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Drosophila laminins act as key regulators of basement membrane assembly and morphogenesis

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Laminins are heterotrimeric molecules found in all basement membranes. In mammals, they have been involved in diverse developmental processes, from gastrulation to tissue maintenance. The *Drosophila* genome encodes two laminin α chains, one β and one γ, which form two distinct laminin trimers. So far, only mutations affecting one or other trimer have been analysed. In order to study embryonic development in the complete absence of laminins, we mutated the gene encoding the sole laminin β chain in *Drosophila*, *LanB1*, so that no trimers can be made. We show that *LanB1* mutant embryos develop until the end of embryogenesis. Electron microscopy analysis of mutant embryos reveals that the basement membranes are absent and the remaining extracellular material appears disorganised and diffuse. Accordingly, abnormal accumulation of major basement membrane components, such as Collagen IV and Perlecan, is observed in mutant tissues. In addition, we show that elimination of *LanB1* prevents the normal morphogenesis of most organs and tissues, including the gut, trachea, muscles and nervous system. In spite of the above structural roles for laminins, our results unravel novel functions in cell adhesion, migration and rearrangement. We propose that while an early function of laminins in gastrulation is not conserved in *Drosophila* and mammals, their function in basement membrane assembly and organogenesis seems to be maintained throughout evolution.

KEY WORDS: Laminin, Basement membrane, Morphogenesis, *Drosophila*

INTRODUCTION

Basement membranes (BMs) are specialised layers of extracellular matrix (ECM) covering the basal side of all epithelia and endothelia, and surrounding muscles, peripheral nerves and other tissues. BMs provide mechanical stability and physical barriers between different cell types and are important for tissue morphogenesis in metazoans. They have been implicated in many processes such as cell differentiation, shape, adhesion, survival and migration (Fessler and Fessler, 1989; Timpl, 1996).

Even though the composition of BMs varies according to tissues and developmental stages, they are mainly composed of two meshworks formed by laminins and collagen IV. They also contain other ECM components, such as Nidogen and proteoglycans. Laminins are a family of large heterotrimeric glycoproteins composed of three non-identical chains, α, β and γ (Timpl et al., 1979). The laminin trimer forms a cross-shaped structure consisting in three short arms, each formed by a different chain, and a long arm composed of the three assembled coiled chains. Data from mammalian cell culture has shown that while the α subunit can be secreted alone as a monomer, secretion of the β and γ chains requires simultaneous expression of all three chains and their assembly into α-β-γ heterotrimers (Yurchenco et al., 1997). Once secreted, laminins form a meshwork by self-assembly through interactions between the three short arms. In developing tissues, laminin assembly also requires the long arm to be tethered to receptors on the cell surface, such as integrins and dystroglycan (Colognato and Yurchenco, 2000).

Laminins are common to tissues of most multicellular metazoans and they are highly conserved across evolution. However, whereas invertebrates possess one to two laminin heterotrimers, mammals possess at least 15, which are formed through combinations of 5α, 4β and 3γ subunits (Aumailley et al., 2005). They differ with respect to their tissue distribution, presumably reflecting diverse biological functions. Thus, whereas null mutations in laminin α1 results in early embryonic lethality (day E7) (Miner et al., 2004), lack of laminin α2 and α3 chains causes, respectively, severe muscular dystrophy and skin blistering both in mice and in humans (Helbling-Leclerc et al., 1995; Pulkkinen and Uitto, 1999). Mice failing to express the laminin β1 or γ1 subunits lack embryonic BMs and do not survive beyond embryonic day 5.5 (E5.5), suggesting some type of compensation between different α subunits (Miner et al., 2004; Smyth et al., 1999).

The *Drosophila* genome encodes only four laminin chains: two α chains (α1,2 and α3,5), one β chain and one γ chain. These form two trimers, lamininA (α3,5; β1; γ1) and lamininW (α1,2; β1; γ1). The first laminin α chain described in *Drosophila*, $\alpha_{3,5}$ (encoded by *Laminin A*, *LanA*), is most similar to vertebrate α_3 and α_5 and is part of the lamininA trimer. Experiments in cell culture have shown that lamininA is likely to bind PS1 integrin (αPS1βPS) and not PS2 integrin (αPS2βPS) (Gotwals et al., 1994). This is supported by the similarity of phenotypes of *LanA* mutant embryos and those lacking the PS1 integrin (Prokop et al., 1998). The second laminin α chain described in *Drosophila*, $\alpha_{1,2}$ (encoded by *wing blister*, *wb*), is most similar to vertebrate α_1 and α_2 chains and is part of the lamininW trimer. $\alpha_{1,2}$ contains an RGD (Arg-Gly-Asp) motif, which is a recognition site for PS2 integrin. Indeed, experiments with *Drosophila* S2 cells in culture have shown that RGD-containing peptides derived from laminin $\alpha_{1,2}$

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can serve as PS2 integrin ligands (Graner et al., 1998). This is further supported by studies showing that lamininW is not recruited to muscle attachment sites in embryos carrying a mutation in the αPS2 subunit affecting the RGD binding site (Devenport et al., 2007).

Only mutations in the α subunits have been described in *Drosophila* so far. Null mutations in the *LanA* gene result in embryonic lethality with defects in somatic muscles, dorsal vessel (heart) and endoderm (Henchcliffe et al., 1993; Yarnitzky and Volk, 1995). The $\alpha_{3,5}$ chain is also required for proper localisation of anteroposterior markers in the oocyte and for normal pathfinding of pioneer axons in the brain (Deng and Ruohola-Baker, 2000; Garcia-Alonso et al., 1996). In addition, hypomorphic mutants and trans-heteroallelic combinations of *LanA* give rise to adult escapers that have disorganised rhabdomeres and display vein defects and wing blistering (Henchcliffe et al., 1993). Mutations in *wb* have wing blisters and defects in the dorsal vessel, trachea, muscles and rhabdomeres (Martin et al., 1999). Mutations in the laminin β subunit (encoded by *Laminin B1*, *LanB1*) or γ subunit (encoded by *laminin B2*, *lanB2*) have not been characterised.

In this work, we have isolated null mutations in *LanB1*, allowing the generation of embryos lacking all laminin function. These embryos develop until the end of embryogenesis, suggesting that in *Drosophila*, as it is the case in nematodes and contrary to mice, laminins are not required for early embryonic morphogenetic events. Our analysis of *LanB1* loss reveals that laminins are required for accumulation of major ECM components, such as Collagen IV and Perlecan (Trol – FlyBase), into BMs. Furthermore, we show that in the absence of the β chain BMs are absent and the remaining extracellular material appears disorganised and diffuse. In addition, our results reveal new functions for laminins in cell adhesion, migration and rearrangement, and identify laminins as essential regulators of the morphogenesis of most organs in *Drosophila*.

MATERIALS AND METHODS

Genetics

The $LanB1^{28a}$ hypomorphic allele was generated by P-element imprecise excision of P{EP}EP2178 using standard procedures. $LanB1^{28a}$ deletes the first non-coding exon and most of the first intron without interrupting the second intron (Fig. 1A).

$LanB1^{1B1}$ and $LanB1^{1P3}$ alleles were generated using a P-GS element insertion in the *LanB1* locus (Toba et al., 1999). The *EP-600* line is inserted 548 bp upstream of the 5′ UTR of the *LanB1* gene (Molnar and de Celis, 2006) (Fig. 1=P2) and when combined with the wing GAL4 driver *638-Gal4* results in visible wing phenotype. To isolate mutations in *LanB1*, we performed an ethane methyl sulphonate (EMS) mutagenesis screen and searched for suppression of the wing phenotype. A total of 9500 *EP-600* chromosomes were screened and two revertant embryonic lethal lines, $LanB1^{1B1}$ and $LanB1^{1P3}$, were isolated. $LanB1^{1B1}$ and $LanB1^{1P3}$ lines encode truncated proteins of 641 and 1143 amino acids, respectively, due to nucleotide changes that result in stop codons (Fig. 1A).

The deficiency $LanB1^{DEF}$ was generated using the Exelixis insertions *d04880* (P3) and *e02263* (P4) (Parks et al., 2004), separated by 11.6 kb including *LanB1* and the 5′ UTR of *CG7134* (Fig. 1A), using standard procedures.

LanB1 alleles were recombined onto *FRT40A*, and germline clones were generated using the FRT/FLP technique (Chou and Perrimon, 1992). In addition, the following lines were used: *Gla/Cyo-actin-GFP* (Bloomington); *5053-Gal4* (Swan et al., 2004). Large clones of *LanB1* mutant cells in the wing were induced in flies of the following genotypes: *638-Gal4 UAS-FLP; FRT40M(2)z f+/FRT40* $LanB1^{1B1}$ and *sal-Gal4UAS-FLP; FRT40M(2)zf+/ FRT40* $LanB1^{1B1}$, using standard procedures.

Immunohistochemistry

Embryos, discs and pupal wings were fixed and stained according to standard procedures and were mounted in Vectashield (Vector Laboratories). Images were collected with a Zeiss Axioplan 2 microscope or a Zeiss LSM 510 confocal.

Primary antibodies used: anti-arm, anti-Cut, anti-FascIII, anti-Hindsight, anti-βPS, anti-2A12 (DHSB); anti-β-gal (Cappel); anti-Collagen IV (Lunstrum et al., 1988); anti-dCREB-A (Andrew et al., 1997); anti-GFP (Molecular Probes); anti-Laminin β, anti-LamininA, anti-$\alpha_{1,2}$ (Kumagai et al., 1997); anti-MEF2 (Hanh Nguyen); anti-Muscle myosin (Kiehart and Feghali, 1986); anti-Pericardin (Chartier et al., 2002); anti-Perlecan (Schneider et al., 2006); anti-Teashirt (Wu and Cohen, 2000); anti-Tiggrin (Fogerty et al., 1994); anti-Serpent (Reuter, 1994). Alexa-fluor-conjugated secondary antibodies used were Alexa fluor 488 (Green), Alexa fluor 568 (red) (Molecular Probes). For non-fluorescent staining, embryos were incubated in biotinylated secondary antibodies followed by incubation with Elite ABC complex (Vector Laboratories) and revealed with DAB (Gibco-BRL). Filamentous actin and nuclei were visualised using Rhodamine-labelled Phalloidin and TO-PRO 3, respectively (Molecular Probes).

Electron microscopy

Ultrastructural analyses were carried out on early stage 17 wild-type and $LanB1^{DEF}$ mutant embryos (Tepass and Hartenstein, 1994b). Four embryos were examined for each genotype. Mutant embryos were selected by examination of midgut morphology, which is grossly abnormal in *LanB1* mutants.

