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Sonic hedgehog-dependent synthesis of laminin α1 controls basement membrane assembly in the myotome

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Basement membranes have essential structural and signalling roles in tissue morphogenesis during embryonic development, but the mechanisms that control their formation are still poorly understood. Laminins are key components of basement membranes and are thought to be essential for initiation of basement membrane assembly. Here, we report that muscle progenitor cells populating the myotome migrate aberrantly in the ventral somite in the absence of sonic hedgehog (Shh) signalling, and we show that this defect is due to the failure to form a myotomal basement membrane. We reveal that expression of *Lama1*, which encodes laminin α1, a subunit of laminin-111, is not activated in *Shh*$^{-/-}$ embryos. Recovery of *Lama1* expression or addition of exogenous laminin-111 to *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos restores the myotomal basement membrane, demonstrating that laminin-111 is necessary and sufficient to initiate assembly of the myotomal basement membrane. This study uncovers an essential role for Shh signalling in the control of laminin-111 synthesis and in the initiation of basement membrane assembly in the myotome. Furthermore, our data indicate that laminin-111 function cannot be compensated by laminin-511.

KEY WORDS: Laminin, Myf5, Sonic hedgehog, Basement membrane, Myogenesis, Myotome, Mouse

INTRODUCTION

Skeletal muscles originate mostly from the dermomyotome, an epithelial structure forming in the dorsal somitic compartment, which contains Pax3-positive progenitor cells for trunk and limb skeletal muscles. The myotome is a transient somitic structure located underneath the dermomyotome. This is where myogenesis is initiated during embryonic development when the myogenic regulatory factors, Myf5, MyoD, MRF4 and myogenin are activated (Pownall et al., 2002). Myotome formation begins when Myf5-positive muscle progenitor cells (MPCs) delaminate from the dorso-medial lip of the dermomyotome, and translocate underneath it to form the epaxial myotome (Denetclaw et al., 1997; Gros et al., 2004; Kahane et al., 1998b; Venters et al., 1999). Previous studies have shown that a first wave of MPCs populate the entire extent of the epaxial myotome, elongate in a bi-directional manner along the rostro-caudal axis and differentiate into myocytes (Gros et al., 2004; Venters et al., 1999). Coinciding with their entry in the myotome, MPCs initiate and upregulate the expression of the laminin receptors, integrin α6β1 and dystroglycan (Anderson et al., 2007; Bajanca et al., 2004; Cachaco et al., 2005), and begin assembling the myotomal basement membrane that separates myotome from sclerotome (Bajanca et al., 2004; Tosney et al., 1994). As development proceeds, a second wave of MPCs sequentially populate the myotome from the caudal, rostral and lateral edges of the dermomyotome and participate in the formation of the central and hypaxial myotomes, respectively (Gros et al., 2004; Kahane et al., 1998a; Kahane et al., 2002). During this phase, basement membrane components are progressively added, leading to the formation of a continuous myotomal basement membrane in rostral somites. This strongly suggests that the myotomal basement membrane has an important structural function that shapes and separates the myotome from the underlying sclerotome. It also acts as a scaffold for the migration of MPCs and non-muscle cells, such as neural crest cells (Tosney et al., 1994). Finally, it might have an important signalling function that controls MPC behaviour. Indeed, no myotomal basement membrane forms in the absence of Myf5, and MPCs migrate aberrantly in the dorsal and ventral somite, adopt non-myogenic cell fates and fail to form a myotome (Tajbakhsh et al., 1996a). Furthermore, blockade of the interaction of laminin with its receptor, α6β1 integrin, in primary MPCs also results in severe disruption in myotome formation (Bajanca et al., 2006). These observations suggest that timely formation of the myotomal basement membrane has a crucial role on the subsequent patterning and growth of the myotome.

Basement membranes are specialised extracellular matrix structures that consist mainly of the glycoproteins laminin and nidogen, of collagen IV, and of the heparan sulphate proteoglycan (HSPG) perlecan, and are key to several cellular processes such as migration, proliferation, differentiation and morphogenesis that control early embryonic development. Laminin is a heterotrimer made of three subunits α, β and γ, which assemble into a cross-shaped macromolecule (Colognato and Yurchenco, 2000). In the mouse embryo, laminin-111 (α1β1γ1, formerly known as laminin 1) and laminin-511 (α5β1γ1, formerly known as laminin 10) are the primary laminins expressed during early stages of myotome formation (Cachaco et al., 2005; Li et al., 2003). Basement membrane assembly remains incompletely elucidated. However, because both laminin and collagen IV can self-assemble to form polymeric chains in vitro (Cheng et al., 1997; Yurchenco and Ruben, 1987), it has been proposed that laminin self-assembly into polymers incorporated into the nascent basement membrane constitutes the first and a prerequisite step for the integration of other basement membrane components (Li et al., 2002). Consistent with this hypothesis, laminin β1 (*Lamb1*) and laminin γ1 (*Lamg1*) mouse knockout embryos and ES embryoid bodies fail to form basement membranes and die at peri-implantation stages (Li et al., 2002; Miner et al., 2004; Smyth et al., 1999). By contrast, collagen IV,

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nidogen-1, nidogen-2 and perlecan mouse mutant embryos have a late embryonic lethality and partially affected basement membranes, which is consistent with roles in basement membrane stability and signalling, but not assembly (Bader et al., 2005; Costell et al., 1999; Poschl et al., 2004). Thus, a current model suggests that nidogen acts as a link between the laminin polymer and the collagen IV polymer, bringing together laminin and collagen IV networks and recruiting perlecan, although perlecan can also directly bind to laminin (Timpl and Brown, 1996).

Basement membranes attach to the cell surface through the laminin receptors, integrins and dystroglycan. In the mouse and chick embryo, α6β1, α1β1 and α7β1 integrins are the primary laminin receptors expressed in the myotome, with α6β1 integrin being the earliest laminin receptor expressed at the surface of primary epaxial MPCs (Bajanca et al., 2004; Bronner-Fraser et al., 1992; Cachaco et al., 2005; Duband et al., 1992; Gullberg et al., 1999). Likewise, dystroglycan is upregulated when MPCs enter the epaxial myotome, and becomes polarised as the myotomal basement membrane assembles (Anderson et al., 2007). Previous studies have led to the hypothesis that dystroglycan and integrin are not involved in basement membrane assembly per se, but anchor and cluster laminin to the cell surface, thus facilitating its polymerisation and stabilising the nascent polymer network (Yurchenco et al., 2004). In addition to its anchoring role to the cell surface, the binding of laminin to its receptors also triggers cell signalling and establishes a link between the actin cytoskeleton and the extracellular matrix (Danen and Sonnenberg, 2003; Winder, 2001).

We have previously shown that *Myf5* expression in primary epaxial MPCs requires sonic hedgehog (Shh) signalling (Borycki et al., 1999; Chiang et al., 1996; Gustafsson et al., 2002). Shh mediates its effect through the Gli family of zinc finger transcription factors, and previous work showed that Gli2 and Gli3 are essential effectors of Shh signals in somites (Buttitta et al., 2003; McDermott et al., 2005). Here, we report that in the absence of Shh signalling, the myotome is abnormally patterned and myotomal cells are abnormally located in the ventral somite. We demonstrate that this defect is due to a failure to assemble a continuous basement membrane. Our genetic and embryological analyses further indicate that Shh signalling controls *Lama1* gene expression, suggesting that laminin-111, but not laminin-511 is crucial for the formation of the myotomal basement membrane. Furthermore, we show that laminin-111 is necessary and sufficient to initiate basement membrane formation, indicating that laminin-111 has specific, non-overlapping role in myotomal basement membrane assembly. Together, our data reveal a novel role for Shh signalling in the control of basement membrane assembly and provide a framework for future studies linking intercellular signalling, actin cytoskeleton architecture and cell behaviour in normal development and in diseased tissues.

MATERIALS AND METHODS

Mouse lines

Gli2 and *Gli3XtJ* mice were maintained as heterozygous and crossed to generate different mutant combination. *Shh* mice (kindly provided by M. Maconochie, MRC Mouse Genetics Unit, Harwell, UK), also maintained as heterozygous, were crossed to *Gli3XtJ* heterozygous mice to generate *Shh;Gli3* double mutant embryos. Embryonic day (E) 0.5 was the day vaginal plugs were found. Embryos were harvested between E9.0 and E10.5 by caesarian section, and genotyping was performed on yolk sac DNA by polymerase chain reaction (PCR) with primers described previously (Chiang et al., 1996; Maynard et al., 2002; Mo et al., 1997).

