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

Jose M. Urbano1,*, Catherine N. Torgler1,4,*, Cristina Molnar2,*, Ulrich Tepass3, Ana López-Varea2, Nicholas H. Brown4, Jose F. de Celis2 and Maria D. Martin-Bermudo1,5,†

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 only 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, α3,5 (encoded by Laminin A, LanA), is most similar to vertebrate α3 and α5 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, α1,2 (encoded by wing blister, wb), is most similar to vertebrate α1 and α2 chains and is part of the lamininW trimer. α1,2 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 α1,2
can serve as PS2 integrin ligands (Graner et al., 1998). This is further supported by studies showing that laminin W 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 α3,5 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 wβ have wing blisters and defects in the dorsal vessel, trachea, muscles and rhabdomeres (Martin et al., 1999). Mutations in the lanin Aβ subunit (encoded by Laminin A1, LanA1) 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 LanB128a hypomorphic allele was generated by P-element imprecise excision of P{EP}EP2178 using standard procedures. LanB128a deletes the first non-coding exon and most of the first intron without interrupting the second intron (Fig. 1A).

LanB11P3 and LanB12P3 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 (Noliar and de Celis, 2006) (Fig. 1A) 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 individual lines were screened and two revertant embryonic lethal lines, LanB11P3, were isolated. A total of 9500 embryos were induced in flies of the following genotypes: (P3) and e02263/P2) and when combined with the wing GAL4 driver 638-Gal4 UAS-FLP; FRT40M 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-d-CREB A (Andrew et al., 1997); anti-GFP (Molecular Probes); anti-Laminin β, anti-Laminin A, anti-α3,5 (Kumagai et al., 1997); anti-MEF2 (Han Nguyen); anti-Muscle myosin (Kiehart and Feghali, 1986); anti-Pericardin (Chartier et al., 2002); anti-Perlecain (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 were used: Alexa fluor 488 (Green), Alexa fluor 656 (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).

**RESULTS**

**Generation of laminin γ1 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 (LanB11P3 and LanB12P3) and a LanB1 deficiency, LanB11P3 (Fig. 1A; Materials and methods). The LanB11P3 and LanB12P3 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 LanB12P3 and LanB11P3 homozygous mutant embryos are equivalent to those of LanB11P3 and LanB12P3 over deficiencies. Analysis of homozygous zygotic LanB11P3 and LanB12P3 mutant embryos revealed that they died at stage 17, as did embryos lacking both zygotic and maternal LanB1. The LanB12P3 allele deletes only the first non-coding exon; therefore, it is likely to affect only the LanB1-RA transcript (Fig. 1A).

In addition, LanB12P3 homozygous mutant animals died at larval stages, demonstrating that it is a hypomorphic allele. Accordingly, viability of LanB12P3 was further reduced in combination with LanB11P3. The LanB12P3 allele eliminated the entire LanB1 gene and also the 5′ UTR of the adjacent CG72143 gene (Fig. 1A), and exhibited the same phenotype as LanB11P3 and LanB12P3.

**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
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\textsuperscript{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-α\textsubscript{3,5} and anti-α\textsubscript{4,2} antibodies. We found that, whereas in wild-type embryos laminin α\textsubscript{3,5} localises to the BM of different tissues, this localisation was lost in LanB1\textsuperscript{def} embryos (compare Fig. 1D with 1E). This is also the case for α\textsubscript{4,2}, which could not be found at BMs or muscle attachment sites in LanB1\textsuperscript{def} embryos (data not shown).

We therefore conclude that during embryogenesis the discrete accumulation of the α\textsubscript{3,5} and α\textsubscript{4,2} 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 (arrowhead) and at muscle attachment sites (arrow). (C) This expression is lost in maternal and zygotic homozygous LanB1\textsuperscript{def} mutant embryos. (D) In wild-type embryos, LanA (red) is found in BMs. (E) This expression is not detected in LanB1\textsuperscript{def} mutant embryos. In all figures, the nuclear marker TO-PRO 3 is in blue. wt, wild type.
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. 4 A), whereas Tiggrin localises predominantly at muscle attachment sites (Fogerty et al., 1994). We

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**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').