RESULTS

Generation of laminin β1 mutant alleles

The *Drosophila* genome contains a single gene encoding a laminin β subunit, *LanB1*. The *LanB1* gene gives rise to two predicted transcripts, *LanB1-RA* and *LanB1-RB*, which encode the same LanB1 protein (Fig. 1A). To analyse *LanB1* requirements during development we generated *LanB1* complete or partial loss-of-function alleles ($LanB1^{1B1}$, $LanB1^{1P3}$ and $LanB1^{28a}$, respectively) and a *LanB1* deficiency, $LanB1^{DEF}$ (Fig. 1A; Materials and methods). The $LanB1^{1B1}$ and $LanB1^{1P3}$ alleles consist of stop codons at positions 641 and 1143, respectively (Fig. 1A). They are most likely null alleles because, even though they encode a small portion of the protein, they lack the crucial coiled-coil region, necessary for their incorporation in a functional laminin trimer (Timpl and Brown, 1994). In addition, the phenotypes of $LanB1^{1B1}$ and $LanB1^{1P3}$ homozygous mutant embryos are equivalent to those of $LanB1^{1B1}$ and $LanB1^{1P3}$ over deficiencies. Analysis of homozygous zygotic $LanB1^{1B1}$ and $LanB1^{1P3}$ mutant embryos revealed that they died at stage 17, as did embryos lacking both zygotic and maternal *LanB1*. The $LanB1^{28a}$ allele deletes only the first non-coding exon; therefore, it is likely to affect only the LanB1-RA transcript (Fig. 1A). In addition, $LanB1^{28a}$ homozygous mutant animals died at larval stages, demonstrating that it is a hypomorphic allele. Accordingly, viability of $LanB1^{28a}$ was further reduced in combination with $LanB1^{def}$. The $LanB1^{def}$ allele eliminated the entire *LanB1* gene and also the 5′ UTR of the adjacent *CG72143* gene (Fig. 1A), and exhibited the same phenotype as $LanB1^{1B1}$ and $LanB1^{1P3}$.

LanB1 expression during embryonic, larval and pupal development

In order to analyse the role of LanB1 during development we first analysed its expression pattern. To investigate the distribution of LanB1 we used a polyclonal anti-LanB1 antibody (Kumagai et al., 1997). We found that LanB1 distribution resembled that of both α subunits (Montell and Goodman, 1989). Thus, at stage 16, LanB1 was detected in BMs surrounding most embryonic tissues, such as

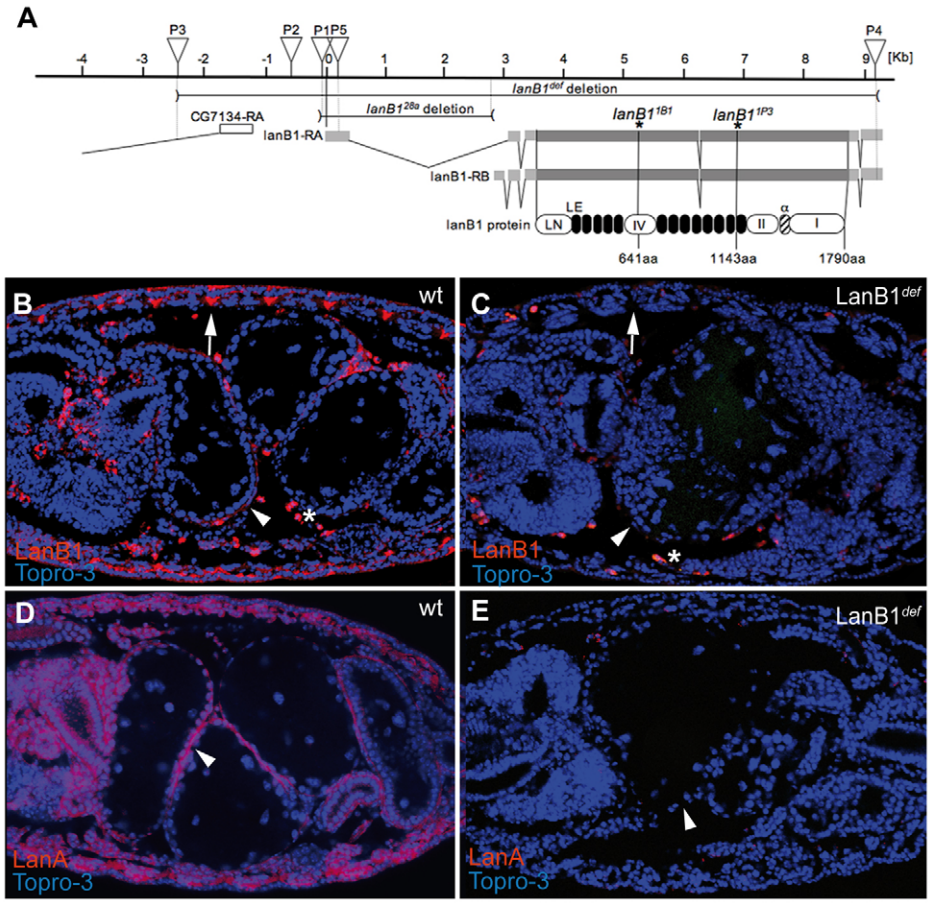

Fig. 1. Molecular description of *LanB1* mutant alleles and distribution of the LanB1 protein. (**A**) Schematic representation of the *LanB1* genomic region. Both laminin β transcripts (LanB1-RA and LanB1-RB) with their coding and untranslated regions are represented in dark and light grey horizontal bars, respectively. The white box represents the 5′ UTR of the gene *CG7134*. The localisations of transposon insertions appear as inverted triangles [P1=*P*(*EP*)*EP2178*, P2=*EP600*, P3=*P*{*XP*}^{d04880}, P4=*PBac*{*RB*}*LanB1*^{e02263}, P5=*l*(*2*)*k05404*]. The horizontal lines correspond to the genomic region deleted in the *LanB1*^{28a} allele and the deficiency *LanB1*^{def}. Asterisks indicate the positions of stop codons in the *LanB1*^{1B1} and *LanB1*^{1P3} alleles. Conserved domains are schematised as follows: white, from left to right, LanB1 N-terminal domain (LN), domain IV, domain II and domain I; black, 13 laminin-type EGF-like domains (LE); and stripped, the alpha domain. In all figures, embryos are oriented with anterior to the left. (**B**) In stage 16 wild-type embryos, LanB1 (red) is found in BMs surrounding most tissues (arrowhead) and at muscle attachment sites (arrow). (**C**) This expression is lost in maternal and zygotic homozygous *LanB1*^{def} mutant embryos. (**D**) In wild-type embryos, LanA (red) is found at BMs. (**E**) This expression is not detected in *LanB1*^{def} mutant embryos. In all figures, the nuclear marker TO-PRO 3 is in blue. wt, wild type.

muscles and gut, as well as being enriched at muscle attachment sites (Fig. 1B). We could not detect this staining in BMs or muscle attachment sites of homozygous maternal and zygotic *LanB1*^{def} mutant embryos, strongly suggesting that this expression pattern corresponds to the endogenous LanB1 subunit (Fig. 1C). We could see some weak scattered signals that were still present in mutant embryos, suggesting they are unspecific (asterisks in Fig. 1B,C).

LanB1 was also conspicuously expressed in BMs in the wing imaginal disc during larval and pupal development (Fig. 2). In third instar wing discs and during the first 4 hours of pupal development, LanB1 was found at the basal side of the epithelium, where it colocalised with integrins (Fig. 2A,B). Later in development, at 20-24 hours after puparium formation (APF), LanB1 became strongly localised to the basal side of the lacuna formed after apposition of dorsal and ventral wing surfaces (Fig. 2C). This localisation was also observed in the developing longitudinal veins during later stages of pupal development (Fig. 2D-F). As the expression of integrins became restricted to the basal side of intervein cells, at 40-44 hours APF (Fristrom et al., 1993), LanB1 and integrins appeared in complementary domains (Fig. 2F).

Basement membrane assembly in the absence of laminin function

Cell culture experiments have shown that mouse and *Drosophila* α chains can be secreted as a single subunit (Kumagai et al., 1997; Yurchenco et al., 1997). To address whether laminin α chains can be detected at BMs in the absence of laminin β in the developing *Drosophila* embryo, we stained *LanB1* mutant embryos with anti-$\alpha_{3,5}$ and anti-$\alpha_{1,2}$ antibodies. We found that, whereas in wild-type embryos laminin $\alpha_{3,5}$ localises to the BM of different tissues, this localisation was lost in *LanB1*^{def} embryos (compare Fig. 1D with 1E). This is also the case for $\alpha_{1,2}$, which could not be found at BMs or muscle attachment sites in *LanB1*^{def} embryos (data not shown). We therefore conclude that during embryogenesis the discrete accumulation of the $\alpha_{3,5}$ and $\alpha_{1,2}$ chains in the extracellular environment requires the presence of the laminin β subunit.

Studies in vertebrates and in *Caenorhabditis elegans* have suggested that laminins are essential for the formation of an initial scaffold, which in turn recruits other BM components, such as Collagen IV and Perlecan (Li, J. et al., 2003; Yurchenco et al., 2004). To determine whether accumulation of BM components is affected by the absence of laminins in *Drosophila*, their distribution in *LanB1* mutant embryos was analysed. In wild-type embryos, Collagen IV is expressed by macrophages and localises to BMs surrounding most tissues, including the gut, brain and ventral nerve cord (VNC) (Natzle et al., 1982; Yasothornsrikul et al., 1997) (Fig. 3A,C). Collagen IV expression was observed in macrophages of *LanB1*^{def} embryos, indicating that laminins are not required for expression of Collagen IV by macrophages (Fig. 3D). However, it was not localised surrounding the gut and VNC (Fig. 3B,D), suggesting that it is no longer able to contribute to the formation of a BM. Furthermore, loss of Collagen IV results in VNC condensation defects (Martinek et al., 2008), and we found that the VNC failed to condense in *LanB1*^{def} embryos (Fig. 3E,F). Perlecan is another major component of BMs surrounding the gut, brain and VNC, in addition to being enriched at muscle attachment sites (Friedrich et al., 2000) (see Fig. S1A,C in the supplementary material). In stage 16 *LanB1* mutant embryos, Perlecan appeared less uniform and loosely associated with the gut,