Immunohistochemistry

Immunofluorescence was essentially performed as described previously (Anderson et al., 2007). Primary antibodies used in this study were mouse anti-β-dystroglycan MANDAG-2 (Pereboev et al., 2001) at a dilution of 1:50, rabbit anti-laminin (L 9393, Sigma) at 1:100, anti-β1-laminin (MAB1905, Chemicon) at 1:50, anti-α1-laminin (MAB1903, Chemicon) at 1:800, anti-α5 laminin (a gift from J. Miner, Washington University, St Louis, MO, USA) at 1:800, anti-Perlecan (MAB1948, Chemicon) at 1:100, anti-Myogenin (F5D, Hybridoma Bank) at 1:100, anti-α6-integrin (CD49f, Abd Serotec, CD49f) at 1:100, anti-Entactin/Nidogen (RT-797-P0, Thermo Fisher Scientific) at 1:100, rabbit anti-collagen IV (AB756P, Chemicon) at 1:100, mouse anti- Pax3 (Hybridoma Bank) at 1:100 and rabbit anti-Myf5 (sc-302, Santa Cruz) at 1:1000. Secondary antibodies included Alexa Fluor 594-conjugated goat anti-mouse (A11005, Molecular Probes), Alexa Fluor 594-conjugated goat anti-rabbit (A11012, Molecular Probes) diluted at 1:500 and FITC-conjugated goat anti-rat (112-095-003, Jackson ImmunoResearch), FITC-conjugated rabbit anti-mouse (315-095-045, Jackson ImmunoResearch), and goat anti-rabbit Cy2 (111-225-144, Jackson ImmunoResearch) at 1:200. Images were captured on a SP1 laser-scanning confocal microscope (Leica), and processed using ImageJ and Photoshop (Adobe) software.

Electron microscopy

Embryonic tissue was fixed at 4°C in 3% EM-grade glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, and post-fixed at 4°C in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The tissue was then serially dehydrated into 100% ethanol, before being equilibrated in propylene oxide, then in Araldite resin containing benzyldimethylamine (BDMA). Ultra-thin sections (80 nm) were cut using a diamond knife (Diatome) and collected onto Formvar-coated, slot and 50-mesh EM-grade copper grids. Sections were stained with a 5% solution of uranyl acetate and with Reynolds solution (Lead citrate). Grids were inspected on a Technai Spirit transmission electron microscope. Images were captured on a Gatan camera and processed using Gatan software and ImageJ.

Cloning of mouse Lama1 and Lama5

Mouse *Lama1* and *Lama5* cDNA fragments were cloned by RT-PCR from E9.5 mouse embryo mRNA using the following primers: *Lama1* forward, 5′-ACCGCAGGACACTCCTGTCAGG-3′ and *Lama1*-reverse, 5′-TTA-CGCGCCGTCTGGTTC-3′; Lama5 forward, 5′-AAGGATCTCCAGG-CGATGCC-3′ and *Lama5* reverse, 5′-TTGTGCCTCCTGGATCTGGG-3′. PCR conditions were 5 minutes at 94ºC followed by 34 cycles 94°C for 1 minute, 58°C or 56°C for 1 minute, 72°C for 1 minute, and a final cycle at 72°C for 10 minutes. PCR fragments were subcloned into the pCRII TOPO vector (Invitrogen) and sequenced (ABI Prism).

In situ hybridisation

Embryos were fixed overnight at 4°C in 4% formaldehyde with 2 mM EGTA in PBS, pH 7.5, rinsed in PTW (PBS + 0.1% Tween-20), and processed for whole-mount in situ hybridisation as described previously (Borycki et al., 1999). Whole-mount images were captured using a Spot digital camera (SPOT INSIGHT Colour) and a Leica stereomicroscope. Transverse sections (80 μm) were obtained from embryos embedded in 2% agarose in PBS, sectioned using a Vibratome 1500, and mounted in Glycergel (Dako). Sections were photographed on a DMR microscope using DIC optics (Leica) and captured using a Leica camera DC300FX.

Explant cultures

Explant culture was performed essentially as described (Bajanca et al., 2006). Briefly, E10.5 $Shh^{-/-}$;$Gli3^{+/-}$ and $Shh^{-/-}$;$Gli3^{-/-}$ mouse embryos were harvested in Dulbecco's modified Eagle's medium-F12 (DMEM-F12, Gibco) supplemented with 4 mM L-glutamine, 1% penicillin-streptomycin, 1% sodium pyruvate and 10% fetal bovine serum (Gibco). Explants were cultured at 37°C in a 5% CO_2 atmosphere for 12 hours. Experimental explants were cultured with 30 μg/ml laminin-111 (Chemicon), control explants were cultured in medium containing the equivalent amount of buffer (5 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA).

RESULTS

Abnormal myotome patterning in the absence of Shh-Gli signalling

The first MPCs to colonise the myotome are derived from the dorso-medial lip of the dermomyotome and express *Myf5* under the control of Shh signalling (Borycki et al., 1999). Soon after their entry into the myotome, MPCs begin to express myogenin (*Myog*) and differentiate (Venters et al., 1999). In the absence of Shh signalling, dorso-medial lip cells of the dermomyotome do not activate *Myf5* and are not specified to the epaxial muscle lineage, resulting in a delay in *Myog* activation and myotome formation (Borycki et al., 1999; McDermott et al., 2005). However by E10.0, myotome formation is visible in $Shh^{-/-}$ embryos, presumably occurring through the second wave of colonisation by MPCs derived from the lateral hypaxial, and perhaps caudal and rostral, domains of the dermomyotome that express *Myf5* under distinct regulatory mechanisms (Hadchouel et al., 2000; Summerbell et al., 2000). Noticeably, the myotome of $Shh^{-/-}$ embryos displays an abnormal arrangement, with cells located in the ventro-medial somite (Borycki et al., 1999). To investigate this defect further, we examined *Myf5* mRNA and protein expression in the myotome of E9.5 mutant embryos for Shh, and for Gli2 and Gli3, which mediate Shh signalling (Fig. 1 and see Fig. S1 in the supplementary material). In wild-type embryos, Myf5-expressing cells delaminated from the dorso-medial lip of the dermomyotome and colonised the epaxial myotome as a thin cell layer located between the sclerotome and dermomyotome (Fig. 1A-C). By contrast, $Shh^{-/-}$ and $Gli2^{-/-}$;$Gli3^{-/-}$ embryos, which fail to activate Myf5 in primary epaxial MPCs and express it with a delay in the second wave of MPCs populating the myotome (Borycki et al., 1999; McDermott et al., 2005), had a marked defect in somite morphology and myotome formation: (1) the dorso-medial lip of the dermomyotome extended ventrally along the neural tube; (2) Myf5-expressing cells migrated aberrantly into the ventral somite and failed to form a regular sheet underneath the dermomyotome (white arrows in Fig. 1E,F,H,I). Intermediate compound $Gli2^{+/-}$;$Gli3^{-/-}$ and $Gli2^{-/-}$;$Gli3^{+/-}$ mutant embryos displayed progressive severity of the defect (see Fig. S1 in the supplementary material). These observations indicate that in the absence of Gli-mediated Shh signalling, the myotome is abnormally patterned and MPCs display aberrant migratory behaviour.

A myotomal basement membrane does not form in the absence of Shh-Gli signalling

The abnormal migration of MPCs into ventral, and to a lesser extent dorsal, somitic domains in $Shh^{-/-}$ and $Gli2^{-/-}$;$Gli3^{-/-}$ embryos evokes the behaviour of MPCs in the absence of Myf5, which is attributed to a loss of myotomal basement membrane (Bajanca et al., 2006; Tajbakhsh et al., 1996b). To address whether the myotome basement membrane forms normally in the absence of Shh or Gli signalling, we examined the expression of laminin in E9.5 embryos using an antibody specific to laminin β1, which is common to all laminins expressed at early stages of myotome formation. In wild-type embryos, laminin is first immunodetected as small speckles in the central somitocoele of newly formed epithelial somites (data not shown) (Anderson et al., 2007; Tosney et al., 1994). As somites mature, laminin is observed in the basement membrane lining the dermomyotome. This precedes the formation of the myotomal basement membrane, which is initiated at the level of hindlimb somites as a discontinuous sheet in E9.5 embryos. A fully assembled continuous sheet of laminin-containing basement membrane in close contact with Myf5-expressing myotomal cells was only visible at the level of forelimb somites (Fig. 1C and see Fig. S1 in the supplementary material). This contrasts with the absence of a continuous myotomal basement membrane observed in $Shh^{-/-}$ and $Gli2^{-/-}$;$Gli3^{-/-}$ embryos and the partial defect observed in $Gli2^{+/-}$;$Gli3^{-/-}$ and $Gli2^{-/-}$;$Gli3^{+/-}$ embryos, which correlates with the degree of ventral dispersion of cells expressing Myf5 and myogenin (white arrows in Fig. 1F,I and Fig. S1 in the supplementary material). However, fragments of laminin polymers that are often associated with Myf5- and myogenin-expressing cells could be detected in these embryos (white arrowheads in Fig. 1F,I), indicating that the failure to form a continuous myotomal basement membrane is not due to a defect in the synthesis or secretion of laminin polymers. It is also worth noting that although there was no myotomal basement membrane in $Shh^{-/-}$ and $Gli2^{-/-}$;$Gli3^{-/-}$ embryos, other basement membranes lining tissues such as the dermomyotome and the neural tube were unaffected (yellow arrowheads in Fig. 1F,I).

