

Laminin β 1 and γ 1 Containing Laminins Are Essential for Basement Membrane Integrity in the Zebrafish Eye

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PURPOSE. In this study recessive zebrafish mutations in the genes encoding laminin β 1 (*lamb1*) and laminin γ 1 (*lamc1*) were used to determine the functions of these laminin proteins during ocular basement membrane formation and during zebrafish eye development.

METHODS. Ocular defects in *lamb1* and *lamc1* mutants were characterized by using a combination of histology, immunohistochemistry, in situ hybridization, and transmission electron microscopy.

RESULTS. The results demonstrated that zebrafish *lamb1* and *lamc1* mutants possess defects in two ocular basement membranes—the lens capsule and the inner limiting membrane—whereas Bruch's membrane is largely unaffected. *lamb1* and *lamc1* mutants possess severe lens dysplasias that result from a compromise in lens capsule integrity. Inner limiting membrane continuity is irregular in these mutants, and these irregularities result in small retinal ectopias that extend from the retina into the interstitial space between the retina and the lens. At late embryonic stages (e.g., 5–7 days after fertilization), retinal lamination defects are also observed in a subset of laminin mutants.

CONCLUSIONS. The results demonstrate that laminin β 1 and γ 1 containing laminins are essential for the integrity of the lens capsule basement membrane and inner limiting membrane in the zebrafish eye. (*Invest Ophthalmol Vis Sci.* 2007;48:2483–2490) DOI:10.1167/iovs.06-1211

Basement membranes are sheets of extracellular matrix (ECM) primarily composed of laminins, type IV collagens, nidogen, perlecan, and agrin and to a lesser extent, type XV and XVIII collagens, SPARC, fibulins, and fibronectin.¹ Laminins are critical for basement membrane assembly in which they are thought to form the nascent ECM scaffold with which other protein components interact.² Laminins are heterotrimeric glycoproteins consisting of individual α , β , and γ subunits. To date, five α , three β , and three γ chains have been identified that combine to form at least 15 distinct heterotrimers.^{3,4} Laminins interact with a variety of cell surface receptors on adjacent cells, and these cell-ECM interactions serve to anchor adherent cells within the three-dimensional scaffold that is required for tissue organization and physiological function. Cell-ECM interactions also orchestrate complex molecular and biochemical changes in adherent cells. These changes

range from the acquisition of cell fates, the regulation of morphogenesis, and the restriction of cell proliferation and growth to the prevention of apoptosis.

Several basement membranes are present in the eye, including the corneal epithelium, the corneal endothelium (Descemet's membrane), the lens capsule, the inner limiting membrane (ILM), and Bruch's membrane. The identities and distributions of protein components expressed in these ocular basement membranes have been described in several organisms.^{5–8} What has not yet been fully elucidated, however, is the degree to which these protein components are necessary in vivo for basement membrane formation and integrity in the eye. Although the genes encoding perlecan, SPARC, several collagen isoforms, and 9 of 11 laminin subunits (α 1-5, β 1, β 2, γ 1, and γ 2) have been disrupted in mice, many of these knockouts lead to embryonic death well before the central nervous system (CNS) has formed, thus preventing detailed analyses of their roles in eye development.³ For those mutant alleles in which embryonic lethality is avoided, ocular basement membrane defects have been reported. For example, in transgenic mice in which only the nidogen binding site of laminin γ 1 has been deleted, ocular basement membranes form, including the ILM, but these structures are weak and often rupture later in embryogenesis.^{9,10} In mice in which the heparan sulfate attachment sites of perlecan have been deleted, lens capsule integrity is compromised, and the lens often ruptures from the capsule.¹²

One of the best current models for a specific basement membrane function in the eye comes from a series of studies in the chick in which the ILM was transiently disrupted at defined periods via enzymatic digestion with collagenase and subsequently was reconstituted by injection of laminin-1/ α -2 macroglobulin at several times thereafter.^{5,9,11} The model that emerges from this work is that an intact ILM is essential for retinal ganglion cell organization, growth, survival, and axonal pathfinding to the optic nerve. On ILM disruption, each of these processes is affected, and, in addition, small retinal ectopias extend through the reconstituted ILM into the vitreous body of the eye. From these studies, it is clear that basement membranes are important in eye development, but having limited in vivo models, we do not yet fully understand how these ECM structures shape its development.

With an interest in how basement membranes facilitate ocular development, our laboratory has begun to use zebrafish laminin β 1 (*lamb1*) and laminin γ 1 (*lamc1*) mutants to study this process. Multiple recessive mutations in zebrafish *lamb1* and *lamc1* have been identified in forward genetic screens performed over the past several years.^{13,14} In screening an existing collection of retroviral insertional mutants for those that possess morphologic defects in eye formation, we noted obvious ocular defects in the insertional alleles of the *lamb1* and *lamc1* mutations.¹⁵ The laminin γ 1 chain is a component of 10/15 laminin heterotrimers whereas the β 1 chain is a component of 6/15 heterotrimers, and thus a significant percentage of all functional laminin proteins are disrupted in *lamb1* and *lamc1* mutants. That the zebrafish embryo is endowed with a maternal supply of protein and mRNA enables homozygous recessive mutants to survive through early embry-

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Supported by a grant from the Knights Templar Eye Foundation (JMG).

Submitted for publication October 9, 2006; revised January 16, 2007; accepted March 20, 2007.

Disclosure: J. Lee, None; J.M. Gross, None

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onic stages—stages at which mammalian embryos lacking these proteins normally expire. We report that laminin $\beta 1$ and $\gamma 1$ are essential in the zebrafish eye for the integrity of the lens capsule basement membrane and the ILM, but not for Bruch's membrane. Defects in the lens capsule basement membrane result in lens dysplasias whereas defects in the ILM result in retinal ectopias and possibly contribute to the retinal lamina- tion defects that are observed in a subset of *lamb1* and *lamc1* mutants.