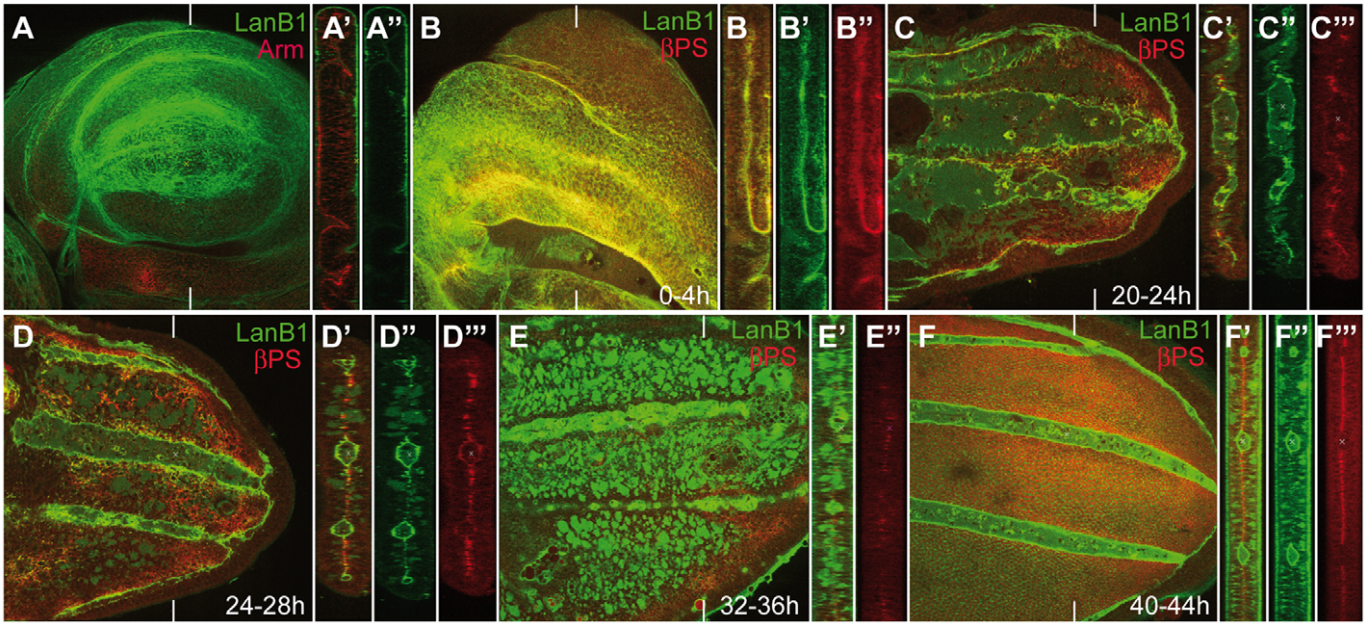

Fig. 2. Localisation of LanB1 during imaginal and pupal wing development. (**A-F‴**) Vertical lines indicate the position of transverse sections shown in the accompanying panels. (A-A″) Basal side of a third instar wing disc stained with LanB1 (green) and Armadillo (red). LanB1 is localised at the basal side of the columnar epithelium and peripodial membrane. (B-B″) In 0- to 4-hour-APF pupal wings, LanB1 (green) and βPS integrin (red) colocalise at the basal surface of all wing epithelial cells. (C-D‴) This localisation persists from 20 to 28 hours APF. LanB1 also localises in the lacunae formed between the dorsal and ventral wing spaces from 20 to 44 hours (C-F‴). (E-F‴) However, from 32 to 44 hours APF, LanB1 and βPS integrin are found in mutually exclusive patterns, with LanB1 being found at the basal surface of veins (D″,F″) and βPS integrin at that of interveins (E″,F‴).

showing a discontinuous distribution (see Fig. S1B in the supplementary material). Furthermore, Perlecan was not detected in the BM surrounding the VNC or brain (see Fig. S1B,D in the supplementary material). By contrast, Perlecan localisation at muscle attachment sites appeared normal (see Fig. S1B in the supplementary material). Together, these data show that laminins are crucial for the assembly of Perlecan and Collagen IV into BMs.

Drosophila has other ECM proteins that are not found in BMs, and instead highlight other forms of ECM, such as the collagen-like protein Pericardin, and Tiggrin (Chartier et al., 2002; Fogerty et al., 1994). Pericardin is concentrated at the basal surface of cardioblasts and around pericardial cells in close proximity to the dorsal ectoderm (Chartier et al., 2002) (Fig. 4A), whereas Tiggrin localises predominantly at muscle attachment sites (Fogerty et al., 1994). We

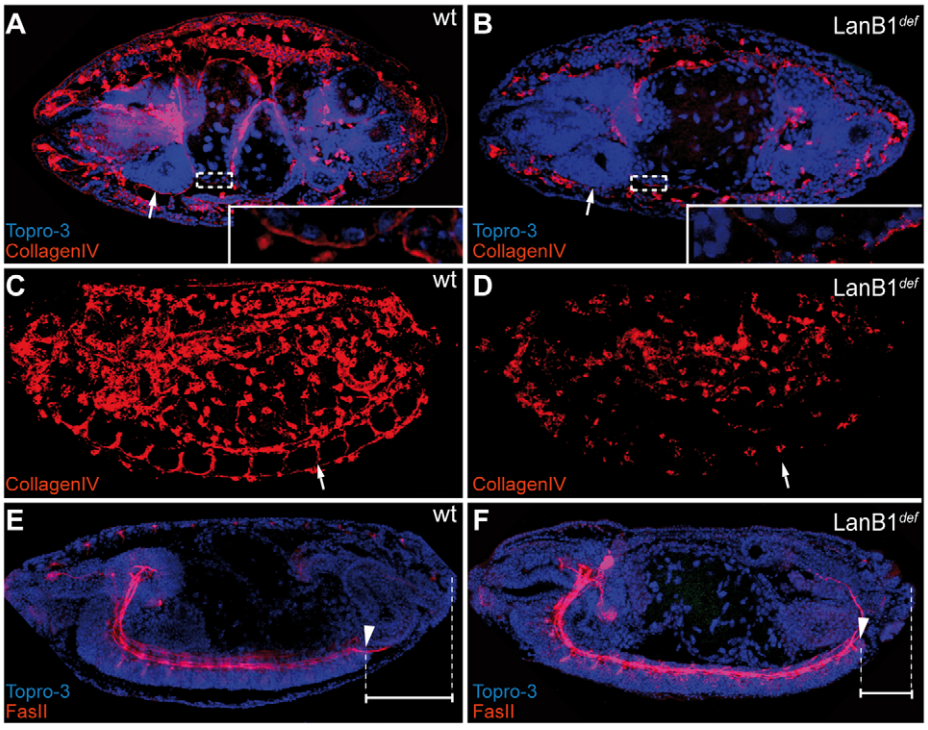

Fig. 3. LanB1 is required for proper localisation of Collagen IV at basement membranes. (**A-F**) Dorsal views of stage 16 embryos (A,B). Lateral views of stage 15 (C,D) and 16 (E,F) embryos. (A,D) In wild-type embryos Collagen IV (red) is found at BMs surrounding the brain (arrow) and midgut (magnification in the white box). (B) This localisation is severely disrupted in *LanB1*^{def} embryos. (C) In addition, Collagen IV localisation in the ventral nerve cord channels of a stage 15 embryo (arrow) is lost in *LanB1*^{def} embryos (D, arrow). (E,F) In stage 16 embryos, the ventral nerve cord, visualised with anti-FasII antibody (red), fails to condense properly in *LanB1*^{def} mutants. White horizontal bars indicate the length of CNS condensation.

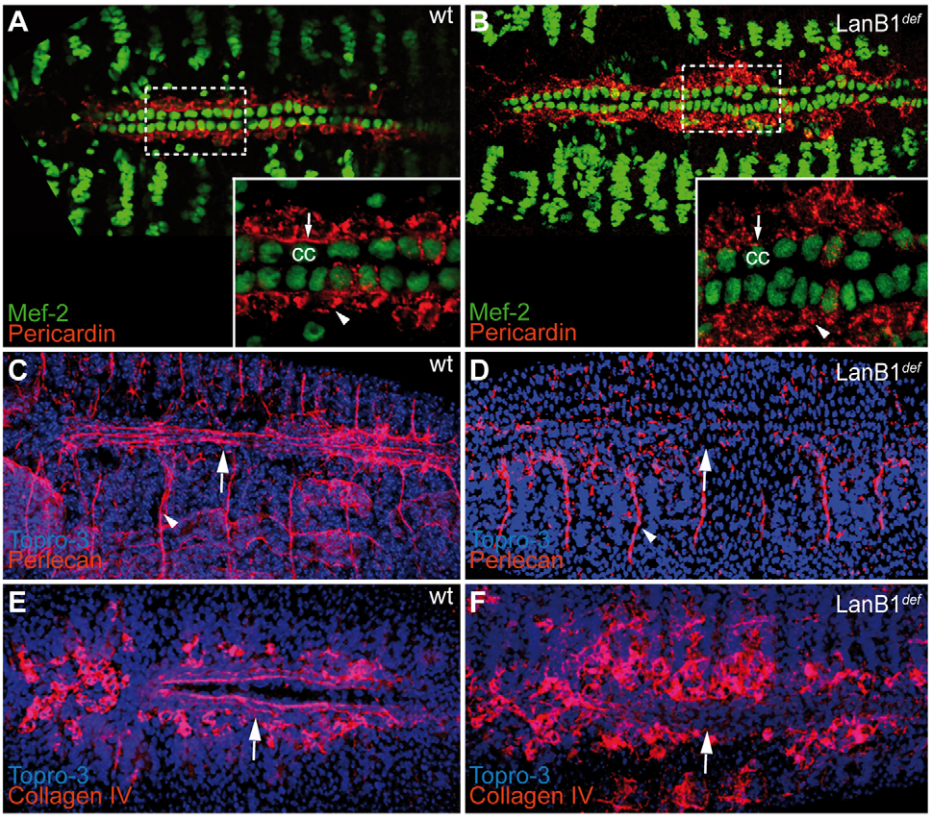

Fig. 4. LanB1 is essential for correct localisation of ECM components during heart morphogenesis. (**A**,**B**) Dorsal view of stage 16 embryos labelled with anti-Pericardin (red) and anti-Mef2 (green) antibodies. (A) In wild-type embryos, Pericardin is localised at the basal surface of cc (arrow) and around pericardial cells (arrowhead). This localisation is disrupted in *LanB1*^{def} embryos (B). (**C-F**) Localisation of other ECM components, such as Perlecan (red in C and D) and Collagen IV (red in E and F), around the heart (arrows) is also altered in *LanB1*^{def} embryos. Perlecan localisation at muscle attachments remains unaffected (C,D, arrowheads).

found that Pericardin did not concentrate at the basal surface of cardioblasts or around pericardial cells in *LanB1*^{def} embryos but appeared diffuse (Fig. 4B). Note that loss of LanB1 caused an irregular morphology of the cardiac tube (Fig. 4B), a phenotype described for loss of individual laminin α chains (Haag et al., 1999; MacMullin and Jacobs, 2006). Moreover, Perlecan and Collagen IV failed to accumulate around cardiac cells in late *LanB1*^{def} mutant embryos (Fig. 4D,F) in contrast to wild type (Fig. 4C,E) (Friedrich et al., 2000; Natzle et al., 1982; Yasothornsrikul et al., 1997). On the contrary, Tiggrin showed a normal distribution at muscle attachment sites (data not shown), similar to that of Perlecan (arrowhead Fig. 4D and see Fig. S1 in the supplementary material). Finally, we found that the main laminin receptors, integrins and dystroglycans, showed a normal distribution in the absence of LanB1 (data not shown).