**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<sup>−/−</sup> embryos. (C) In addition, Collagen IV localisation in the ventral nerve cord channels of a stage 15 embryo (arrow) is lost in LanB1<sup>−/−</sup> 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<sup>−/−</sup> mutants. White horizontal bars indicate the length of CNS condensation.
found that Pericardin did not concentrate at the basal surface of cardioblasts or around pericardial cells in \textit{LanB1\textsuperscript{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 \(\alpha\) chains (Haag et al., 1999; MacMullin and Jacobs, 2006). Moreover, Perlecan and Collagen IV failed to accumulate around cardiac cells in late \textit{LanB1\textsuperscript{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 \textit{LanB1} further supports the conclusion that laminins are essential for BM organisation in \textit{Drosophila} embryos (Fig. 5). Late-stage \textit{LanB1\textsuperscript{def}} embryos showed either no BMs or unorganised and diffuse ECM material associated with the surfaces of several tissues, such as the visceral musclecutaneous 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 \textit{Drosophila}, the analysis of mutations in individual \(\alpha\) 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; Ronne 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 \textit{LanB1\textsuperscript{BI}} mutant embryos.

The \textit{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 epithilium (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 \(\alpha_{3,5}\) function (Martin et al., 1999). In addition, although apical basolateral 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 \textit{LanB1\textsuperscript{BI}} 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 \textit{LanB1} mutants (Fig. 6C,D). These results suggest that both laminin trimers act redundantly during midgut morphogenesis.

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 \textit{LanB1\textsuperscript{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 \textit{LanB1\textsuperscript{def}} embryos. Perlecan localisation at muscle attachments remains unaffected (C,D, arrowheads).
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 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 α1,2 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. 6E), the visceral branch also failed to migrate along the vm in LanB1 mutant embryos (arrowhead in Fig. 6H).

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 α1,2 has been involved in the process of germ-band retraction (Schöck 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 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
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, LanB128a 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 LanB11B1 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 Malpighian 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 α1,2 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 LanB1181. We observed that, in addition to the ventral oblique muscles, several other muscles detached from tendon cells and adopted a round shape in LanB1181 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 LanB1181 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 α1,2 or laminin α3,5 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 LanB1281/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 LanB1181 and LanB11P3 flies. Large dorsal or ventral clones (33 and 23 clones, respectively) differentiated normal cuticle, and the wing
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 \( \beta \) 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 \( \beta \) subunit encoded by the *Drosophila* genome has allowed us to study laminin requirements during development. We show that in absence of laminin \( \beta \), other laminin subunits as well as major BM components fail to assemble into BMs. Our analysis reveals new functions for laminins in cellular adhesion, migration and rearrangement during organogenesis.

**Laminin \( \beta \) 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 \( \beta \) and \( \gamma \) chain is first assembled intracellularly before incorporation of an \( \alpha \) 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 \( \beta \) or \( \gamma \) subunits no functional laminin trimers could be exported. Our experiments showing that in laminin \( \beta \) 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...
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. LamBt−/− 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 annelids, the basal matrix forms shortly before gastrulation, whereas in nematodes, and 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 β1,2.

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
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 α1,3 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 permissive, laminins 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 Tigrin 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.

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 (Yurchenko 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 Tigrin or Perlecan, between muscle and tendon cells in the absence of laminins support this conclusion.

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 LanB1def/FRT40 M, z; UAS-FLP (B) and sal-Gal4; FRT40 LanB1def/FRT40 Mダga (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.
RGD-containing fragments of the extracellular proteins tiggin, ten-m, and D-laminin 2. J. Biol. Chem. 273, 18235-18241.