MATERIALS AND METHODS

Animals

All embryos were obtained from the natural spawning of heterozygous carriers setup in pair-wise crosses. Embryos were collected and raised at 28.5°C according to the method of Westerfield¹⁶ and were staged according to Kimmel et al.¹⁷ Alleles used in these studies were *lamb1*^{bt1113b} and *lamc1*^{bt3890}; however, similar phenotypes were observed in the other insertional alleles of *lamc1* (*lamc1*^{bt2557} and *lamc1*^{bt1193}¹³). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with those provisions established at the University of Texas at Austin governing animal use and care.

Histology

Mutants and wild-type siblings were collected and fixed overnight at 4°C in a solution of 1% (wt/vol) paraformaldehyde, 2.5% glutaraldehyde, and 3% sucrose in PBS. They were washed three times for five minutes each in PBS and refixed for 90 minutes at 4°C in a 2% OsO₄ solution, washed three times for 5 minutes each in PBS at room temperature (RT) and dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, and two times in 100%). Embryos were further dehydrated two times for 10 minutes each in propylene oxide and infiltrated 1 to 2 hours in a 50% propylene oxide/50% Epon/Araldite mixture (Polysciences, Inc., Warrington, PA). Embryos were then incubated overnight at RT in 100% Epon/Araldite resin with caps open to allow for propylene oxide evaporation and resin infiltration, embedded, and baked at 60°C for 2 to 3 days. Sections of 1 to 1.25 μ m were cut, mounted on glass slides, and stained in a 1% methylene blue/1% borax solution. The sections were mounted (DPX; Electron Microscopy Sciences; Hatfield, PA) and photographed on a microscope (DMRB; Leica, Deerfield, IL) equipped with a digital camera (Retiga EXi; QImaging, Burnaby, BC, Canada). Images were then processed (Photoshop 5.0; Adobe Systems, San Jose, CA).

Transmission Electron Microscopy

For transmission electron microscopy (TEM), the embryos were processed as described for histology, and transverse sections were then cut through the central retina, 60 to 80 nm in thickness, and mounted on slot grids. The sections were stained with lead citrate and uranyl acetate. Images were obtained on a transmission electron microscope (80 kV; EM 208; Philips, Eindhoven, The Netherlands) via a digital camera (HR 1MB; Advantage; AMT Imaging, Danvers, MA). Images were then processed using (Photoshop CS; Adobe Systems).

Immunohistochemistry

Embryos were collected and fixed overnight at 4°C in a solution of 4% paraformaldehyde and 3% sucrose in PBS. The embryos were washed at RT three times for 5 minutes each in PBS and then processed immediately for wholemount immunohistochemistry or infiltrated by 35% sucrose for 1 to 2 hours at RT for cryosectioning.

Wholemount embryos were washed in PBS/0.1% Tween-20 (PBST) and permeabilized for 12 minutes with 100% acetone prechilled to -20°C. The embryos were washed three times at RT with PBST and digested with proteinase K (10 μ g/mL diluted in PBST) for 12 to 30 minutes, depending on age. They were then washed with PBST, re-

fixed in 4% paraformaldehyde (PFA) for 10 minutes at RT, washed three times in PBST, and blocked for 1 hour at RT in 2% normal goat serum (NGS) and 1% dimethylsulfoxide (DMSO) in PBST (block). The embryos were then incubated overnight at 4°C in anti-laminin-111 antibody diluted 1:400 in block. They were washed five times for 30 minutes each in PBST and incubated overnight at 4°C in biotin-SP-conjugated, affinity-purified F(ab')₂ goat anti-rabbit IgG diluted 1:500 in block. The embryos were then washed five times for 30 minutes each in PBST and incubated 3 hours at RT in avidin-biotin peroxidase complex reagent (ABC Reagent; Vector Laboratories, Burlingame, CA). They were washed three times for 30 minutes in PBST and then developed for 10 to 30 minutes with diaminobenzidine reagent (DAB; Sigma-Aldrich, St. Louis, MO). After development, the embryos were fixed briefly in 4% PFA and either cleared through a series of glycerol steps or cryosectioned as below. Wholemount embryos were imaged with a stereomicroscope (MZ16FA; Leica) mounted with a color camera (QImaging). Cryosections from these embryos were imaged on a microscope (DMLB; Leica) equipped with a camera (DFC 320; Leica).

Embryos for cryosectioning were embedded in plastic molds containing TBS cryopreservation media (Triangle Biomedical Sciences, Durham, NC). Cryosections 10 μ m in thickness were cut and affixed to gelatin coated slides. For immunohistochemistry, after drying for 1 to 2 hours at RT, the slides were lined with a hydrophobic marker (PAP pen), rehydrated briefly in PBS and blocked for 1 to 2 hours in 5% NGS. Primary antibodies, diluted in 5% NGS, were added, and the slides were incubated overnight at 4°C. The slides were then washed in PBS at RT two times for 5 minutes each and one time for 30 minutes and incubated in secondary antibody, diluted in 5% NGS, for 90 minutes at RT. Slides were washed three times for 15 minutes each in PBS at RT and mounted in mounting medium containing 4',6'-diamino-2-phenylindole (DAPI; Vector Laboratories, Inc.). The following antibodies and dilutions were used: 1d1 (1:30), 5e11 (1:100), zn8 (1:100), caz (1:500), and goat anti-mouse Cy3 secondary (1:500). Imaging was performed on a laser scanning confocal microscope (PASCAL; Carl Zeiss Meditec, Thornwood, NY). Three to five optical sections (1 μ m in thickness) were collected and projected using confocal software (Carl Zeiss Meditec, Inc.).

In Situ Hybridization

Hybridizations were performed, essentially as described by Jowett and Lettice,¹⁸ by using digoxigenin-labeled antisense probes. *lamb1* cDNA construct was kindly provided by Derek Stemple (Sanger Institute, Cambridge, UK)¹⁴ and a 775-bp fragment of the *lamc1* 3'-UTR (untranslated region) was cloned from cDNA generated from 48 hours postfertilization (hpf) zebrafish embryos, ligated into a PCR cloning vector (pGEMT-EASY; Promega, Madison, WI) and used for probe synthesis (cloning details available on request).