To confirm the absence of myotomal basement membrane defect, we examined interlimb somites from E9.5 wild-type and $Shh^{-/-}$ somites using transmitted electron microscopy (Fig. 2). In wild-type

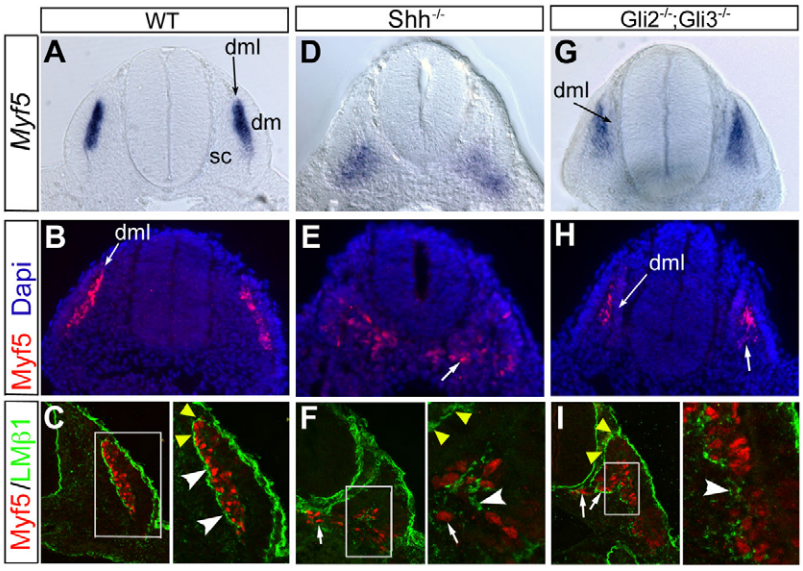

Fig. 1. Myotome patterning defect in the absence of Shh-Gli signalling correlates with loss of a laminin-containing myotomal basement membrane. (**A-I**) Myf5 (red) and β1 laminin (green) expression examined by whole-mount in situ hybridisation (A,D,G) and immunohistochemistry (B,C,E,F,H,I) in rostral somites of E9.5 wild-type (A-C), $Shh^{-/-}$ (D-F) and $Gli2^{-/-}$;$Gli3^{-/-}$ (G-I) mouse embryos. (A-C) Wild-type embryos. (D-F) $Shh^{-/-}$ embryos. (G-I) $Gli2^{-/-}$;$Gli3^{-/-}$ embryos. Dm, dermomyotome; dml, dorso-medial lip of the dermomyotome; sc, sclerotome. White arrows show ventral Myf5-positive cells in E,H,F and I. White arrowheads indicate short fragments of assembled laminin in F,I. Yellow arrowheads indicate the basement membrane around the dorsal and ventral lips of the dermomyotome in C,F,I. Note also the ventral extension of the dml. Magnification: ×200 and ×400 (right-hand panels in C,F,I).

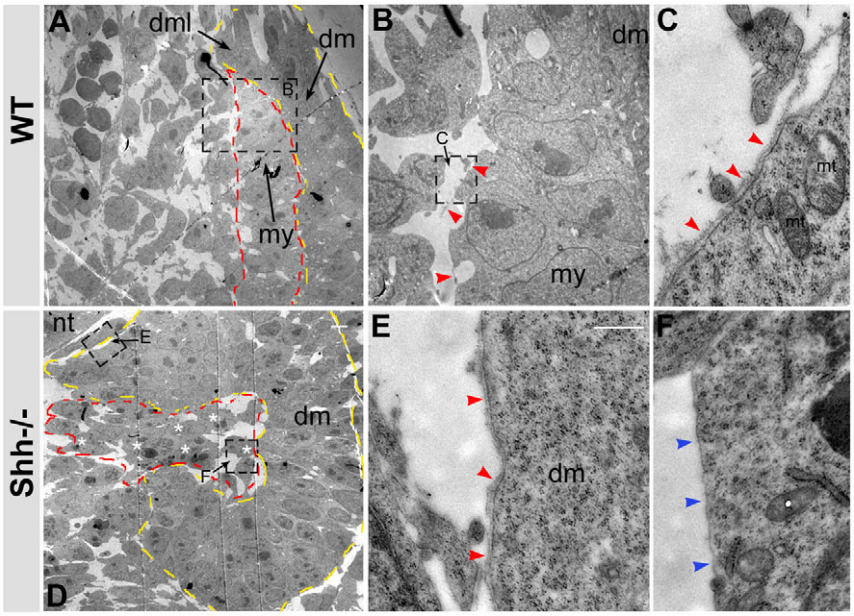

Fig. 2. The myotomal basement membrane fails to form in *Shh* mutant embryos. (**A-F**) E9.5 wild-type (A-C) and *Shh*$^{-/-}$ (D-F) interlimb somites were examined by transmitted electron microscopy. (A) Wild-type somites (×1150). (B) Higher magnification (×4500) of framed area in A shows a continuous basement membrane deposited at the surface of myotomal cells (red arrowheads). (C) High magnification of framed area in B (×34,000) confirms the presence of a myotomal basement membrane (red arrowheads). (D) *Shh*$^{-/-}$ somites with myotomal cells in the ventral somite (white asterisks) (×1150). (E,F) High magnification (×34,000) of framed areas in D. (E) A basement membrane forms at the basal surface of dermomyotomal cells (red arrowheads), (F) but not at the surface of myotomal cells (blue arrowheads). The yellow dashed line indicates the dermomyotome. The red dashed line marks the myotome. dm, dermomyotome; dml, dorso-medial lip of dermomyotome; my, myotome; nt, neural tube; mt, mitochondria. Scale bars: 5 μm in D; 0.05 μm in E.

embryos, a continuous basement membrane was visible in the medial myotome (red arrowheads in Fig. 2B,C). By contrast, no recognisable basement membrane structure (blue arrowheads in Fig. 2F) was detected at the surface of the myotomal cells that accumulate in the centre of the dermomyotome-like structure and in the ventral somite of *Shh*$^{-/-}$ embryos (denoted by white asterisks in Fig. 2D), although a normal basement membrane formed on the basal side of the dermomyotome (red arrowheads in Fig. 2E) and around the neural tube (data not shown).

Basement membrane components are synthesised and secreted in the absence of Shh

To establish whether the failure to form a myotomal basement membrane results from a defect in the synthesis of a basement membrane component in the absence of Shh-Gli signalling, we examined the distribution of collagen IV, perlecan and nidogen in E9.5 *Shh*$^{-/-}$ (see Fig. S2 in the supplementary material) and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ (data not shown) embryos. Consistent with the sequential assembly of laminin and collagen IV polymer networks through the incorporation of nidogen and perlecan, wild-type embryos displayed a continuous distribution of collagen IV, perlecan and nidogen in forelimb somites (see Fig. S2A,C,E in the supplementary material). By contrast, collagen IV, perlecan and nidogen immunostaining presented a clump-like distribution that was often associated with myogenic cells in *Shh*$^{-/-}$ embryos (see Fig. S2B,D,F in the supplementary material), indicating that collagen IV, perlecan and nidogen are synthesised and secreted, but have not been incorporated into a laminin network.

Laminin and collagen IV monomers can self-assemble in vitro, although it is believed that assembly into nascent networks is facilitated in vivo through the binding to integrins and dystroglycans that act to recruit and cluster monomers at the cell surface (Henry and Campbell, 1998; Li et al., 2002; Lohikangas et al., 2001; Moore et al., 2002). To address whether laminin and collagen network formation is affected as a result of loss of integrin and/or dystroglycan expression, we performed immunofluorescence detection on forelimb somites using antibodies against α6 integrin and β-dystroglycan (Fig. 3). α6β1 integrin is the earliest laminin receptor detected in MPCs during early embryonic development (Bajanca et al., 2004). As previously described, integrin α6 was present at the surface of dermomyotomal cells (yellow arrowheads in Fig. 3A,D), and was upregulated in Myf5-positive myotomal cells in E9.5 wild-type embryos (white arrows in Fig. 3A,D). This expression remained unchanged in all single and compound *Gli2*;*Gli3* and in *Shh* mutant embryos (Fig. 3B,C,E,F and see Fig. S3 in the supplementary material), although there was an increasing disorganisation of myotomal cells in *Gli2*$^{-/-}$;*Gli3*$^{+/-}$, *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ and *Shh*$^{-/-}$ embryos (white arrows in Fig. 3B,C,E,F and Fig. S3 in the supplementary material). These observations establish that the secondary wave of Myf5-expressing MPCs that enter the myotome in the absence of Shh-Gli signalling express α6β1 integrin.