Ultrastructural analysis of embryos mutant for *LanB1* further supports the conclusion that laminins are essential for BM organisation in *Drosophila* embryos (Fig. 5). Late-stage *LanB1*^{def} embryos showed either no BMs or unorganised and diffuse ECM material associated with the surfaces of several tissues, such as the visceral musculature surrounding the midgut or hindgut, the Malphigian tubules or the perineural cells of the central nervous system (CNS; Fig. 5).

Taken together, these data suggest that laminins are essential for normal assembly of matrix components into BMs surrounding most tissues, but not for localisation of the specialised matrix found at muscle attachment sites, even though lamininW is a component of this matrix.

Laminins are required for the migration of different cell populations

The ECM promotes cell migration in many systems (Perris and Perissinotto, 2000). In *Drosophila*, the analysis of mutations in individual α chains has revealed that they are required for the migration of the dorsal tracheal trunk cells (Martin et al., 1999; Stark et al., 1997), but not for the migration of the endodermal midgut primordium (Martin et al., 1999). By contrast, integrins were shown to regulate the migration of most cell populations in the embryo, including the trachea, endoderm, salivary gland and macrophages (Boube et al., 2001; Bradley et al., 2003; Huelsmann et al., 2006; Martin-Bermudo et al., 1999; Roote and Zusman, 1995). There are at least two ways to explain this. On the one hand, different cells might use other ECM substrates besides laminins for their migration. On the other hand, both laminin trimers might play redundant functions in some migratory events, with the absence of one of them being compensated by the other. In order to distinguish between these two possibilities, we analysed the migration of these cell populations in *LanB1*^{1B1} mutant embryos.

The *Drosophila* midgut is composed of two cell layers: the endoderm and the visceral mesoderm. The midgut endoderm arises from two separated primordia situated in the anterior and posterior halves of the embryo, which migrate over the visceral mesoderm (vm) to form a continuous epithelium (Reuter et al., 1993; Skaer, 1993; Tepass and Hartenstein, 1994a). Towards the end of embryogenesis, the midgut constricts and lengthens to form a convoluted tube (Skaer, 1993). None of these processes are affected in absence of laminin $\alpha_{1,2}$ function (Martin et al., 1999). In addition, although apical basal polarity of endoderm is initially affected in the absence of laminin $\alpha_{3,5}$, midgut migration, constriction and elongation are also normal (Yarnitzky and Volk, 1995). By contrast, here we show that migration of midgut endodermal cells in *LanB1*^{1B1} embryos was delayed, so that anterior and posterior endoderm cells did not contact each other at stage 13, when wild-type cells have already met (Fig. 6A,B). Furthermore, by stage 15, multilayered clumping of the endoderm was observed, and formation of constrictions and midgut elongation was not seen in *LanB1* mutants (Fig. 6C,D). These results suggest that both laminin trimers act redundantly during midgut morphogenesis.

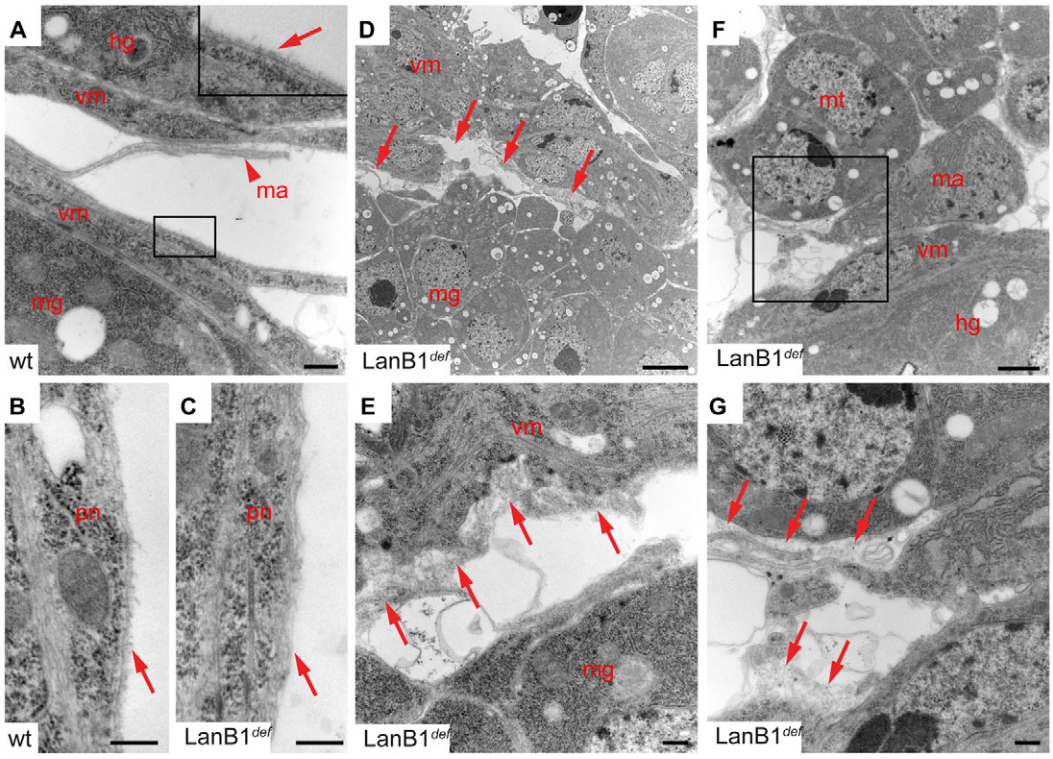

Fig. 5. Ultrastructural analysis of embryos lacking LanB1. (**A-G**) Wild-type (A,B) and *LanB1*^{def} mutant (C-G) embryos at early stage 17. (A) Basal surfaces of the midgut (mg) and hindgut that are closely associated with a layer of visceral musculature (vm). All tissue surfaces exposed to the haemolymph show a uniform layer of BM (arrow in inset). A lamellipodium of a macrophage is also seen that is not accumulating a BM (arrowhead). (B) The CNS is surrounded by a layer of perineural cells that is covered by BM in wild type. (C) In *LanB1* mutant embryos only residual ECM material is detected. (D) Gaps between mg and vm cells are apparent in *LanB1* mutants (arrows), often filled with unorganised and diffuse ECM material (E, arrows). (F) Section of Malphigian tubule (mt), hindgut with associated vm and a macrophage in *LanB1* mutant embryo (F). (G) Close up of boxed region in F. Arrows point to diffuse ECM material at the surface of the mt and vm. Scale bars: 1 μm in F; 2 μm in D; 100 nm in A,C,E,G. hg, hindgut; ma, macrophage; mg, midgut; mt, Malphigian tubule; pn, perineural cell; vm, visceral musculature.

Integrins have also been shown to regulate migration of embryonic macrophages and tracheal cells (Boube et al., 2001; Huelsmann et al., 2006). In wild type, macrophages from each pole of the embryo migrate along the VNC towards each other, so that by stage 13 they surround the entire midline of the VNC (Fig. 6E). By contrast, macrophages in *LanB1*^{1B1} mutant embryos failed to migrate along the VNC (Fig. 6F). Inhibition of macrophages migration blocks VNC condensation (Olofsson and Page, 2005), a defect we also observed in *LanB1* mutant embryos (Fig. 3F).

The tracheal or respiratory system of *Drosophila* arises from the embryonic placodes that invaginate and migrate in stereotyped directions to form a branched network of epithelial tubes (Manning and Krasnow, 1993). Previous analyses have shown that embryos lacking laminin $\alpha_{1,2}$ show prominent gaps in the dorsal tracheal trunk (Martin et al., 1999). Here, we found that elimination of *LanB1* enhanced the migration defects found in *wb* mutant embryos. Thus, in addition to gaps in the dorsal trunk (arrow in Fig. 6H), the visceral branch also failed to migrate along the vm in *LanB1* mutant embryos (arrowhead in Fig. 6H).

The salivary gland primordium forms a placode of columnar epithelial cells in the ventral ectoderm of parasegment two, which upon invagination turns and migrate along the vm (Bradley et al., 2003). In embryos lacking the PS1 and PS2 integrins, salivary cells invaginated but failed to migrate over the vm (Bradley et al., 2003). Similarly, the salivary glands of embryos lacking *LanB1* remained at the point of contact with the vm (Fig. 6J).

In conclusion, our analysis of *LanB1* mutant embryos reveals new functions for laminins in regulating cell migration during embryonic development. These migration defects resemble those previously described for integrin-compromised embryos (Huelsmann et al., 2006; Martin-Bermudo et al., 1999; Roote and Zusman, 1995; Boube et al., 2001). These results identify laminins as key ECM components regulating integrin-dependent cell-migration processes.

