defects.



Figure 10. Embryo in the germband stage with TC16062 gene silenced showing an irregular border of the embryo.



Figure 11. Embryo in the germband stage with TC3342 gene silenced showing a flat germband.



Figure 12. Embryo in the in a late germband stage with TC5676 gene silenced showing a germband retraction defect.



Figure 13. Embryo in the in a late germband stage with TC5546 gene silenced showing dorsal closure defects.



Figure 14. Embryo in the in a late germband stage with TC3007 gene silenced showing the serosal nuclei irregular spaced.