As we previously showed (Anderson et al., 2007), β-dystroglycan is upregulated in wild-type MPCs as they enter the myotome (white arrow in Fig. 3G). Likewise, *Shh*$^{-/-}$ and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$, as well as in intermediate compound mutant myotomal cells expressed β-dystroglycan (white arrows in Fig. 3H,I and Fig. S3 in the supplementary material), indicating that the secondary wave of MPCs that populate the myotome in the absence of Shh signalling upregulates and maintains dystroglycan and integrin expression. To confirm that the laminin receptors α6β1 integrin and dystroglycan were functional, we performed immunohistochemistry on wild-type and *Shh*$^{-/-}$ embryos using antibodies against α-dystroglycan (the subunit that binds laminin) and the activated form of β1 integrin (CD29) (see Fig. S4 in the supplementary material). We found that in *Shh*$^{-/-}$ embryos (see Fig. S4B,D in the supplementary material), as in wild-type embryos (see Fig. S4A,C in the supplementary material), α-dystroglycan and CD29 immunodetection was localised at the cell surface of Myf5-expressing myotomal cells, despite their aberrant location in the ventral somite (see Fig. S4D in the supplementary material).

These observations demonstrate that despite the synthesis of basement membrane components and the expression of functional laminin receptors, MPCs are unable to assemble a continuous myotomal basement membrane in the absence of Shh signalling.

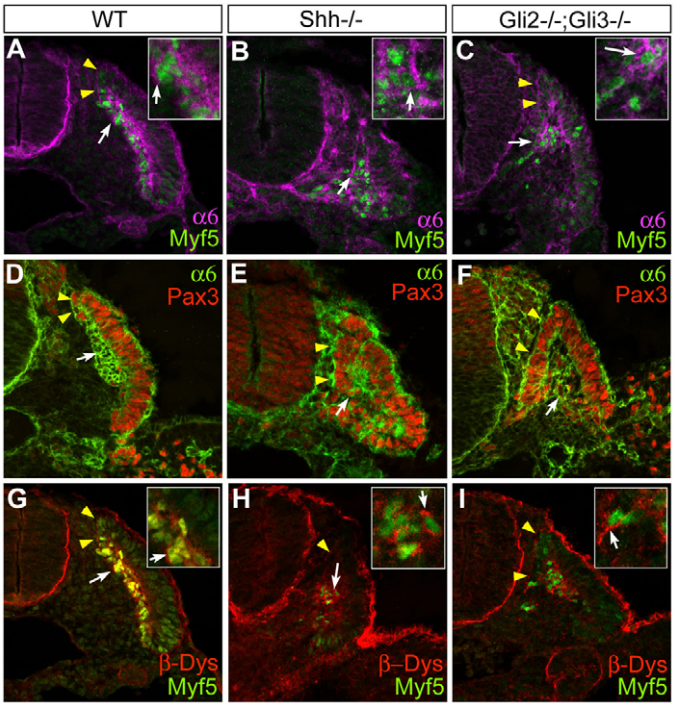

Fig. 3. The laminin receptors, α6β1 integrin and dystroglycan, are expressed in *Shh* mutant embryos. (**A-I**) α6 integrin (purple in A-C, green in D-F) and β-dystroglycan (G-I, red) protein expression in relation to Myf5 (A-C,G-I, green) or Pax3 (D-F, red) distribution was examined by immunohistochemistry and confocal imaging in forelimb somites of E9.5 wild-type (A,D,G), *Shh*$^{-/-}$ (B,E,H), and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ (C,F,I) embryos. (E,F) Note the increased number of Pax3-positive cells in the myotome in *Shh*$^{-/-}$ and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$. (G-I) Note the misorientation of myotomal cells in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ embryos (white arrow in I). Yellow arrowheads indicate receptor expression in dermomyotomal cells. White arrows show receptor expression in myotomal cells. Magnification: ×400; ×630 in insets.

Laminin-111 expression is impaired in the absence of Shh signalling

As mentioned above, laminin-111 (α1β1γ1) and laminin-511 (α5β1γ1) are the sole laminins synthesised during early myotome formation and differ by the type of α-chain used (Bajanca et al., 2006). We have already established that β1 laminin is synthesised in *Shh*$^{-/-}$ and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ embryos (see Fig. 1), indicating that at least one of these two laminins is produced. However, it is possible that the absence of myotomal basement membrane in *Shh* and *Gli* mutant somites is due to a defect in the synthesis of a single laminin subunit. To test this possibility, we performed in situ hybridisation with RNA probes that specifically recognise genes encoding α1 (*Lama1*) and α5 (*Lama5*) laminins. Consistent with previous observations (Miner et al., 2004), abundant *Lama1* mRNA was detected in the pre-somitic mesoderm and in newly formed somites of wild-type embryos (Fig. 4A). As somites mature, *Lama1* is downregulated in the dorsal somite and remained expressed at low levels in the sclerotome (Fig. 4A,A′). Noticeably, *Lama1* expression was lost in somites and neural tube of *Shh*$^{-/-}$ embryos, although expression was unaffected in the pre-somitic mesoderm and mesonephros (Fig. 4B,B′). In contrast to *Lama1*, was not expressed in the pre-somitic mesoderm, and was activated immediately before somite formation (Fig. 4C). In somites, *Lama5* mRNA was localised to the apical side of dorso-medial, as well as rostral and caudal lip cells in the dermomyotome (Fig. 4C′), although low levels of expression were also detected in the somitocoele of newly formed somites (data not shown). *Lama5* somitic expression was unchanged in *Shh*$^{-/-}$ embryos, although it was mispatterned because of the ventral extension of the dermomyotome (Fig. 4D,D′). This contrasts with the loss of *Lama5* expression in the neural tube of *Shh*$^{-/-}$ embryos, which probably results from the dorsalisation of the neural tube in these embryos. Thus, the absence of Shh signalling impairs transcriptional activation of *Lama1*, but not *Lama5*, in somites. Consistent with this finding, immunodetection of laminin α1 was lost in sclerotome and myotome, and was greatly reduced in the neural tube of *Shh*$^{-/-}$ embryos (compare Fig. 4B″ with 4A″), whereas polymeric fragments containing laminin α5 are detected in *Shh*$^{-/-}$ somites (Fig. 4C″,D″). As epaxial muscle progenitor cells located in the dorso-medial lip of the dermomyotome primarily expressed *Lama5* and not *Lama1*, our data raise the interesting hypothesis that sclerotome-derived laminin-111 is crucial to initiate assembly of the myotomal basement membrane and that laminin-511 is not sufficient to initiate basement membrane assembly in the myotome.

Myotomal basement membrane assembly is partially restored in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos

The failure to produce laminin α1 in *Shh*$^{-/-}$ newly formed somites is reminiscent of other somitic defects previously characterised in *Shh*-null embryos that affect both the ventral sclerotomal and the dorsal dermomyotomal compartments (Borycki et al., 1999; Buttitta et al., 2003; Chiang et al., 1996; McDermott et al., 2005). Thus, it is possible that the failure to activate *Lama1* expression in *Shh*$^{-/-}$ somites is secondary to the lack of cell fate specification of somitic cells rather than a direct effect of Shh signalling on laminin-111 production. Interestingly, cell fate specification is recovered in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ compound mutant embryos, owing to the removal of the Gli3 repressor function unleashed in the absence of Shh. Thus, Myf5 activation in epaxial MPCs and early epaxial myotome formation is recovered (McDermott et al., 2005), and Pax1 activation and sclerotome formation is restored (Buttitta et al., 2003). Furthermore, cell death that occurs in the ventral sclerotomal compartment in *Shh*$^{-/-}$ somites is abrogated (Borycki et al., 1999; Litingtung and Chiang, 2000). Thus, we assessed whether basement membrane assembly and *Lama1* expression was restored in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos. In E9.5 *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos, epaxial *Myf5* expression is only partially restored and remains delayed (McDermott et al., 2005), no continuous basement membrane is observed, and *Lama1* expression is absent in interlimb somites (see Fig. S5 in the supplementary material). In E9.5 and E10.0 *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos, sclerotomal *Pax1* expression and epaxial *Myf5* activation are fully restored and apoptosis is prevented (Borycki et al., 1999; Buttitta et al., 2003; McDermott et al., 2005), resulting in the recovery of the primary wave of MPCs in the myotome (asterisk in Fig. 5D-F and Fig. S5 in the supplementary material). Consistent with this observation, laminin β1 immunoreactivity, which was scattered in *Shh*$^{-/-}$ embryos (Fig. 5A-C), displayed a higher degree of organisation in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos (white arrows in Fig. 5D-F). However, the myotomal basement membrane in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos was qualitatively poor and presented numerous disruptions at hindlimb and interlimb levels (compare Fig. 5D,E with 5G,H), leading to Myf5-positive MPCs aberrantly located in the ventral somite (red arrows in Fig. 5D). Interestingly, a nearly continuous basement membrane was present at forelimb level in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos (white arrows in