RESULTS

Zebrafish *lamb1* and *lamc1* Mutants

At 24 hpf, when compared with wild-type siblings, recessive zebrafish *lamb1* and *lamc1* mutants presented with a short, dorsally curved body axis (Fig. 1A). Indeed, previous studies have demonstrated that laminin-containing ECMs are necessary for embryonic axis elongation and for basement membrane deposition in the notochord.¹⁴ In the eye, *lamb1* and *lamc1* insertional mutants presented with retinal containment defects resembling colobomas (Fig. 1B).¹⁵ Colobomas varied in incidence and severity between *lamb1* and *lamc1* mutants; however, in more than 80% of the mutants with colobomas, they were present bilaterally.

Zebrafish *lamb1* and *lamc1* mutants also showed obvious lens abnormalities (Fig. 1A). When observed at 5 days postfertilization (dpf), lens dysplasia was observed in ~30% of *lamb1* mutants and ~75% of *lamc1* mutants. Like colobomas, lens dysplasias were also bilateral in nearly all affected mutants.

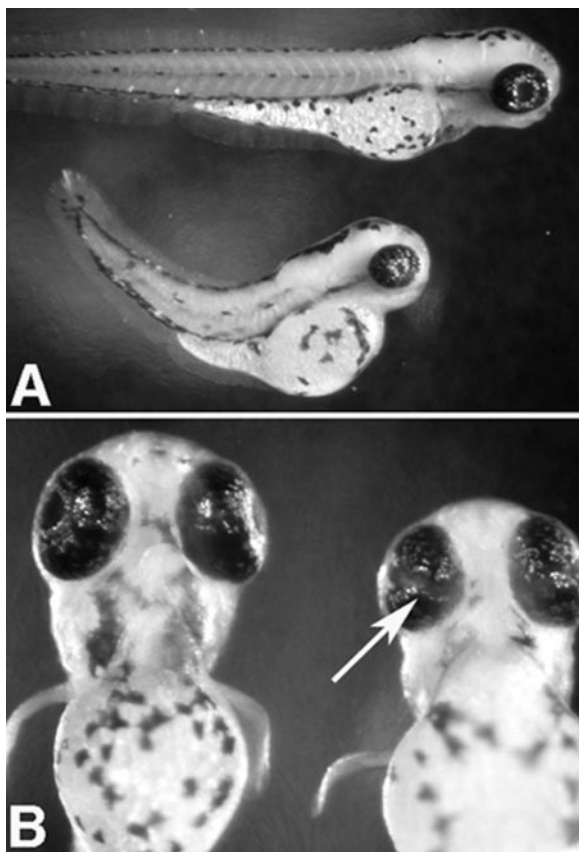


FIGURE 1. Zebrafish laminin mutants. Recessive laminin mutant embryos displayed several overt phenotypic characteristics at 5 dpf. (A) Lateral view of wild-type (*top*) and *lamc1* mutant (*bottom*) embryos. *lamc1* mutants were significantly shorter than their wild-type siblings and displayed prominent dorsal curvature. (B) Ventral view. *lamc1* mutants showed obvious lens dysplasias and colobomas (*arrow*). *lamb1* mutants were phenotypically indistinguishable from *lamc1* mutants (data not shown).

Histologic examination of laminin mutant eyes at 3 dpf indicated the degree to which lens formation was disrupted in these mutants (Figs. 2A, 2E). Lenses were not anchored in the anterior chamber of the eye as they were in a wild-type embryo (Fig. 2A) but were instead found within the retinal neuroepithelium (Fig. 2E). Lens tissue was severely disorganized in these mutants with obvious abnormalities in fiber cell differentiation. In several *lamb1* and *lamc1* embryos, the dysplastic lens had begun to fragment into two or more distinct pieces (Fig. 2E and data not shown). Lens tissue was rapidly cleared from mutant retinas, with significantly less tissue observed at 5 dpf (Fig. 2F) and often, by 7 dpf, no traces of the dysplastic lens were found in the mutant eye (Fig. 2G). Immunohistochemical examination of a lens-specific protein in zebrafish, the z1-1 antigen, highlighted the lens dysplasia and the significant degree of morphologic abnormalities in lens fiber organization in these mutants (Figs. 2D, 2H).

***lamb1*, *lamc1*, and Laminin-111 Expression in the Zebrafish Eye**

To gain a better understanding of how laminin $\beta 1$ and $\gamma 1$ containing basement membranes facilitate eye development in zebrafish, we first wanted to determine the normal distribution of laminin mRNAs and protein in the zebrafish eye. In situ hybridization for *lamb1* and *lamc1* mRNAs revealed that these were expressed in the lens, retina, and RPE of the developing

zebrafish eye (Fig. 3). Laminin-111 (formerly known as laminin-1⁴) is composed of $\alpha_1\beta_1\gamma_1$ laminin subunits and that polyclonal antiserum derived against mouse laminin-111 specifically cross-reacts with the zebrafish protein.^{14,19} In the absence of antisera that specifically cross-react with the zebrafish laminin $\beta 1$ and $\gamma 1$ subunit proteins, we used laminin-111 antiserum as a means to determine some of the ocular regions that express the laminin $\beta 1$ and $\gamma 1$ proteins. At 24 hpf, strong laminin-111 expression was observed in the choroid fissure, the lens capsule, and the ILM (Figs. 4A, 4B). Expression was maintained throughout the next 48 hours of development in the lens capsule, the ILM, and the RPE/Bruch's membrane region of the eye (Figs. 4C-F). At 48 and 72 hpf, strong laminin-111 expression was also observed in the optic nerve (Figs. 4D, 4F), and recent observations indicate that laminin-111 functions as a guidance cue during the pathfinding of retinal ganglion cell axons to the optic tectum.^{20,21}

The Lens Capsule Basement Membrane Is Disrupted in *lamb1* and *lamc1* Mutants

Given the high degree of lens dysplasia observed in laminin mutants and the observation that the laminin $\beta 1$ and γ mRNAs, and laminin-111 protein is highly expressed by the lens capsule (Figs. 3, 4), we next sought to examine directly the lens capsule formation in *lamb1* and *lamc1* mutants, to determine whether defects in this basement membrane might underlie the lens phenotype observed in these mutants. TEM images of the lens capsule in wild-type and *lamc1* mutant embryos at 72 hpf are shown in Figure 5. At this time point of zebrafish eye development, the wild-type lens capsule was approximately 30 nm in width and spanned the entire circumference of the lens (Fig. 5A). By comparison, in laminin mutant embryos, little to no lens capsule basement membrane tissue was observed (Fig. 5B).