Laminins regulate coordinated cell movement during organogenesis

The laminin $\alpha_{1,2}$ has been involved in the process of germ-band retraction (Schock and Perrimon, 2003). Next, we decided to test whether laminins were required for the regulation of other coordinated cell movement driving epithelial morphogenesis during organogenesis. The proventriculus serves as a valve that regulates food passage from the foregut into the midgut. The foregut epithelium folds back on itself twice to form the valve of the proventriculus during late embryogenesis (Fig. 7A,A′, arrow) (Pankratz and Hoch, 1995). The late steps of cells moving inwards require integrins (Pankratz and Hoch, 1995). In addition, it has been shown that the region inside the proventriculus is rich in ECM components (King, 1988). Here, we found that the multilayered architecture of the proventriculus failed to form in *LanB1*^{1B1} mutants (Fig. 7B,B′, arrow). Defects in proventriculus formation prevent food from entering the midgut (Pankratz and Hoch, 1995). This is assayed by feeding larvae with yeast paste containing red dye and assessing whether food is

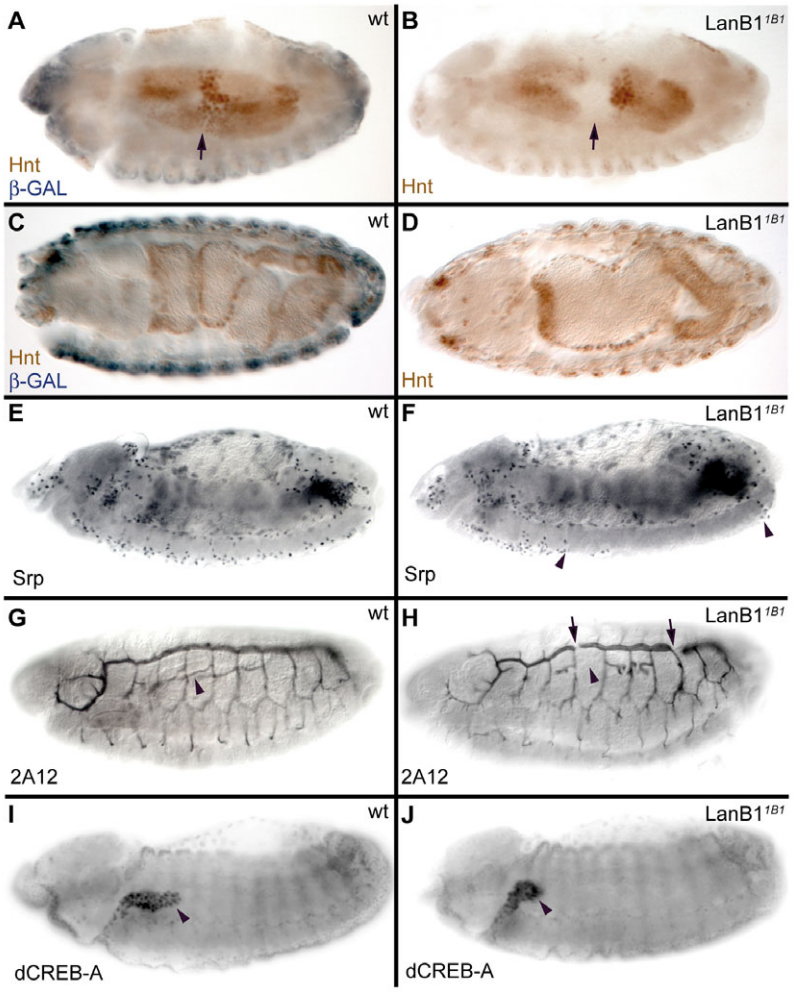

Fig. 6. Elimination of LanB1 function affects the migration of different cell populations. (**A-D**) Embryos were labelled with anti-Hindsight (Hnt, brown) and anti-β-gal (blue) antibodies to visualise the midgut (mg) cells and to mark the balancer chromosome, respectively. (A,B) The mg primordia has completed its migration and met in the centre of stage 13 wild-type embryos (A, arrow). By contrast, migration is delayed in *LanB1* mutant embryos (B, arrow). (C) Although mg constrictions are formed in stage 15 wild-type embryos, they are absent in *LanB1*^{1B1} mutant embryos (D). (**E**,**F**) In stage 13 wild-type embryos, macrophages have migrated and covered the VNC (E). This migration fails in *LanB1*^{1B1} embryos (F, arrowheads). (**G-J**) Similarly, migration of the visceral (H, arrowhead) and dorsal (H, arrow) branches of the trachea and salivary glands (J, arrowhead) is also affected in *LanB1*^{1B1} embryos.

pumped along the alimentary duct (Pankratz and Hoch, 1995). In wild-type larvae, the midgut and hindgut turn red after only 10 minutes of feeding (Fig. 7C). By contrast, *LanB1*^{28a} larvae showed no food accumulation even after 1 hour (Fig. 7D).

During development of the Malpighian tubules, tubes first grow by cell division of the so-called principal cells. Tubules are first short and thick with six to ten cells encircling the lumen. Then, tubules elongate by cell rearrangement and by insertion of the stellate cells (Fig. 7E, arrow) (Jung et al., 2005). In contrast to wild type, the Malpighian tubules of *LanB1*^{1B1} mutant embryos were abnormally short and of irregular thickness (Fig. 7F, arrow). This failure in tube elongation was not a consequence of stellate cells failing to incorporate in the tubules (insets in Fig. 7E,F). These findings suggest that laminins are required for cell rearrangement during Malphigian tubule morphogenesis.

Laminins are required for cell adhesion during mesoderm development and wing morphogenesis

Despite strong expression of laminins in all muscle attachment sites, only mild defects in the attachment of ventral oblique muscles were reported for laminin $\alpha_{1,2}$ mutants (Martin et al., 1999). This weak phenotype could be explained by redundancy between both laminin trimers. Thus, we decided to examine muscle defects in *LanB1*^{1B1}. We observed that, in addition to the ventral oblique muscles, several other muscles detached from tendon cells and adopted a round shape in *LanB1*^{1B1} mutants (data not shown). We focused our subsequent analysis on a single ventral longitudinal muscle [VL1, nomenclature according to Bate (Bate, 1990)]. This muscle can be visualised using the VL1-specific 5053Gal4 line (Swan et al., 2004) (Fig. 8A). In wild-type embryos, VL1 muscles were attached in 99.8% of cases analysed (n=180 segments; Fig. 8A,A′). However, in *LanB1*^{1B1} mutants, 11.1% of VL1 muscles were detached (n=180; Fig. 8B,B′). In addition, whereas muscle and epithelial cells of the midgut are normally closely associated with each other (Fig. 8C, Fig. 5A), gaps were apparent in *LanB1* mutants, suggesting a failure of adhesion between these two layers (Fig. 5D,E, Fig. 8D). We could also observe that although visceral circular muscles fused to binucleated cells as in wild type (Fig. 5D), they failed to stretch along the dorsoventral axis. Finally, missarrangement of longitudinal visceral muscles along the dorsoventral axis was also observed (Fig. 8B).

Mutations disrupting the laminin $\alpha_{1,2}$ or laminin $\alpha_{3,5}$ chains cause wing blisters (Henchcliffe et al., 1993; Martin et al., 1999; Woodruff and Ashburner, 1979). Accordingly, we found that flies carrying wing cells mutant for *LanB1* showed a strong wing blister phenotype (Fig. 9B,C). Wing blisters were also observed in *LanB1*^{28a}/*l*(*2*)*k05404* adults (data not shown). To further analyse LanB1 requirements for cell adhesion between dorsal and ventral wing surfaces, somatic clones were induced in *LanB1*^{1B1} and *LanB1*^{1P3} flies. Large dorsal or ventral clones (33 and 23 clones, respectively) differentiated normal cuticle, and the wing

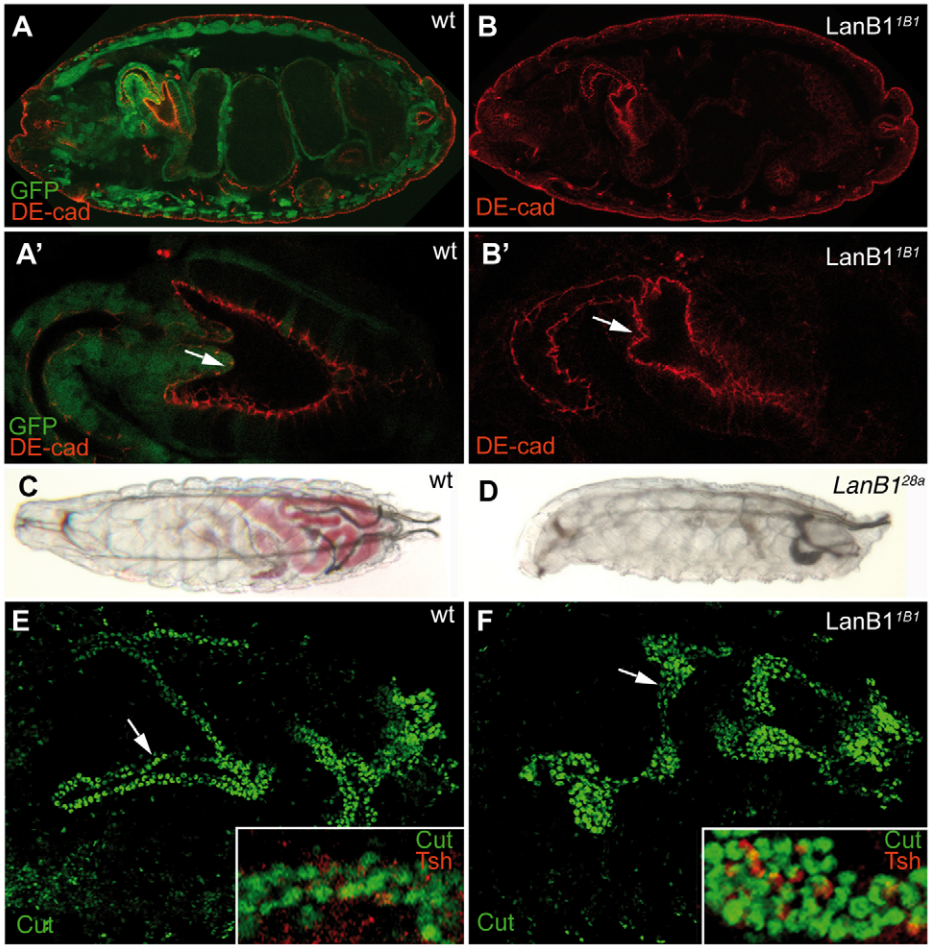

Fig. 7. Defects in tubulogenesis in LanB1 mutants. (**A-B'**) Embryos labelled with anti-DE-cadherin (DE-cad, red) and anti-GFP (green) antibodies to visualise the proventricular cells and to mark the balancer chromosome, respectively. (A',B') Magnifications of the proventriculus shown in A and B. (A,A') In wild-type embryos, proventricular cells have moved inward by stage 16 (arrow in A'). (B,B') This movement fails in homozygous *LanB1*^{1B1} embryos (arrow in B'). (**C**,**D**) Second instar larvae that have been fed with yeast containing red dye. Whereas wild-type larvae can feed normally and show a coloured gut (C), homozygous *LanB1*^{28a} larvae do not (D). (**E**) Wild-type Malpighian tubules of stage 17 embryos consist of four long tubes, as seen with a principal cell marker, anti-Cut antibody (green). (**F**) *LanB1*^{1B1} mutant Malpighian tubules are short and fat, but like the wild type contain principal (green) and stellate (red) cells (E,F, white boxes).

surfaces perfectly adhered to each other (not shown). By contrast, clones including mutant dorsal and ventral cells resulted in local blisters, only when they overlapped. They were localised between the L3 and L5 veins (Fig. 9D-F). Altogether, these results support a non-cell-autonomous requirement for laminin β during wing morphogenesis, most likely because laminins secreted by wild-type cells on either side of the wing can compensate for the loss of laminins on the opposite side. The blister phenotype strongly resembles defects associated with mutations in integrins, suggesting that laminins function as integrin ligands in the cell-adhesion system that maintains dorsal and ventral wing epithelia together.