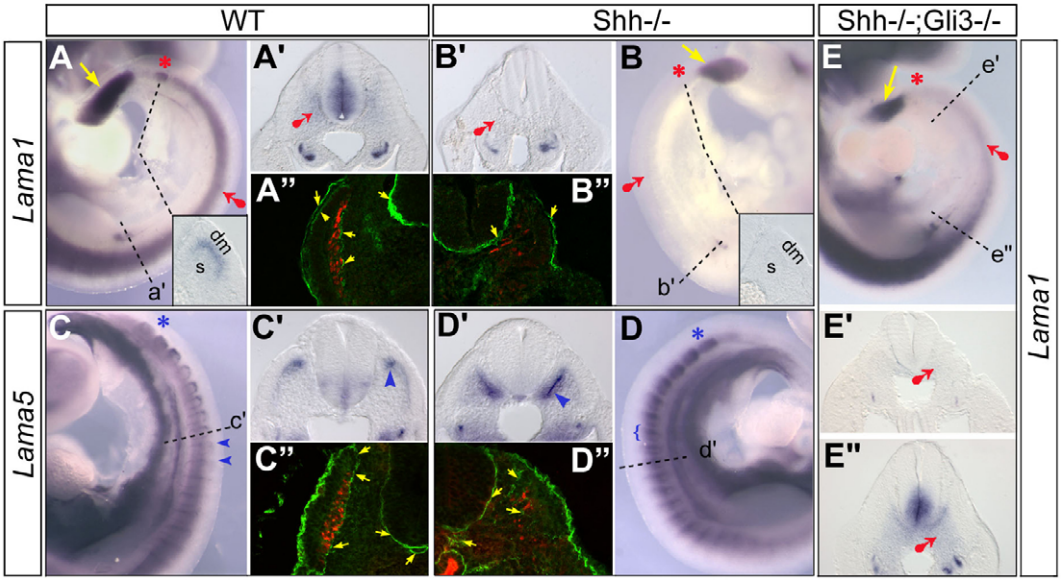

Fig. 4. Laminin α1 expression is disrupted in the absence of Shh signalling. (**A-E″**) Whole-mount in situ hybridisation was performed on E9.5 wild-type (A,A′,C,C′), *Shh*$^{-/-}$ (B,B′,D,D′) and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (E,E′,E″) mouse embryos using DIG-labelled RNA probes for *Lama1* (A,B,E) and *Lama5* (C,D). Laminin α1 (A″) and α5 (C″) were also detected by immunofluorescence. (A,A′) *Lama1* expression in wild-type embryos. (A″) Laminin α1 immunodetection in wild-type interlimb somites. (B,B′) *Lama1* expression in *Shh*$^{-/-}$ embryos. Transverse sections of newly-formed (inset in A and B) and interlimb (A′,B′) somites are shown. (B″) Laminin α1 immunodetection in *Shh*$^{-/-}$ interlimb somites. (C,C′) *Lama5* expression in wild-type embryos. (C″) Laminin α5 immunodetection in wild-type interlimb somites. (D,D′) *Lama5* expression in *Shh*$^{-/-}$ embryos. Transverse sections of interlimb somites (C′,D′) are shown. (D″) Laminin α5 immunodetection in wild-type interlimb somites. (E,E′,E″) *Lama1* expression in *Gli3*$^{-/-}$;*Shh*$^{-/-}$ embryos. The position of transverse sections shown in E′ and E″ is indicated. Yellow arrows and red asterisks show *Lama1* expression in the pre-somitic mesoderm and newly-formed somites, respectively. Red arrows indicate sites of expression affected in the absence of Shh. Blue asterisks indicate *Lama5* expression in newly formed somites, and blue arrowheads show dermomyotomal expression. s, sclerotome; dm, dermomyotome. Magnification: ×250 (A-E) and ×400 (A′-E′,A″-E″).

Fig. 5F), which coincides with the progressive recovery of *Lama1* transcription (Fig. 4E), suggesting that basement membrane assembly can occur with a delay in the absence of Shh and Gli3, and that laminin α1 is the limiting factor for the formation of the myotomal basement membrane.

Exogenous laminin-111 is sufficient to restore myotomal basement membrane formation

To address whether laminin-111 is necessary and sufficient for myotomal basement membrane formation, we tested whether providing exogenous laminin-111 to *Shh*$^{-/-}$;*Gli3*$^{+/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos was sufficient to restore the myotomal basement membrane. E10.5 wild-type, *Shh*$^{-/-}$;*Gli3*$^{+/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos were cultured for 12 hours in the presence of 30 μg/ml laminin-111 and analysed by immunofluorescence for the presence of a myotomal basement membrane (Fig. 6). Consistent with our previous observations (Fig. 5 and see Fig. S5 in the supplementary material), *Shh*$^{-/-}$;*Gli3*$^{+/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ posterior somites failed to form a continuous myotomal basement membrane when cultured in control medium (Fig. 6A,B). However, we observed that myotomal basement membrane formation was restored in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos cultured in the presence of 30 μg/ml of purified laminin-111 (n=3; Fig. 6C,D). Despite this, we observed aberrantly located myotomal cells that must have migrated ventrally before the addition of laminin. It is worth noting that no continuous myotomal basement membrane was observed in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ somites cultured in the presence of exogenous laminin-111 (data not shown). This observation suggests that the dosage of laminin-111 in somites is crucial for myotomal basement membrane assembly, and in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos, laminin-111 concentration is sub-optimal for myotomal basement membrane assembly. Together, these data demonstrate that laminin-111 is crucial to initiate assembly of the myotomal basement membrane.

DISCUSSION

In addition to their structural role, basement membranes have an important role in the control of cell behaviour, in particular in the control of cell proliferation and differentiation, cell polarity and migration, as well as modulating cell signalling. The laminin-rich basement membrane that forms and separates the myotome from the underlying sclerotome during somite maturation regulates muscle and non-muscle cell behaviour. Indeed, neural crest cells use the myotomal basement membrane during their migration into somites (Tosney et al., 1994). Furthermore, muscle progenitor cells display aberrant migration when the myotomal basement membrane fails to form in *Myf5*$^{-/-}$ embryos (Tajbakhsh et al., 1996b) or when α6β1 laminin binding is prevented (Bajanca et al., 2006; Tajbakhsh et al., 1996b). Yet, the mechanisms that control myotomal basement membrane formation remain elusive. Here, we report on the failure to form a myotomal basement membrane in the absence of Shh signalling, which leads to abnormal muscle progenitor cell migration and cell differentiation. We show that Shh signalling is required for laminin-111 expression and that in the absence of laminin-111, laminin-511 is unable to compensate and mediate the assembly of the myotomal basement membrane. Our findings indicate that laminin-111 is the rate-limiting factor in basement membrane assembly in the myotome. This study identifies for the first time a

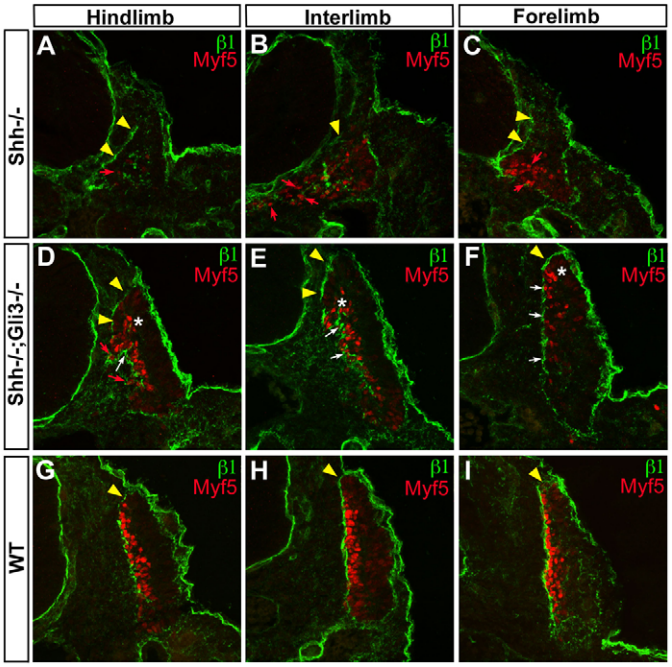

Fig. 5. Progressive recovery of myotomal basement membrane in *Gli3*$^{-/-}$;*Shh*$^{-/-}$ embryos. (**A-I**) β1 laminin subunit (green) and Myf5 (red) protein distribution examined by immunohistochemistry and confocal imaging in somites of E10.0 *Shh*$^{-/-}$ (A-C), *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (D-F), and wild-type (G-I) embryos. (A-C) *Shh*$^{-/-}$ embryos. (D-F) *Shh*$^{-/-}$;*Gli3*$^{-/-}$ somites. Note the recovery of epaxial Myf5 activation at all axial levels (white asterisk). (G-I) Wild-type embryos. Yellow arrowheads indicate the dermomyotomal basement membrane. Red arrows show abnormally located myotomal cells. White arrows indicate sites of partially assembled basement membrane. Magnification: ×400.