Retinal Lamination Is Abnormal in a Subset of *lamb1* and *lamc1* Mutants

During the large-scale insertional mutant screening in which we first identified ocular defects in *lamb1* and *lamc1* mutants, we did not note any obvious histologic phenotypes in retinal development in these mutants.¹⁵ As we were performing additional histology in this study, however, we noted several laminin mutant embryos that possessed ectopic laminae in their inner retinas (Fig. 2G, arrows). Ectopic laminae were observed histologically in approximately 25% of both *lamb1* and *lamc1* mutants at 5 and 7 dpf. Ectopic laminae were not limited to those mutants with lens dysplasias, but were also observed in those mutants in which the lens was still anchored in the anterior chamber (Fig. 6 and data not shown).

To further characterize this retinal lamination phenotype and the organization/cell type composition of the ectopic laminae, we next performed immunohistochemical localization of several marker proteins expressed by retinal neuron subtypes that are located in distinct retinal laminae. The results of these analyses are shown in Figure 6 for two of the four retinal marker proteins assayed: 5e11, an antigen expressed by amacrine cells (Figs. 6A-C) and zpr-1, an antigen expressed by red/green double cones (Figs. 6D-F).²² In wild-type embryos, expression of 5e11 was limited to the amacrine cells of the inner nuclear layer (INL) and to the so-called displaced amacrine cells found on the scleral side of the ganglion cell layer (GCL; Fig. 6A). In most laminin mutants, expression was also limited to these regions (Fig. 6B); however, in a subset of mutants, an ectopic patch of cells was observed in the inner retina, vitread to the normal GCL and sclerad to the anterior chamber (Fig. 6C). Expression of zpr-1 is limited in wild-type embryos to the outer segments of the red-green double cones

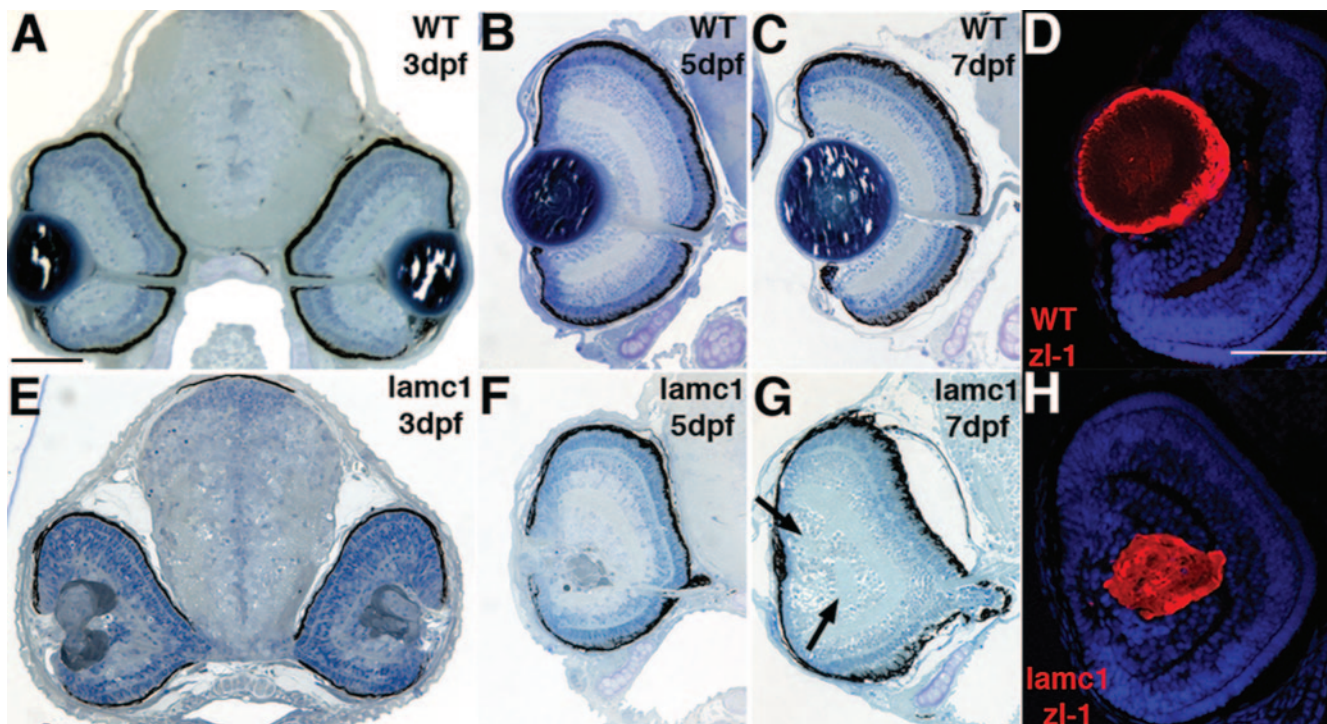


FIGURE 2. Laminin mutants exhibited complex ocular phenotypes. (A) Histologic examination of wild-type eyes at 3 dpf in comparison to *lamc1* mutants (E) indicated the severe degree of lens dysplasia and coloboma present in the mutants. The lens had ruptured from the lens capsule and was located ectopically within the retina. Lens morphology was abnormal and fiber cell differentiation appeared to be impaired. Between 5 (B) and 7 (C) dpf, whereas the wild-type lens continued to grow and mature, the dysplastic lens in *lamc1* was cleared from the retina (F, G). At 7 dpf, laminin mutants showed retinal lamination defects where ectopic cellular laminae can be observed in the innermost region of the retina (arrows), vitread to the normal GCL. Immunohistochemical localization of zl-1, a lens-specific marker protein, highlights the lens abnormalities in laminin mutant embryos. Dorsal is up in all panels. Scale bar, 100 μ m.