DISCUSSION

The isolation of loss-of-function mutations in the single laminin β subunit encoded by the *Drosophila* genome has allowed us to study laminin requirements during development. We show that in absence of laminin β, other laminin subunits as well as major BMs components fail to assemble into BMs. Our analysis reveals new functions for laminins in cellular adhesion, migration and rearrangement during organogenesis.

Laminin β is essential for basement membrane formation

Data from cell-culture experiments suggest that only laminin trimers are secreted extracellularly. The current model is that a transitional dimeric configuration composed of a β and γ chain is first assembled intracellularly before incorporation of an α chain allows secretion (Goto et al., 2001; Kumagai et al., 1997; Morita et al., 1985; Peters et al., 1985). This model predicts that in absence of the laminin β or γ subunits no functional laminin trimers could be exported. Our experiments showing that in laminin β mutant embryos the laminin $\alpha_{3,5}$ and laminin $\alpha_{1,2}$ chains are not present at BMs or muscle attachment sites fully support this model.

The network of collagen IV was thought to provide an initial scaffold that incorporates other BM components, including laminins, nidogens and perlecan (Timpl et al., 1981; Yurchenco and Furthmayr, 1984). However, analysis of *collagen IV* mutants in mice and *C. elegans* showed that collagen IV was in fact dispensable for deposition and initial assembly of BMs (Guo et al., 1991; Poschl et al., 2004). Similarly, in *Drosophila* embryos lacking collagen IV or SPARC, a collagen IV interacting protein, the association of laminin and perlecan with cell surfaces was not affected until late embryogenesis (Martinek et al., 2008). However, when examined the other way around, genetic and developmental studies in the mouse and in *C. elegans* have demonstrated that laminins are essential for BM assembly in these two species (reviewed by Sasaki et al., 2004). Here, we show, at both microscopic and ultrastructural levels, that this is also the case in *Drosophila*. Thus, the crucial role of laminins as a scaffold for recruitment of BM components is conserved throughout animal evolution.

In contrast to BMs, we found that laminins are not required for assembly of ECM components at the specialised matrix present at muscle attachment sites. This could be explained by the fact that

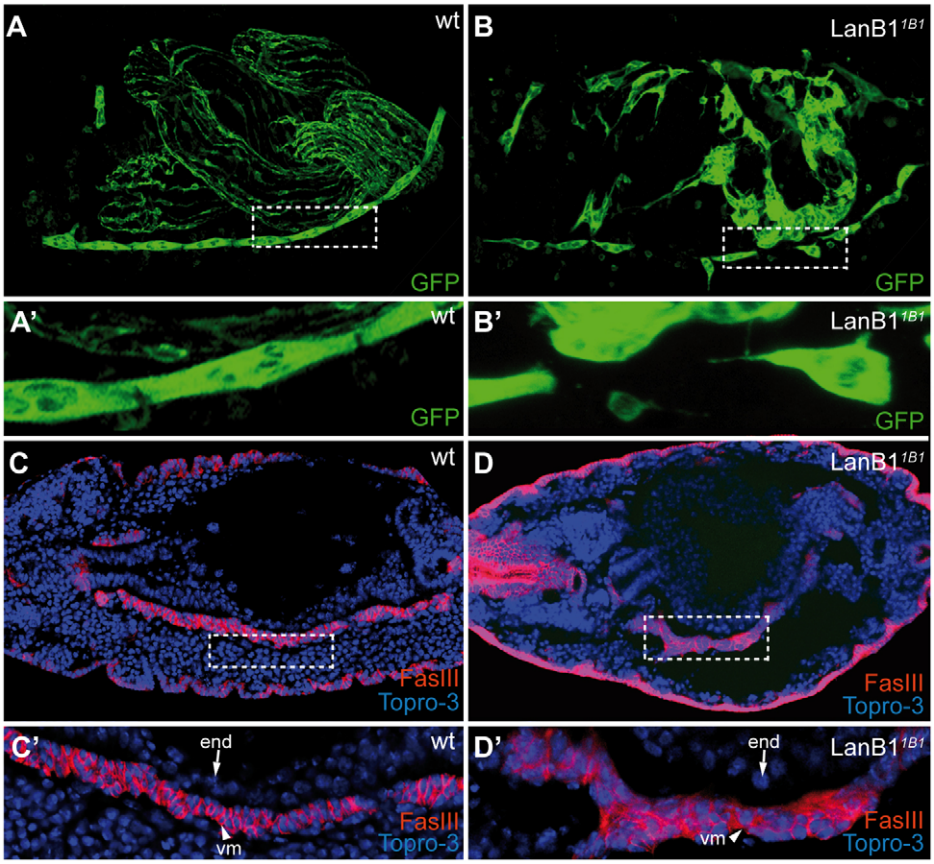

Fig. 8. Role of LanB1 during mesoderm development. (**A-B'**) Stage 17 wild-type (A) and homozygous *LanB1*^{1B1} (B) embryos expressing GFP in the VL1 and in longitudinal visceral musculature (vm). (A',B') Magnifications of the white boxes in A and B, respectively. Whereas all VL1 muscles are properly attached in wild-type embryos (A,A'), this attachment is disrupted in homozygous *LanB1*^{1B1} embryos (B,B'). (**C-D'**) Stage 13 wild-type (C) and homozygous *LanB1*^{1B1} (D) embryos stained with anti-FasIII (red) antibody to visualise the circular vm. (C',D') Magnifications of the white boxes in C and D, respectively. In the absence of LanB1 the circular vm (vm, arrowhead) detaches from the endoderm (end, arrow) and looks disorganised (compare C' with D').

assembly of this matrix clearly differs from assembly of BMs, as it is mainly mediated by cell-cell interactions (Martin-Bermudo and Brown, 2000). An alternative explanation might be that transmembrane receptors, such as integrins, could promote association of the tendon matrix to the cell surface independently of laminin networks. Our findings that integrins become localised to muscle attachment sites in the absence of laminins and integrins are essential for lamininW recruitment (Devenport et al., 2007) support this idea.

Laminins are not required for gastrulation in *Drosophila*

In mouse, laminin β1 is required for embryo implantation and gastrulation. *Lamb1*$^{-/-}$ embryos lack BMs and do not survive beyond embryonic day 5.5 (Miner et al., 2004). Similarly, in sea urchin embryos, injections with antibodies to laminin α chain inhibit gastrulation and spicule formation (Benson et al., 1999). However, RNAi inhibition of either β, γ or both α laminin genes in *C. elegans* did not impair embryonic development before the elongation stage (Kao et al., 2006), at which stage mutant embryos stopped developing and displayed severe defects in BM integrity and tissue development. These different requirements for laminins during early stages of embryonic development could be explained by considering that in sea urchins and amniotes the basal matrix forms shortly before gastrulation, whereas in nematodes, as well as in insects and amphibians, a basal lamina develops only at the end of gastrulation (Stern, 2004). An alternative explanation could be that laminins are required for epithelialisation and while in mammals this process precedes gastrulation, in nematodes, gastrulation precedes epithelialisation (for a review, see Li, S. et al., 2003). In *Drosophila* mutations in either of the two laminin trimers result in late embryonic lethality. However, the role of laminins during gastrulation in *Drosophila* has remained an open question, as each trimer could compensate for the absence of the other. Here, we show that removal of all laminins in *Drosophila* does not affect gastrulation, demonstrating that in *Drosophila*, and contrary to the mouse (Miner et al., 2004), the integrity of BMs is not crucial for this developmental event. Taken altogether, we conclude that, although the late functions during organogenesis are well conserved, an early function for laminins is absent in *Drosophila*.

New roles for laminins during embryogenesis

During midgut morphogenesis, both the migration of endodermal cells and the subsequent transition to a polarised epithelium depend on the association of the endoderm with the visceral mesoderm. Laminins are deposited between these two cell layers, yet the overall morphogenesis of the midgut, including migration, midgut constriction, tube elongation and adhesion of the endoderm to the visceral mesoderm, was shown to occur normally in mutants for the *Drosophila* laminin α3,5 chain (Yarnitzky and Volk, 1995). This led to the proposal that these processes might be mediated by other substrates. We show here that this other substrate is the second laminin, as all these processes are affected in *LanB1* mutant embryos. Whether this represents a unique function for the lamininW or requires both laminins awaits the analysis of these processes in embryos lacking just laminin $\alpha_{1,2}$.

Experiments in different model systems, such as the chick, axolotl and mouse, have demonstrated a role for laminins in the migration of different cell populations (Tzu and Marinkovich, 2008). One of the best-studied processes is the migration of the neural crest, which appears to migrate in response to heterogeneity in the ECM that forms their migration substrate. Collectively, these studies propose

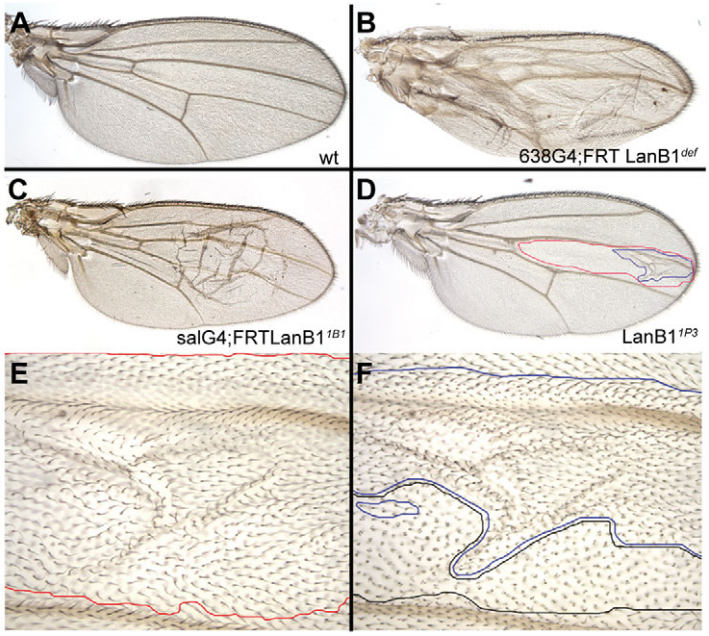

Fig. 9. LanB1 requirements in the wing. (**A**) Wild-type fly wing. (**B**,**C**) Generation of large territories of *LanB1* mutant cell in flies of genotype *638-Gal4; FRT40 LanB1^{def}/FRT40 M(2)z; UAS-FLP* (B) and *sal-Gal4; FRT40 LanB1^{1B1}/FRT40 M(2)z* (C) results in dorsoventral adhesion defects. (**D**) Wing blisters are formed only when LanB1 function is removed from both the ventral (blue line) and dorsal (red line) side of the wing. (**E**,**F**) Higher magnification of the ventral and dorsal sides of the wing shown in D.