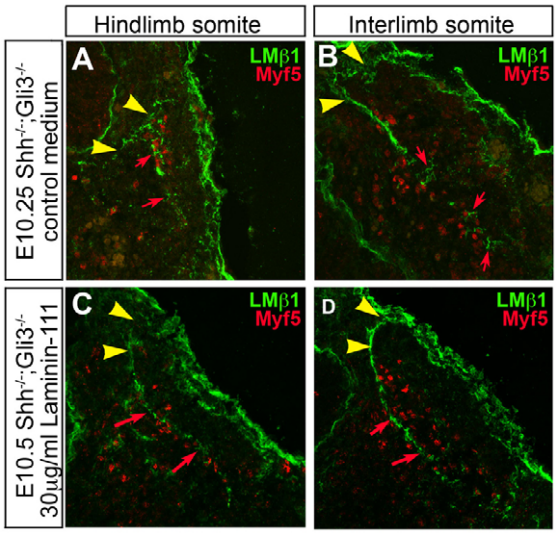

Fig. 6. Exogenous laminin-111 restores myotomal basement membrane formation. (**A-D**) Laminin (green) and Myf5 (red) distribution in E10.5 *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos cultured for 12 hours in control medium (A,B) or in the presence of laminin-111 (30 μg/ml) (C,D). (A) No myotomal basement membrane forms in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ hindlimb somites, whereas (B) a higher degree of organisation is observed in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ interlimb somites in control medium. By contrast, *Shh*$^{-/-}$;*Gli3*$^{-/-}$ hindlimb (C) and interlimb (D) somites form a myotomal basement membrane when cultured in the presence of laminin-111 (red arrows). Yellow arrowheads indicate the dermomyotomal basement membrane. Magnification: ×200.

direct link between the developmental signal Shh and the synthesis of a basement membrane component that regulates important cell behaviour, such as migration and differentiation. This finding might have important implications in the development of other Shh-dependent organs, and in our understanding of disease mechanisms associated with Shh signalling, including those in degenerative and neoplasmic disorders.

Shh signalling controls the formation of the myotomal basement membrane, independently of Myf5

The first mesodermal cells to be specified to the muscle lineage in the embryo are the epaxial MPCs born in the dorso-medial lip of the dermomyotome. Their specification is dependent on Myf5 activity, because neither MyoD nor Mrf4 are expressed at this stage (E8.5) (Ott et al., 1991; Sassoon et al., 1989; Summerbell et al., 2002), and requires both Shh and Wnt signalling (Borello et al., 2006; Borycki et al., 1999). The second wave of MPCs that contribute to the myotome express Myf5 independently of Shh signalling (Borycki et al., 1999), because a distinct enhancer, other than the Shh-dependent epaxial enhancer, controls this expression (Buchberger et al., 2003; Carvajal et al., 2001; Hadchouel et al., 2003; Hadchouel et al., 2000; Summerbell et al., 2000). *Myf5*$^{nlacZ/nlacZ}$ embryos lack a myotomal basement membrane (Tajbakhsh et al., 1996b) as a result of loss of α6β1 integrin expression in epaxial MPCs (Bajanca et al., 2006). Since epaxial MPCs fail to activate Myf5 in *Shh*$^{-/-}$ embryos, it was possible that, as suggested before (Cinnamon et al., 2001; Kahane et al., 2007), a population of Myf5-expressing pioneer cells emanating from the dorsal medial lip of the dermomyotome was essential for myotomal basement membrane formation. However, our data do not favour this possibility, because we observed no correlation between the recovery of Myf5-expressing pioneer cells (and the presence of α6β1 integrin on these Myf5-positive cells) in *Gli3*$^{-/-}$;*Shh*$^{-/-}$ embryos and recovery of the myotomal basement membrane assembly. Instead, we observed a striking parallel between the timing of *Lama1* expression and the gradual recovery of basement membrane assembly in the myotome of *Gli3*$^{-/-}$;*Shh*$^{-/-}$ embryos. A similar correlation is observed between *Lama1* expression and the extent of myotomal basement membrane defect found in *Gli2*;*Gli3* and *Gli3*;*Shh* compound mutant embryos. This indicates that the primary cause for myotomal basement membrane disruption in *Shh*$^{-/-}$ somites is not the loss of epaxial pioneer MPCs, because although those are present in posterior *Gli3*$^{-/-}$;*Shh*$^{-/-}$ somites, no myotomal basement membrane forms. Rather, our results indicate that failure in laminin assembly in *Shh*$^{-/-}$ embryos is due to the absence of laminin-111. This implies that although Myf5 and α6β1 integrin are necessary for the formation of the myotomal basement membrane (Bajanca et al., 2006), they are not sufficient in the absence of laminin-111, and levels of laminin-111 in somites dictate the degree of basement membrane assembly in the myotome. Consistent with this observation, ectopic addition of laminin-111 to *Gli3*$^{-/-}$;*Shh*$^{-/-}$ explant cultures improves

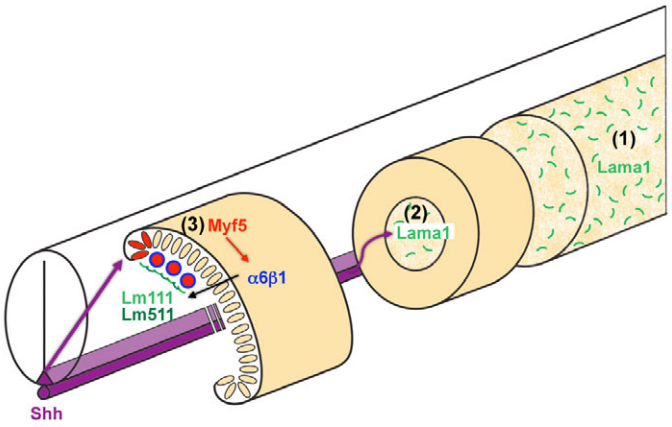

Fig. 7. Model for myotomal basement membrane formation. (1) Laminin α1 (green) is synthesised independently of Shh in the pre-somitic mesoderm. (2) Laminin α1 is synthesised under the control of Shh signalling (purple arrow) in the paraxial mesoderm at the time somites form and assembles with β1 and γ1 laminin chains into a laminin-111 network in the sclerotome/somitocoele. (3) Under Shh signalling, dorso-medial lip cells in the dermomyotome activate Myf5 (orange), which is required to upregulate the expression of the laminin receptor, α6β1 integrin (blue), in MPCs that delaminate and populate the myotome. Dorso-medial lip cells also synthesise laminin α5, which presumably begin to polymerise. However, laminin-511 cannot initiate myotomal basement membrane assembly. This requires laminin-111, which we hypothesise interacts with higher affinity to a receptor molecule present at the surface of MPCs through its LG domains. Once initiated, the myotomal basement membrane incorporates laminin-511 and other extracellular matrix components such as collagen IV, perlecan and nidogen to form a continuous, stable myotomal basement membrane.

considerably the degree of laminin assembly. Thus, we propose the following model that reveals a two-step requirement for Shh signalling for myotomal basement membrane formation (Fig. 7): (1) Shh signalling controls *Lama1* expression in newly formed somites and in the sclerotome, allowing for the gradual accumulation of laminin-111 protein in the sclerotome, which is required to initiate myotomal basement assembly; (2) Shh is also essential for the activation of Myf5 in epaxial MPCs, which in turn controls the expression of the laminin receptor α6β1 integrin.

Our data indicate also that the source of laminin-111 is local and probably originates from the somite. Indeed, *Lama1* is expressed at high levels in the pre-somitic mesoderm (Fig. 4A), but in contrast to somites, this expression is not affected in *Shh*$^{-/-}$ embryos (Fig. 4B). Thus, laminin-111 produced in the pre-somitic mesoderm probably does not contribute to the myotomal basement membrane and cannot compensate for the loss of somitic laminin-111 in *Shh*$^{-/-}$ embryos. We suggest that it is incorporated in basement membranes surrounding somites or the neural tube that are not affected in *Shh*$^{-/-}$ embryos. This would explain why in the absence of *Lama5* and *Lama1* expression, a basement membrane forms around the neural tube. Alternatively, another laminin α-subunit might be expressed and compensate for the loss of α1 and α5 laminins. In somites, *Lama1* mRNA is abundant in the first two or three newly formed somites and is then present at low levels in the sclerotome. This is consistent with our observation that laminin β1 is found in the somitocoele, and indicates that laminin-111 is synthesised and secreted by sclerotomal cells, and assembled and incorporated into the myotomal basement membrane via its interaction with laminin receptors expressed at the surface of myotomal cells. The evolutionary significance of this interdependent relationship between sclerotome and myotome might lie in the increasing bearing of the axial skeleton on the development of skeletal muscles in amniotes, and might represent a novel feature in evolution. Indeed, amphibians and fish have a limited sclerotome, and the basement membrane forming at intersomitic boundaries acts as a myotomal boundary. Despite this divergence, there is a remarkable conservation in the requirement for Shh signalling in laminin assembly and incorporation into the myotomal boundary basement membrane in the zebrafish (Henry et al., 2005), suggesting an ancestral role for Hedgehog signalling in basement membrane assembly.