(Fig. 6D). Expression similar to that in wild-type embryos was observed in most laminin mutants (Fig. 6E), but again, in a subset of mutants, a small patch of ectopic red/green cones was observed in the inner retina (Fig. 6F). These ectopic cones were always vitread to the normal GCL and also to the ectopic IPL and INL. Similar results were obtained for rods and bipolar cells (data not shown). These immunohistochemical data indicate that ectopic laminae are composed of all retinal cell types and that they are organized in an inside-out fashion in the inner retina of laminin mutants.

Defects of the ILM in *lamb1* and *lamc1* Mutants

The ILM expressed high levels of laminin-111 protein (Fig. 4) and we hypothesized that defects in the ILM may underlie the retinal lamination phenotypes observed in these mutants. The ILM is composed in part of Müller glia end feet that serve as a scaffold onto which other ECM components are deposited.^{23,24} Carbonic anhydrase (caz) is a useful protein marker for Müller glia and their processes in zebrafish.²⁵ When compared with wild-type embryos, both *lamb1* (data not shown) and *lamc1* (Figs. 7A–C) mutants showed abnormalities in Müller glia processes. Müller end feet did not form a distinct inner retinal boundary as they did in wild-type embryos (Figs. 7A–C). In laminin mutants, Müller end feet localized to an abnormally wide region of the inner retina, and some Müller processes terminated aberrantly to regions within the retina proper, rather than to its internal limit (Fig. 7B). Müller end feet defects were observed in laminin mutants with intact lenses (Fig. 7B) and those with lens dysplasia (Fig. 7C), indicating that the physical insult resulting from lens displacement is not likely to underlie these ILM defects.

For a more direct examination of ILM formation in laminin mutants, we next performed TEM (Figs. 7D, 7E). At 48 hpf, the ILM was easily identified in wild-type embryos as a distinct tissue boundary between the retina and the lens (Fig. 7D). In laminin mutants, although regions of intact ILM can be observed (Fig. 7E), the ILM was not uniform through the inner retina and in many mutants, small retinal ectopias extended from the retina into the interstitial space between the retina and the lens (Fig. 7E, arrowhead). Thus, the integrity of the ILM was compromised in *lamb1* and *lamc1* mutants.

Bruch's Membrane Is Unaffected in Zebrafish Laminin Mutants

Laminin-111 was distributed to Bruch's membrane in the zebrafish eye (Fig. 4), and laminin β 1 and γ 1, as well as several other subtypes of laminin proteins, have been shown to be expressed in Bruch's membrane in other organisms.^{6,8,26} We examined Bruch's membrane by TEM at 3 dpf (Fig. 8) and 7 dpf (data not shown) in *lamb1* and *lamc1* mutants, to determine whether it was also affected in these mutants. We specifically examined Bruch's membrane regions outside of the area affected by the coloboma. Surprisingly, when compared with wild-type siblings we did not note any obvious differences in Bruch's membrane in *lamb1* or *lamc1* mutants (Fig. 8). A normal Bruch's membrane boundary was observed between the RPE and the choriocapillaris in laminin mutants, and this boundary appeared very similar to that observed in wild-type siblings.

DISCUSSION

An understanding of the *in vivo* roles of specific laminin subunits in regulating the development of distinct regions of the

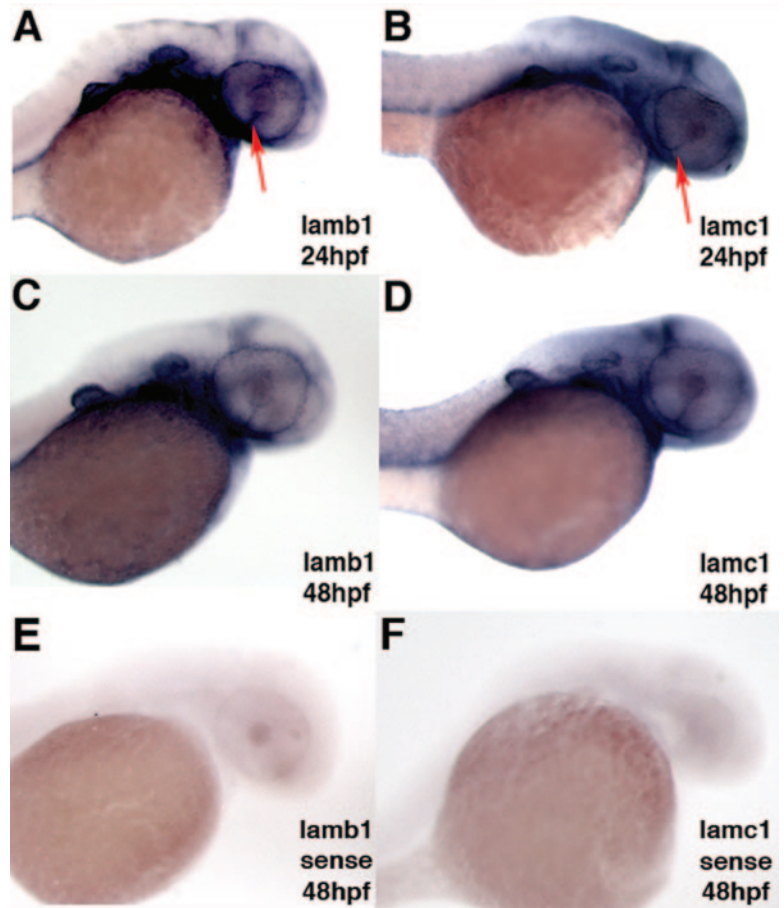


FIGURE 3. *lamb1* and *lamc1* distribution in the zebrafish eye. Wholemount in situ hybridization for *lamb1* (A, C) and *lamc1* (B, D) mRNAs at 24 and 48 hpf. Both laminin gene products were distributed in the lens, retina, and RPE of the embryonic zebrafish. Both gene products were enriched in the retina/RPE at the choroid fissure (A, B, arrows). Sense control probes *lamb1* (E) and *lamc1* (F) showed low levels of background staining at 24 hpf (data not shown) or 48 hpf. Dorsal is up and anterior to the right in all panels.