that neural crest migration may be governed by the relative ratio of permissive ECM components, such as fibronectin and laminin, versus non-permissive ECM components, such as chondroitin sulphate proteoglycans. Other ECM molecules, such as vitronectin, perlecan and several collagen types, seem to play a neutral role in this process (Henderson and Copp, 1997; Perris and Perissinotto, 2000). In *Drosophila*, despite the implication of integrins in most migratory processes during embryogenesis, a clear role for laminins in regulating cell migration has remained elusive. To date, only mutations in the laminin $\alpha_{1,2}$ were shown to result in gaps in the tracheal dorsal trunk (Martin et al., 1999). Here, we show that laminins are required for all integrin-dependent migrations described so far, including that of the endoderm, macrophages, salivary glands and trachea visceral branches. These results identify the laminins as being either the key integrin ligands regulating cell migration during *Drosophila* embryogenesis, or essential for recruitment of key ligands into the migration substrate. In this new scenario, several questions now arise. What cells provide the laminins? Do other *Drosophila* ECM molecules, such as Collagen IV or Perlecan, permit or inhibit cell migration? Are other laminin receptors beside integrins involved in cell migration in *Drosophila*? Here, we show that in the absence of laminins, other BM components, such as Collagen IV and Perlecan, are not deposited around the VNC, and macrophages no longer migrate along this path. Inhibition of haemocyte migration impairs ECM deposition around the VNC (Olofsson and Page, 2005). Thus, as macrophages need matrix components to migrate and BM deposition around the VNC requires macrophage migration, it is tempting to speculate that macrophages might be able to deposit their own matrix molecules for migration, making them independent of the matrix of the environment. For example, human keratinocytes deposit laminin 332 to promote their linear migration (Frank and Carter, 2004).

Molecular mechanisms for laminin function

Laminins can interact with different types of receptors, including integrins, α-dystroglycan, sulphated carbohydrates (sulphatides, heparin, heparan sulphates and HNK-1) and the Lutheran antigen (Yurchenco et al., 2004). Several of the defects seen in *LanB1* mutants are remarkably similar to those reported for loss of integrin function, including defects in: adhesion between the wing surfaces; macrophage and tracheal cell migration; proventriculus morphogenesis, elongation and formation of constrictions during gut development; and adhesion between visceral mesoderm and endoderm (Bradley et al., 2003; Martin-Bermudo et al., 1999; Pankratz and Hoch, 1995; Stark et al., 1997). These similarities suggest that laminins use integrins as their main receptors to mediate cellular responses in these processes. By contrast, several defects observed in laminin mutant embryos are weaker than those observed in integrin mutants – for example, the attachment of muscles to tendon cells. Thus, whereas in integrin mutant embryos all somatic muscles detach, we found that in *LanB1* mutant embryos only a small proportion of muscles detached. These results suggest that other ECM components besides laminins can perform integrin-mediated attachment between muscles and tendon cells, and this is supported by the muscle detachment in embryos lacking thrombospondin (Chanana et al., 2007; Subramanian et al., 2007). The normal distribution of ECM molecules, such as Tiggrin or Perlecan, between muscle and tendon cells in the absence of laminins support this conclusion.

Our results are consistent with the idea that in *Drosophila*, as in mouse, laminins play a central role in organising the specialised ECM present at BMs and that this may represent a first important step for BM formation (Li et al., 2002). The establishment of a laminin-based BM scaffold is then crucial for many different cellular processes governing morphogenesis of most organs and tissues. Future studies are needed to address how laminins can perform such different developmental functions, including strong adhesion between different layers but also weak adhesion to allow cell migration. The information derived from these studies should help to understand the pathology of diseases related to abnormal laminin functions.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/24/4165/DC1

References

Andrew, D. J., Baig, A., Bhanot, P., Smolik, S. M. and Henderson, K. D. (1997). The Drosophila dCREB-A gene is required for dorsal/ventral patterning of the larval cuticle. *Development* **124**, 181-193.

Aumailley, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Yamada, Y. and Yurchenco, P. D. (2005). A simplified laminin nomenclature. *Matrix Biol.* **24**, 326-332.

Bate, M. (1990). The embryonic development of larval muscles in *Drosophila*. *Development* **110**, 791-804.

Benson, S., Page, L., Ingersoll, E., Rosenthal, E., Dungca, K. and Signor, D. (1999). Developmental characterization of the gene for laminin alpha-chain in sea urchin embryos. *Mech. Dev.* **81**, 37-49.

Boube, M., Martin-Bermudo, M. D., Brown, N. and Casanova, J. (2001). Specific tracheal migration is mediated by complementary expression of cell surface proteins. *Genes Dev.* **15**, 1554-1562.

Bradley, P. L., Myat, M. M., Comeaux, C. A. and Andrew, D. J. (2003). Posterior migration of the salivary gland requires an intact visceral mesoderm and integrin function. *Dev. Biol.* **257**, 249-262.

Chanana, B., Graf, R., Koledachkina, T., Pflanz, R. and Vorbruggen, G. (2007). AlphaPS2 integrin-mediated muscle attachment in Drosophila requires the ECM protein Thrombospondin. *Mech. Dev.* **124**, 463-475.

Chartier, A., Zaffran, S., Astier, M., Semeriva, M. and Gratecos, D. (2002). Pericardin, a Drosophila type IV collagen-like protein is involved in the morphogenesis and maintenance of the heart epithelium during dorsal ectoderm closure. *Development* **129**, 3241-3253.

Chou, T. B. and Perrimon, N. (1992). Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. *Genetics* **131**, 643-653.

Colognato, H. and Yurchenco, P. D. (2000). Form and function: the laminin family of heterotrimers. *Dev. Dyn.* **218**, 213-234.

Deng, W.-M. and Ruohola-Baker, H. (2000). LamininA is required for follicle cell-oocyte signaling that leads to establishment of the anterior-posterior axis in *Drosophila*. *Curr. Biol.* **10**, 683-686.

Devenport, D., Bunch, T. A., Bloor, J. W., Brower, D. L. and Brown, N. H. (2007). Mutations in the Drosophila alphaPS2 integrin subunit uncover new features of adhesion site assembly. *Dev. Biol.* **308**, 294-308.

Fessler, J. H. and Fessler, L. I. (1989). *Drosophila* extracellular matrix. *Annu. Rev. Cell Biol.* **5**, 309-339.

Fogerty, F. J., Fessler, L. I., Bunch, T. A., Yaron, Y., Parker, C. G., Nelson, R. E., Brower, D. L., Gullberg, D. and Fessler, J. H. (1994). Tiggrin, a novel *Drosophila* extracellular matrix protein that functions as a ligand for *Drosophila* alpha PS2 beta PS integrins. *Development* **120**, 1747-1758.

Frank, D. E. and Carter, W. G. (2004). Laminin 5 deposition regulates keratinocyte polarization and persistent migration. *J. Cell Sci.* **117**, 1351-1363.

Friedrich, M. V., Schneider, M., Timpl, R. and Baumgartner, S. (2000). Perlecan domain V of Drosophila melanogaster. Sequence, recombinant analysis and tissue expression. *Eur. J. Biochem.* **267**, 3149-3159.

Fristrom, D., Wilcox, M. and Fristrom, J. (1993). The distribution of PS integrins, Laminin A and F-actin during key stages in *Drosophila* wing development. *Development* **117**, 509-523.

Garcia-Alonso, L., Fetter, R. D. and Goodman, C. S. (1996). Genetic analysis of Laminin A in Drosophila: extracellular matrix containing laminin A is required for ocellar axon pathfinding. *Development* **122**, 2611-2621.

Goto, A., Aoki, M., Ichihara, S. and Kitagawa, Y. (2001). alpha-, beta- or gamma-chain-specific RNA interference of laminin assembly in Drosophila Kc167 cells. *Biochem. J.* **360**, 167-172.

Gotwals, P. J., Fessler, L. I., Wehril, M. and Hynes, R. O. (1994). *Drosophila* PS1 integrin is a laminin receptor and differs in ligand specificity from PS2. *Proc. Natl. Acad. Sci. USA* **91**, 11447-11451.

Graner, M. W., Bunch, T. A., Baumgartner, S., Kerschen, A. and Brower, D. L. (1998). Splice variants of the Drosophila PS2 integrins differentially interact with RGD-containing fragments of the extracellular proteins tiggrin, ten-m, and D-laminin 2. *J. Biol. Chem.* **273**, 18235-18241.

Guo, X. D., Johnson, J. J. and Kramer, J. M. (1991). Embryonic lethality caused by mutations in basement membrane collagen of C. elegans. *Nature* **349**, 707-709.

Haag, T. A., Haag, N. P., Lekven, A. C. and Hartenstein, V. (1999). The role of cell adhesion molecules in Drosophila heart morphogenesis: faint sausage, shotgun/DE-cadherin, and laminin A are required for discrete stages in heart development. *Dev. Biol.* **208**, 56-69.

Helbling-Leclerc, A., Zhang, X., Topaloglu, H., Cruaud, C., Tesson, F., Weissenbach, J., Tome, F. M., Schwartz, K., Fardeau, M., Tryggvason, K. et al. (1995). Mutations in the laminin alpha 2-chain gene (LAMA2) cause merosin-deficient congenital muscular dystrophy. *Nat. Genet.* **11**, 216-218.

Henchcliffe, C., Garcia-Alonso, L., Tang, J. and Goodman, C. S. (1993). Genetic analysis of laminin A reveals diverse functions during morphogenesis in Drosophila. *Development* **118**, 325-337.

Henderson, D. J. and Copp, A. J. (1997). Role of the extracellular matrix in neural crest cell migration. *J. Anat.* **191**, 507-515.

Huelsmann, S., Hepper, C., Marchese, D., Knoll, C. and Reuter, R. (2006). The PDZ-GEF Dizzy regulates cell shpe of migrating macrophages via Rap1 and integrins in the Drosophila embryo. *Development* **133**, 2915-2924.

Jung, A. C., Denholm, B., Skaer, H. and Affolter, M. (2005). Renal tubule development in *Drosophila*: a closer look at the cellular level. *J. Am. Soc. Nephrology* **16**, 322-328.

Kao, G., Huang, C. C., Hedgecock, E. M., Hall, D. H. and Wadsworth, W. G. (2006). The role of the laminin beta subunit in laminin heterotrimer assembly and basement membrane function and development in C. elegans. *Dev. Biol.* **290**, 211-219.

Kiehart, D. P. and Feghali, R. (1986). Cytoplasmic myosin from *Drosophila melanogaster*. *J. Cell Biol.* **103**, 1517-1525.