Differential requirement for Gli2 and Gli3 in myotomal basement membrane assembly

It remains now to establish whether Shh controls *Lama1* expression directly or indirectly. *Lama1*, which encodes laminin α1, spans about 150 kb and contains 63 exons. The differential effect of loss of Shh on pre-somitic and somitic *Lama1* expression suggests that at least two distinct enhancers may control paraxial mesoderm expression of *Lama1*. Alternatively, it is possible that the same enhancer controls both sites of expression, because we showed that the pre-somitic mesoderm is not competent to respond to Shh signalling owing to the absence of Gli proteins (Borycki et al., 2000; McDermott et al., 2005). Two studies have unveiled a putative promoter or enhancer region upstream of exon 1 of *Lama1* (Niimi et al., 2003; Piccinni et al., 2004). Further work is required to establish whether Shh-Gli signalling affects this transcriptional activity directly. Nevertheless, our data indicate that *Lama1* expression is differentially sensitive to Gli2 and Gli3, and to loss of Gli activator and repressor function. First, the myotomal basement membrane is more severely affected in *Gli2*$^{-/-}$;*Gli3*$^{+/-}$ embryos than in *Gli2*$^{+/-}$;*Gli3*$^{-/-}$ embryos, suggesting that *Lama1* expression in somites is primarily controlled by Gli2. Second, *Lama1*, similarly to *Pax1* and *Myf5*, is a Shh-dependent somitic gene (Borycki et al., 1999; Chiang et al., 1996; Fan et al., 1995). However, unlike *Pax1* and *Myf5*, which are immediately restored in *Gli3*$^{-/-}$;*Shh*$^{-/-}$ embryos (Buttitta et al., 2003; McDermott et al., 2005), *Lama1* expression is only recovered after a delay. Thus, *Lama1* transcription is more sensitive to loss of Gli activator function than to the presence of Gli repressor activity.

Laminin-111 is required for myotomal basement membrane assembly

Our data indicate that despite being expressed in the dorso-medial lip of the dermomyotome, which contains muscle progenitor cells, laminin-511 is not sufficient to initiate basement membrane formation in the absence of laminin-111 in *Shh*$^{-/-}$ embryos. This suggests that laminin-111 has specific characteristics enabling it to initiate myotomal basement membrane formation, which laminin-511 lacks. Yet, the inability of laminin-511 to compensate for the loss of laminin-111 in myotomal basement membrane assembly is not due to the absence of the laminin N-terminal (LN) domain implicated in laminin self-assembly (Colognato and Yurchenco, 2000), because all three chains of laminin-511 (i.e. α5β1γ1) contain an LN domain. Thus, a possible hypothesis is that laminin-511 compensates for the loss of laminin-111 in some instances (Miner et al., 2004), but not in the myotome. It remains to be established what characteristic(s) differ between laminin α1 and laminin α5. Although it remains possible that the larger N-terminal arm of laminin α5 (compared with that of laminin α1) affects its function,

the prevailing model is that the five laminin globular (LG) domains located at the C-terminal of laminin α chains have a crucial function in basement membrane assembly (McKee et al., 2007). These LG domains mediate cell-matrix interactions, with LG1-3 binding integrins and LG4-5 binding α-dystroglycan, heparin and sulphatides (Andac et al., 1999; Li et al., 2002; Miner and Yurchenco, 2004; Talts et al., 1999; Taraboletti et al., 1990). Thus, one possibility is that LG domains differ between α1 and α5 laminins and confer specific functions, as documented by others (Li et al., 2002; Scheele et al., 2005). Alternatively, laminin α5 LG domains might have different affinity than laminin α1 LG domains for a cell surface receptor expressed by MPCs (Nishiuchi et al., 2003; Yu and Talts, 2003). Further studies testing the ability of chimeric α-chains to rescue myotomal basement membrane assembly would help elucidate the identity of the laminin α1 domain(s) required.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/20/3495/DC1

References

Andac, Z., Sasaki, T., Mann, K., Brancaccio, A., Deutzmann, R. and Timpl, R. (1999). Analysis of heparin, alpha-dystroglycan and sulfatide binding to the G domain of the laminin alpha1 chain by site-directed mutagenesis. *J. Mol. Biol.* **287**, 253-264.

Anderson, C., Winder, S. J. and Borycki, A. G. (2007). Dystroglycan protein distribution coincides with basement membranes and muscle differentiation during mouse embryogenesis. *Dev. Dyn.* **236**, 2627-2635.

Bader, B. L., Smyth, N., Nedbal, S., Miosge, N., Baranowsky, A., Mokkapati, S., Murshed, M. and Nischt, R. (2005). Compound genetic ablation of nidogen 1 and 2 causes basement membrane defects and perinatal lethality in mice. *Mol. Cell. Biol.* **25**, 6846-6856.

Bajanca, F., Luz, M., Duxson, M. J. and Thorsteinsdottir, S. (2004). Integrins in the mouse myotome: developmental changes and differences between the epaxial and hypaxial lineage. *Dev. Dyn.* **231**, 402-415.

Bajanca, F., Luz, M., Raymond, K., Martins, G. G., Sonnenberg, A., Tajbakhsh, S., Buckingham, M. and Thorsteinsdottir, S. (2006). Integrin alpha6beta1-laminin interactions regulate early myotome formation in the mouse embryo. *Development* **133**, 1635-1644.

Borello, U., Berarducci, B., Murphy, P., Bajard, L., Buffa, V., Piccolo, S., Buckingham, M. and Cossu, G. (2006). The Wnt/beta-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. *Development* **133**, 3723-3732.

Borycki, A. G., Brunk, B., Tajbakhsh, S., Buckingham, M., Chiang, C. and Emerson, C. P., Jr (1999). Sonic hedgehog controls epaxial muscle determination through Myf5 activation. *Development* **126**, 4053-4063.

Borycki, A., Brown, A. M. and Emerson, C. P., Jr (2000). Shh and Wnt signaling pathways converge to control Gli gene activation in avian somites. *Development* **127**, 2075-2087.

Bronner-Fraser, M., Artinger, M., Muschler, J. and Horwitz, A. F. (1992). Developmentally regulated expression of alpha 6 integrin in avian embryos. *Development* **115**, 197-211.

Buchberger, A., Nomokonova, N. and Arnold, H. H. (2003). Myf5 expression in somites and limb buds of mouse embryos is controlled by two distinct distal enhancer activities. *Development* **130**, 3297-3307.

Buttitta, L., Mo, R., Hui, C. C. and Fan, C. M. (2003). Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. *Development* **130**, 6233-6243.

Cachaco, A. S., Pereira, C. S., Pardal, R. G., Bajanca, F. and Thorsteinsdottir, S. (2005). Integrin repertoire on myogenic cells changes during the course of primary myogenesis in the mouse. *Dev. Dyn.* **232**, 1069-1078.

Carvajal, J. J., Cox, D., Summerbell, D. and Rigby, P. W. (2001). A BAC transgenic analysis of the Mrf4/Myf5 locus reveals interdigitated elements that control activation and maintenance of gene expression during muscle development. *Development* **128**, 1857-1868.

Cheng, Y. S., Champliaud, M. F., Burgeson, R. E., Marinkovich, M. P. and Yurchenco, P. D. (1997). Self-assembly of laminin isoforms. *J. Biol. Chem.* **272**, 31525-31532.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function. *Nature* **383**, 407-413.

Cinnamon, Y., Kahane, N., Bachelet, I. and Kalcheim, C. (2001). The sub-lip domain-a distinct pathway for myotome precursors that demonstrate rostral-caudal migration. *Development* **128**, 341-351.

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

Costell, M., Gustafsson, E., Aszodi, A., Morgelin, M., Bloch, W., Hunziker, E., Addicks, K., Timpl, R. and Fassler, R. (1999). Perlecan maintains the integrity of cartilage and some basement membranes. *J. Cell Biol.* **147**, 1109-1122.

Danen, E. H. and Sonnenberg, A. (2003). Integrins in regulation of tissue development and function. *J. Pathol.* **201**, 632-641.

Denetclaw, W. F., Jr, Christ, B. and Ordahl, C. P. (1997). Location and growth of epaxial myotome precursor cells. *Development* **124**, 1601-1610.

Duband, J. L., Belkin, A. M., Syfrig, J., Thiery, J. P. and Koteliansky, V. E. (1992). Expression of alpha 1 integrin, a laminin-collagen receptor, during myogenesis and neurogenesis in the avian embryo. *Development* **116**, 585-600.