vertebrate CNS has been elusive, owing to the early lethality of many of the laminin subunit knockout mice.³ In zebrafish *lamb1* and *lamc1* mRNAs, as well as laminin-111 protein, are

provided maternally¹⁴ and thus, early embryonic defects are circumvented such that later functional requirements for these proteins become apparent. The results of our study demon-

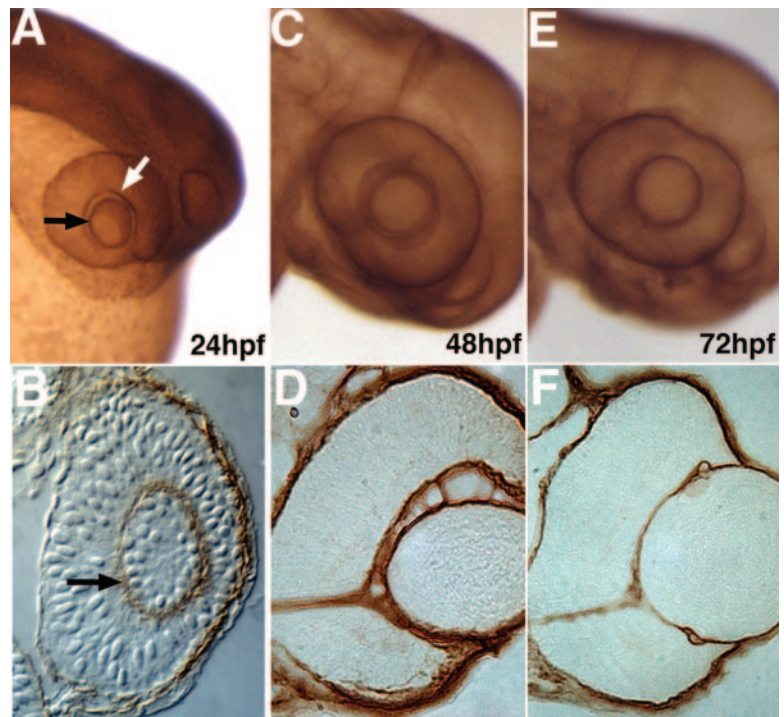


FIGURE 4. Laminin-111 distribution in the zebrafish eye. Laminin-111 ($\alpha_1\beta_1\gamma_1$) protein distribution assayed through the early stages of eye development in wholemount embryos (A, C, E) and in cryosections from wholemount embryos (B, D, F). (A, B) At 24 hpf laminin-111 was distributed to the choroid fissure, the lens capsule basement membrane (A, B, black arrow), and the ILM (A, white arrow). Laminin-111 protein at 48 hpf (C, D) and 72 hpf (E, F) is distributed to the lens capsule, the ILM, the retinal pigment epithelium, Bruch's membrane, the sclera, and the optic nerve. Dorsal is up in all panels.

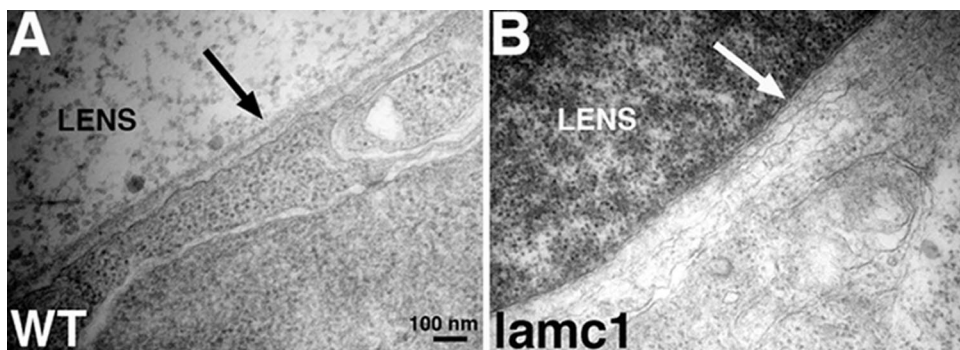


FIGURE 5. Zebrafish laminin mutants have defects in the lens capsule basement membrane. TEM analysis of a wild-type (A) and *lamc1* (B) mutant embryos at 72 hpf. (A) In wild-type embryos, the lens capsule was ~30 nm in thickness and spanned the circumference of the lens (arrow). (B) In contrast, in laminin mutant embryos the lens capsule was barely discernible (arrow). Scale bar, 100 nm.

strate that laminin $\beta 1$ and $\gamma 1$ containing laminins are required for the maintained integrity of the lens capsule basement membrane and the ILM of the zebrafish eye. Indeed, in further support of our conclusions is a study characterizing the ocular and behavioral defects in *N*-ethyl-*N*-nitrosourea (ENU)-induced mutations of *lama1* (laminin $\alpha 1$), *lamb1*, and *lamc1*.²⁷ Similar basement membrane defects in the lens capsule and ILM were observed in these ENU mutants. In addition, two recent reports have implicated laminin $\alpha 1$ in zebrafish ocular development. In one of these, the expression of zebrafish *lama1* was knocked down using a morpholino antisense oligonucleotide which resulted in embryos with lens and anterior segment abnormalities similar to those observed in *lamb1* and *lamc1* mutants.¹⁹ In the second report, three mutant alleles of *lama1* were identified and specific defects in anterior segment and retinal development were described, also similar to those described herein for *lamb1* and *lamc1* mutants.²⁸ Together, these studies demonstrate a critical role for laminin-containing basement membranes *in vivo* for the development and maintenance of distinct regions of the zebrafish eye, and directly implicate the laminin $\alpha 1$, $\beta 1$, and $\gamma 1$ subunits in these processes. Further studies are necessary to determine to what degree these observations transfer to the mammalian eye.