King, D. G. (1988). Cellular organization and peritrophic membrane formation in the cardia (proventriculus) of Drosophila melanogaster. *J. Morphol.* **196**, 253-282.

Kumagai, C., Kadowaki, T. and Kitagawa, Y. (1997). Disulfide-bonding between Drosophila laminin beta and gamma chains is essential for alpha chain to form alpha betagamma trimer. *FEBS Lett.* **412**, 211-216.

Li, J., Tzu, J., Chen, Y., Zhang, Y. P., Nguyen, N. T., Gao, J., Bradley, M., Keene, D. R., Oro, A. E., Miner, J. H. et al. (2003). Laminin-10 is crucial for hair morphogenesis. *EMBO J.* **22**, 2400-2410.

Li, S., Harrison, D., Carbonetto, S., Fassler, R., Smyth, N., Edgar, D. and Yurchenco, P. D. (2002). Matrix assembly, regulation, and survival functions of laminin and its receptors in embryonic stem cell differentiation. *J. Cell Biol.* **157**, 1279-1290.

Li, S., Edgar, D., Fassler, R., Wadsworth, W. and Yurchenco, P. D. (2003). The role of laminin in embryonic cell polarization and tissue organization. *Dev. Cell* **4**, 613-624.

Lunstrum, G. P., Bachinger, H. P., Fessler, L. I., Duncan, K. G., Nelson, R. E. and Fessler, J. H. (1988). Drosophila basement membrane procollagen IV. I. Protein characterization and distribution. *J. Biol. Chem.* **263**, 18318-18327.

MacMullin, A. and Jacobs, J. R. (2006). Slit coordinates cardiac morphogenesis in Drosophila. *Dev. Biol.* **293**, 154-164.

Manning, G. and Krasnow, M. A. (1993). Development of the Drosophila tracheal system. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), vol. I, pp. 609-685. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Martin, D., Zusman, S., Li, X., Williams, E. L., Khare, N., DaRocha, S., Chiquet-Ehrismann, R. and Baumgartner, S. (1999). *wing blister*, a new *drosophila* Laminin a chain required for cell adhesion and migration during embryonic and imaginal development. *J. Cell Biol.* **145**, 191-201.

Martin-Bermudo, M. D. and Brown, N. (2000). The localized assembly of extracellular matrix ligands requires cell-cell contact. *J. Cell Sci.* **113**, 3715-3723.

Martin-Bermudo, M. D., Alvarez-Garcia, I. and Brown, N. H. (1999). Migration of the *drosophila* primordial midgut cells requires coordination of diverse PS integrin functions. *Development* **126**, 5161-5169.

Martinek, N., Shahab, J., Saathoff, M. and Ringuette, M. (2008). Haemocyte-derived SPARC is required for collagen-IV-dependent stability of basal laminae in Drosophila embryos. *J. Cell Sci.***121**, 1671-1678.

Miner, J. H., Li, C., Mudd, J. L., Go, G. and Sutherland, A. E. (2004). Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. *Development* **131**, 2247-2256.

Molnar, C. and de Celis, J. F. (2006). Independent roles of Drosophila Moesin in imaginal disc morphogenesis and hedgehog signalling. *Mech. Dev.* **123**, 337-351.

Montell, D. J. and Goodman, C. S. (1989). Drosophila laminin: sequence of B2 subunit and expression of all three subunits during embryogenesis. *J. Cell Biol.* **109**, 2441-2453.

Morita, A., Sugimoto, E. and Kitagawa, Y. (1985). Post-translational assembly and glycosylation of laminin subunits in parietal endoderm-like F9 cells. *Biochem. J.* **229**, 259-264.

Natzle, J. E., Monson, J. M. and McCarthy, B. J. (1982). Cytogenetic location and expression of collagen-like genes in *Drosophila*. *Nature* **296**, 368-371.

Olofsson, B. and Page, D. T. (2005). Condensation of the central nervous system in embryonic Drosophila is inhibited by blocking hemocyte migration or neural activity. *Dev. Biol.* **279**, 233-243.
Pankratz, M. J. and Hoch, M. (1995). Control by epithelial morphogenesis by cell signaling and integrin molecules in the *Drosophila* foregut. *Development* **121**, 1885-1898.
Parks, A. L., Cook, K. R., Belvin, M., Dompe, N. A., Fawcett, R., Huppert, K., Tan, L. R., Winter, C. G., Bogart, K. P., Deal, J. E. et al. (2004). Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat. Genet.* **36**, 288-292.
Perris, R. and Perissinotto, D. (2000). Role of the extracellular matrix during neural crest cell migration. *Mech. Dev.* **95**, 3-21.
Peters, B. P., Hartle, R. J., Krzesicki, R. F., Kroll, T. G., Perini, F., Balun, J. E., Goldstein, I. J. and Ruddon, R. W. (1985). The biosynthesis, processing, and secretion of laminin by human choriocarcinoma cells. *J. Biol. Chem.* **260**, 14732-14742.
Poschl, E., Schlotzer-Schrehardt, U., Brachvogel, B., Saito, K., Ninomiya, Y. and Mayer, U. (2004). Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. *Development* **131**, 1619-1628.
Prokop, A., Martin-Bermudo, M. D., Bate, M. and Brown, N. H. (1998). In *Drosophila* embryos, the absence of the PS integrins or laminin A affects the extracellular adhesion of hemiadherens and neuromuscular junctions, but not their intracellular assembly. *Dev. Biol.* **196**, 58-76.
Pulkkinen, L. and Uitto, J. (1999). Mutation analysis and molecular genetics of epidermolysis bullosa. *Matrix Biol.* **18**, 29-42.
Reuter, R. (1994). The gene serpent has homeotic properties and specifies endoderm versus ectoderm within the Drosophila gut. *Development* **120**, 1123-1135.
Reuter, R., Grunewald, B. and Leptin, M. (1993). A role for mesoderm in endodermal migration and morphogenesis in Drosophila. *Development* **119**, 1135-1145.
Roote, C. E. and Zusman, S. (1995). Functions for PS integrins in tissue adhesion, migration, and shape changes during early embryonic development in *Drosophila*. *Dev. Biol.* **169**, 322-336.
Sasaki, T., Fassler, R. and Hohenester, E. (2004). Laminin: the crux of basement membrane assembly. *J. Cell Biol.* **164**, 959-963.
Schneider, M., Khalil, A. A., Poulton, J., Castillejo-Lopez, C., Egger-Adam, D., Wodarz, A., Deng, W. M. and Baumgartner, S. (2006). Perlecan and Dystroglycan act at the basal side of the Drosophila follicular epithelium to maintain epithelial organization. *Development* **133**, 3805-3815.
Schock, F. and Perrimon, N. (2003). Retraction of the Drosophila germ band requires cell-matrix interaction. *Genes Dev.* **17**, 597-602.
Skaer, H. (1993). The alimentary canal. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 941-1012. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Smyth, N., Vatansever, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M. and Edgar, D. (1999). Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. *J. Cell Biol.* **144**, 151-160.
Stark, K. A., Yee, G. H., Roote, C. E., Williams, E. L., Zusman, S. and Hynes, R. O. (1997). A novel a integrin subunit associates with bPS and functions in tissue morphogenesis and movement during *Drosophila* development. *Development* **124**, 4583-4594.
Stern, C. D. (2004). *Gastrulation: From Cells to Embryo*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Subramanian, A., Wayburn, B., Bunch, T. and Volk, T. (2007). Thrombospondin-mediated adhesion is essential for the formation of the myotendinous junction in Drosophila. *Development* **134**, 1269-1278.
Swan, L. E., Wichmann, C., Prange, U., Schmid, A., Schmidt, M., Schwarz, T., Ponimaskin, E., Madeo, F., Vorbruggen, G. and Sigrist, S. J. (2004). A glutamate receptor-interacting protein homolog organizes muscle guidance in Drosophila. *Genes Dev.* **18**, 223-237.
Tepass, U. and Hartenstein, V. (1994a). Epithelium formation in the Drosophila midgut depends on the interaction of endoderm and mesoderm. *Development* **120**, 579-590.
Tepass, U. and Hartenstein, V. (1994b). The development of cellular junctions in the Drosophila embryo. *Dev. Biol.* **161**, 563-596.
Timpl, R. (1996). Macromolecular organization of basement membranes. *Curr. Opin. Cell Biol.* **8**, 618-624.
Timpl, R. and Brown, J. C. (1994). The laminins. *Matrix Biol.* **14**, 275-281.
Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M. and Martin, G. R. (1979). Laminin-a glycoprotein from basement membranes. *J. Biol. Chem.* **254**, 9933-9937.
Timpl, R., Wiedemann, H., van Delden, V., Furthmayr, H. and Kuhn, K. (1981). A network model for the organization of type IV collagen molecules in basement membranes. *Eur. J. Biochem.* **120**, 203-211.
Toba, G., Ohsako, T., Miyata, N., Ohtsuka, T., Seong, K. H. and Aigaki, T. (1999). The gene search system. A method for efficient detection and rapid molecular identification of genes in Drosophila melanogaster. *Genetics* **151**, 725-737.
Tzu, J. and Marinkovich, M. P. (2008). Bridging structure with function: structural, regulatory, and developmental role of laminins. *Int. J. Biochem. Cell Biol.* **40**, 199-214.
Woodruff, R. C. and Ashburner, M. (1979). The genetics of a small autosomal region of Drosophila melanogaster containing the structural gene for alcohol dehydrogenase. II. Lethal mutations in the region. *Genetics* **92**, 133-149.
Wu, J. and Cohen, S. M. (2000). Proximal distal axis formation in the Drosophila leg: distinct functions of teashirt and homothorax in the proximal leg. *Mech. Dev.* **94**, 47-56.
Yarnitzky, T. and Volk, T. (1995). Laminin is required for heart, somatic muscles, and gut development in the *Drosophila* embryo. *Dev. Biol.* **169**, 609-618.
Yasothornsrikul, S., Davis, W. J., Cramer, G. D. A. K. and Dearolf, C. R. (1997). viking:identification and characterization of a second type IV collagen in Drosophila. *Gene* **198**, 17-25.
Yurchenco, P. D. and Furthmayr, H. (1984). Self-assembly of basement membrane collagen. *Biochemistry* **23**, 1839-1850.
Yurchenco, P. D., Quan, Y., Colognato, H., Mathus, T., Harrison, D., Yamada, Y. and O'Rear, J. J. (1997). The alpha chain of laminin-1 is independently secreted and drives secretion of its beta- and gamma-chain partners. *Proc. Natl. Acad. Sci. USA* **94**, 10189-10194.
Yurchenco, P. D., Amenta, P. S. and Patton, B. L. (2004). Basement membrane assembly, stability and activities observed through a developmental lens. *Matrix Biol.* **22**, 521-538.