Fan, C. M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A. and Tessier-Lavigne, M. (1995). Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81**, 457-465.

Gros, J., Scaal, M. and Marcelle, C. (2004). A two-step mechanism for myotome formation in chick. *Dev. Cell* **6**, 875-882.

Gullberg, D., Tiger, C. F. and Velling, T. (1999). Laminins during muscle development and in muscular dystrophies. *Cell Mol. Life Sci.* **56**, 442-460.

Gustafsson, M. K., Pan, H., Pinney, D. F., Liu, Y., Lewandowski, A., Epstein, D. J. and Emerson, C. P., Jr (2002). Myf5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification. *Genes Dev.* **16**, 114-126.

Hadchouel, J., Tajbakhsh, S., Primig, M., Chang, T. H., Daubas, P., Rocancourt, D. and Buckingham, M. (2000). Modular long-range regulation of Myf5 reveals unexpected heterogeneity between skeletal muscles in the mouse embryo. *Development* **127**, 4455-4467.

Hadchouel, J., Carvajal, J. J., Daubas, P., Bajard, L., Chang, T., Rocancourt, D., Cox, D., Summerbell, D., Tajbakhsh, S., Rigby, P. W. et al. (2003). Analysis of a key regulatory region upstream of the Myf5 gene reveals multiple phases of myogenesis, orchestrated at each site by a combination of elements dispersed throughout the locus. *Development* **130**, 3415-3426.

Henry, C. A., McNulty, I. M., Durst, W. A., Munchel, S. E. and Amacher, S. L. (2005). Interactions between muscle fibers and segment boundaries in zebrafish. *Dev. Biol.* **287**, 346-360.

Henry, M. D. and Campbell, K. P. (1998). A role for dystroglycan in basement membrane assembly. *Cell* **95**, 859-870.

Kahane, N., Cinnamon, Y. and Kalcheim, C. (1998a). The cellular mechanism by which the dermomyotome contributes to the second wave of myotome development. *Development* **125**, 4259-4271.

Kahane, N., Cinnamon, Y. and Kalcheim, C. (1998b). The origin and fate of pioneer myotomal cells in the avian embryo. *Mech. Dev.* **74**, 59-73.

Kahane, N., Cinnamon, Y. and Kalcheim, C. (2002). The roles of cell migration and myofiber intercalation in patterning formation of the postmitotic myotome. *Development* **129**, 2675-2687.

Kahane, N., Ben-Yair, R. and Kalcheim, C. (2007). Medial pioneer fibers pattern the morphogenesis of early myoblasts derived from the lateral somite. *Dev. Biol.* **305**, 439-450.

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.

Litingtung, Y. and Chiang, C. (2000). Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nat. Neurosci.* **3**, 979-985.

Lohikangas, L., Gullberg, D. and Johansson, S. (2001). Assembly of laminin polymers is dependent on beta1-integrins. *Exp. Cell Res.* **265**, 135-144.

Maynard, T. M., Jain, M. D., Balmer, C. W. and LaMantia, A. S. (2002). High-resolution mapping of the Gli3 mutation extra-toes reveals a 51.5-kb deletion. *Mamm. Genome* **13**, 58-61.

McDermott, A., Gustafsson, M., Elsam, T., Hui, C. C., Emerson, C. P., Jr and Borycki, A. G. (2005). Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation. *Development* **132**, 345-357.
McKee, K. K., Harrison, D., Capizzi, S. and Yurchenco, P. D. (2007). Role of laminin terminal globular domains in basement membrane assembly. *J. Biol. Chem.* **282**, 21437-21447.
Miner, J. H. and Yurchenco, P. D. (2004). Laminin functions in tissue morphogenesis. *Annu. Rev. Cell Dev. Biol.* **20**, 255-284.
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.
Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H., Chik, K. W., Shi, X. M., Tsui, L. C., Cheng, S. H. et al. (1997). Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**, 113-123.
Moore, S. A., Saito, F., Chen, J., Michele, D. E., Henry, M. D., Messing, A., Cohn, R. D., Ross-Barta, S. E., Westra, S., Williamson, R. A. et al. (2002). Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. *Nature* **418**, 422-425.
Niimi, T., Hayashi, Y. and Sekiguchi, K. (2003). Identification of an upstream enhancer in the mouse laminin alpha 1 gene defining its high level of expression in parietal endoderm cells. *J. Biol. Chem.* **278**, 9332-9338.
Nishiuchi, R., Murayama, O., Fujiwara, H., Gu, J., Kawakami, T., Aimoto, S., Wada, Y. and Sekiguchi, K. (2003). Characterization of the ligand-binding specificities of integrin alpha3beta1 and alpha6beta1 using a panel of purified laminin isoforms containing distinct alpha chains. *J. Biochem.* **134**, 497-504.
Ott, M. O., Bober, E., Lyons, G., Arnold, H. and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development* **111**, 1097-1107.
Pereboev, A. V., Ahmed, N., thi Man, N. and Morris, G. E. (2001). Epitopes in the interacting regions of beta-dystroglycan (PPxY motif) and dystrophin (WW domain). *Biochim. Biophys. Acta* **1527**, 54-60.
Piccinni, S. A., Bolcato-Bellemin, A. L., Klein, A., Yang, V. W., Kedinger, M., Simon-Assmann, P. and Lefebvre, O. (2004). Kruppel-like factors regulate the Lama1 gene encoding the laminin alpha1 chain. *J. Biol. Chem.* **279**, 9103-9114.
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.
Pownall, M. E., Gustafsson, M. K. and Emerson, C. P., Jr (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu. Rev. Cell Dev. Biol.* **18**, 747-783.
Sassoon, D., Lyons, G., Wright, W. E., Lin, V., Lassar, A., Weintraub, H. and Buckingham, M. (1989). Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. *Nature* **341**, 303-307.
Scheele, S., Falk, M., Franzen, A., Ellin, F., Ferletta, M., Lonai, P., Andersson, B., Timpl, R., Forsberg, E. and Ekblom, P. (2005). Laminin alpha1 globular domains 4-5 induce fetal development but are not vital for embryonic basement membrane assembly. *Proc. Natl. Acad. Sci. USA* **102**, 1502-1506.
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.
Summerbell, D., Ashby, P. R., Coutelle, O., Cox, D., Yee, S. and Rigby, P. W. (2000). The expression of Myf5 in the developing mouse embryo is controlled by discrete and dispersed enhancers specific for particular populations of skeletal muscle precursors. *Development* **127**, 3745-3757.
Summerbell, D., Halai, C. and Rigby, P. W. (2002). Expression of the myogenic regulatory factor Mrf4 precedes or is contemporaneous with that of Myf5 in the somitic bud. *Mech. Dev.* **117**, 331-335.
Tajbakhsh, S., Bober, E., Babinet, C., Pournin, S., Arnold, H. and Buckingham, M. (1996a). Gene targeting the myf-5 locus with nlacZ reveals expression of this myogenic factor in mature skeletal muscle fibres as well as early embryonic muscle. *Dev. Dyn.* **206**, 291-300.
Tajbakhsh, S., Rocancourt, D. and Buckingham, M. (1996b). Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in myf5 null mice. *Nature* **384**, 266-270.
Talts, J. F., Andac, Z., Gohring, W., Brancaccio, A. and Timpl, R. (1999). Binding of the G domains of laminin alpha1 and alpha2 chains and perlecan to heparin, sulfatides, alpha-dystroglycan and several extracellular matrix proteins. *EMBO J.* **18**, 863-870.
Taraboletti, G., Rao, C. N., Krutzsch, H. C., Liotta, L. A. and Roberts, D. D. (1990). Sulfatide-binding domain of the laminin A chain. *J. Biol. Chem.* **265**, 12253-12258.
Timpl, R. and Brown, J. C. (1996). Supramolecular assembly of basement membranes. *BioEssays* **18**, 123-132.
Tosney, K. W., Dehnbostel, D. B. and Erickson, C. A. (1994). Neural crest cells prefer the myotome's basal lamina over the sclerotome as a substratum. *Dev. Biol.* **163**, 389-406.
Venters, S. J., Thorsteinsdottir, S. and Duxson, M. J. (1999). Early development of the myotome in the mouse. *Dev. Dyn.* **216**, 219-232.
Winder, S. J. (2001). The complexities of dystroglycan. *Trends Biochem. Sci.* **26**, 118-124.
Yu, H. and Talts, J. F. (2003). Beta1 integrin and alpha-dystroglycan binding sites are localized to different laminin-G-domain-like (LG) modules within the laminin alpha5 chain G domain. *Biochem. J.* **371**, 289-299.
Yurchenco, P. D. and Ruben, G. C. (1987). Basement membrane structure in situ: evidence for lateral associations in the type IV collagen network. *J. Cell Biol.* **105**, 2559-2568.
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.