In *lamb1* and *lamc1* mutants, deficiencies in the lens capsule manifest as lens dysplasias in which the lens ruptures through the lens capsule and becomes ectopically localized within the retina. Lens morphogenesis is also abnormal in

these mutants, most likely reflecting an inability of differentiating lens fibers to associate properly with the lens capsule and undergo terminal differentiation. Elongating fibers are known to interact with laminin proteins in the lens capsule,²⁹ and they do so through $\beta 1$ -subunit containing integrins.³⁰ This cell-ECM interaction between differentiating lens fiber cells and the lens capsule not only enables their directed migration along the inner surface of the lens capsule but it also is thought to regulate their terminal differentiation into transparent fibers.

Zebrafish *lamb1* and *lamc1* mutant retinas contained Müller glia projections that terminate prematurely in the retina rather than at its internal limit (Fig. 7). TEM analysis indicated that regions of compromised ILM integrity were present in the mutant eyes and retinal ectopias extended from the retina and into the interstitial space between the retina and the lens (Fig. 7). These phenotypes are strikingly similar to those obtained in chick after collagenase digestion of the ILM¹¹ and also resemble defects observed in integrin- $\alpha 6$ -knockout mice.³¹ Together, these observations strongly suggest that the binding of retinal neurons to an intact ILM is essential for the continued maintenance of retinal integrity.

In studies by Semina et al.,¹⁹ it was demonstrated that surgical removal of the zebrafish lens at 24 hpf does not result in overt defects in retinal development; and, specifically, removal of the lens did not lead to defects in retinal lamination. Thus, the lamination defects observed in our *lamb1* and *lamc1* mutants are not likely to result from lens dysplasias and we

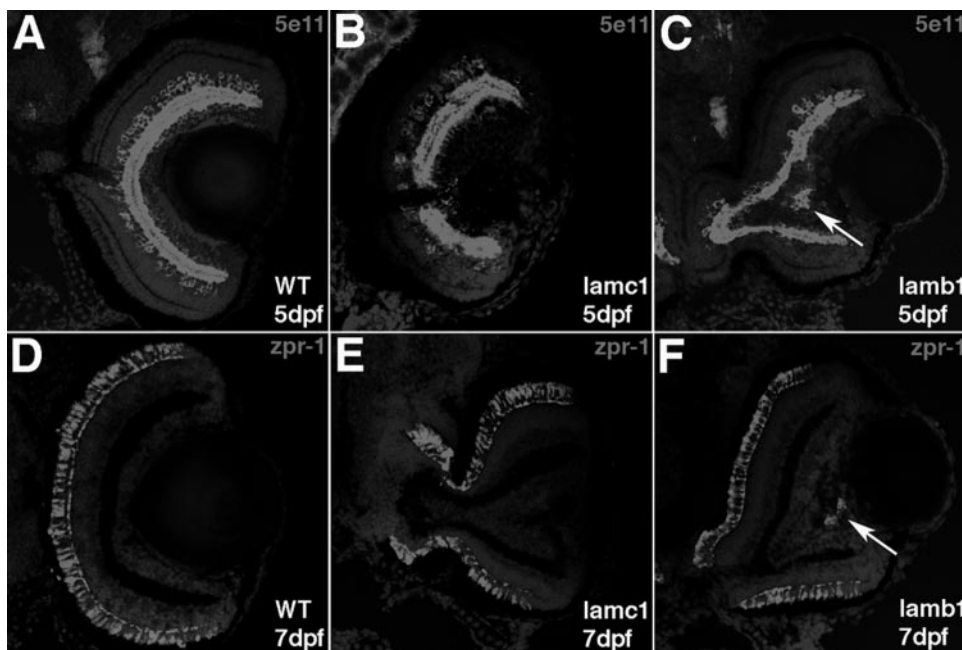
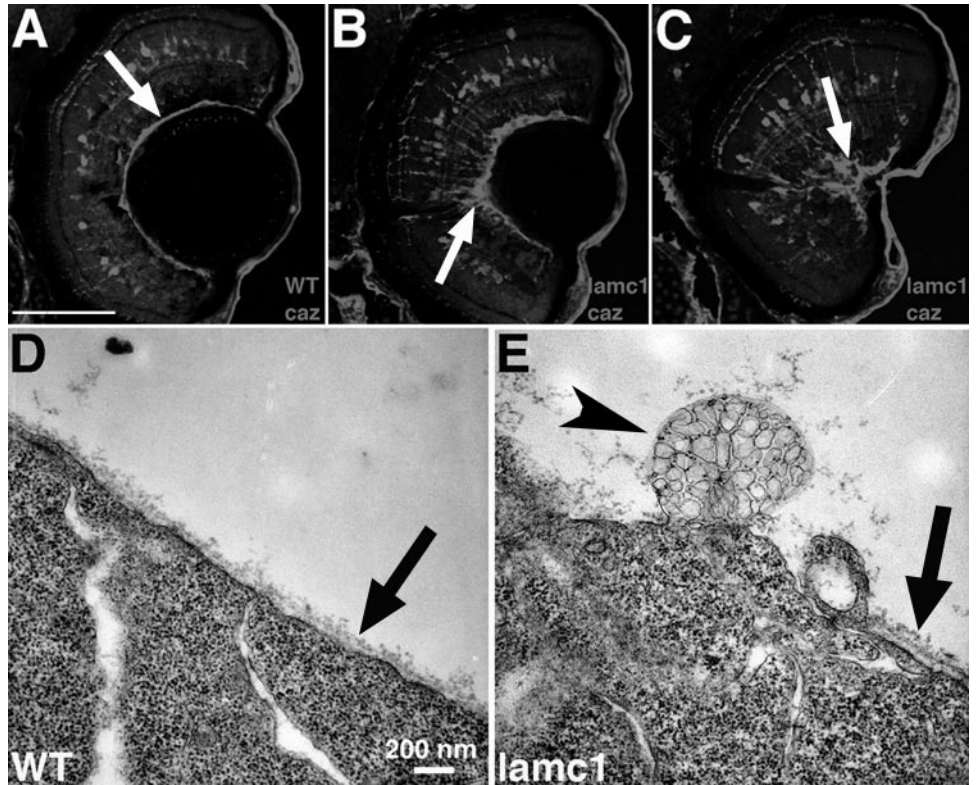


FIGURE 6. A subset of zebrafish laminin mutants showed defects in retinal lamination. Immunohistochemical localization of the 5e11 antigen in amacrine cells at 5 dpf (A-C) and *zpr-1* in red/green cones at 7 dpf (D-F). In wild-type embryos (A) and most laminin mutants (B), amacrine cells were located in the INL with processes extending into the IPL. As well, displaced amacrine cells were found in the scleral side of the GCL. (C) In contrast, a subset of laminin mutants showed abnormal positioning of amacrine cells with ectopic patches located in the inner retina, vitread to the GCL (arrow). Similarly, red/green cones were distributed to the ONL in wild-type (D) and most laminin mutant embryos (E); however, in a subset of mutants, an ectopic patch of red/green cones was also observed in the inner retina (arrow).

FIGURE 7. The ILM is abnormal in laminin mutants. (A) Carbonic anhydrase (*caz*) is expressed by the Müller glia of wild-type embryos. Müller processes extend from the cell body to the ILM of the retina (*arrow*). (B) In laminin mutants in which the retina remains anchored in the anterior chamber, Müller processes extend normally to the inner retina but form a much thicker internal boundary, and some terminate prematurely within the retina rather than at its limit (*arrow*). (C) In laminin mutants with lens dysplasias, Müller processes were severely disrupted and accumulated in a mass within the retina (*arrow*). (D, E) TEM analysis of the ILM in wild-type (D) and in lens-containing laminin mutant embryos at 48 hpf. In wild-type embryos the ILM formed as a distinct tissue boundary between the retina and the lens (*arrow*). (E) In laminin mutants, regions of ILM were observed (*arrow*), but the overall integrity of the ILM was compromised, and small retinal ectopias extended from the retina into the interstitial space between the retina and the lens (*arrowhead*). Scale bars: (A–C) 100 μ m; (D, E) 200 nm. Dorsal is up in (A) to (C).

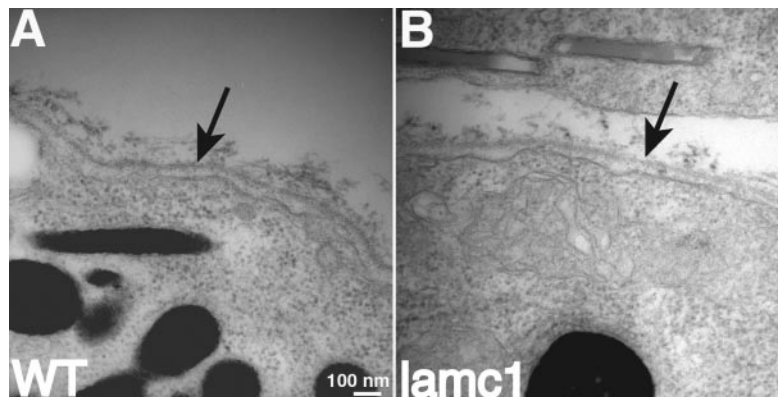


suspect that defects in ILM formation may underlie these lamination abnormalities. It is well known that cell fate specification and retinal lamination require the reception and precise integration of molecular cues that dictate neuronal subtypes and the laminar position of differentiated neurons.³² Lamination defects in *lamb1* and *lamc1* mutants may therefore reflect cell fate changes affecting retinal progenitor cells at the ciliary marginal zone of the retina, or they may reflect an inability of newly differentiated neurons to receive or respond to positional cues in the microenvironment that dictate final laminar location. Basement membranes are sites of deposition for signaling proteins and they are sites of direct interaction between a cell and its extracellular environment. Secreted growth factors are expressed at the ciliary marginal zone,³³ and loss-of-function assays for several laminin-binding receptors expressed in the retina indicate that they are required for its normal lamination.^{31,34,35} Deficiencies in the ILM of *lamb1* and *lamc1* mutants could feasibly disrupt the deposition of a secreted growth factor or the ability of a newly differentiated retinal neuron to migrate to its appropriate laminar destination. Fur-

ther studies designed to identify the molecular basis of these defects promise to shed some light on the role of the ILM in regulating retinal histogenesis and lamination, as well as the role of laminin proteins therein.

In our TEM studies, we noted no defects in Bruch's membrane in *lamb1* or *lamc1* mutants outside of the region encompassed by the coloboma (Fig. 8). This finding was contrary to our expectations and warrants further discussion. Defects in choroid fissure closure can be observed in laminin mutants as early as 22 hpf (data not shown), which suggests that the inability to contain the retina within the optic cup is not the result of rupture through a weak Bruch's membrane, but more likely is the result of defects in early aspects ocular morphogenesis. As the lateral edges of the optic vesicle begin to fuse during the early phases of ocular morphogenesis, the vesicle dramatically invaginates such that a bilayered optic cup forms.³⁶ Fusion between the prospective RPE and retina occurs along the proximodistal axis of the ventral optic cup at the choroid fissure, and this fusion is critical for the containment of the retina and RPE within the optic cup. Laminin dependent

FIGURE 8. Bruch's membrane appears to be normal in laminin mutants. TEM analysis of Bruch's membrane at 72 hpf in wild-type (A) and laminin mutant embryos (B). (A) Bruch's membrane formed as a distinct boundary between the RPE and choroid (*arrow*). (B) In laminin mutants, no overt defects in Bruch's membrane were observed outside of the region affected by the coloboma (*arrow*). Scale bar, 100 nm.



cell-ECM interactions facilitate several morphogenetic movements in vertebrate embryos, and in support of this hypothesis we have shown that *lamb1*, *lamc1*, and laminin-111 are all enriched at the choroid fissure in the zebrafish eye (Figs. 3, 4). A direct analysis of choroid fissure closure in wild-type and laminin mutant zebrafish, as well as the identification of laminin-binding receptors expressed within the choroid fissure, should prove interesting for furthering our understanding of the retinal containment defects in these mutants.

Acknowledgments

The authors thank John Mendenhall and Louise Trakimas for advice on TEM; Connor Oberst for technical assistance; Adam Amsterdam and Nancy Hopkins for laminin mutants; and Oliver Biehlmaier, Stephan Neuhaus, Brian Perkins, and John Wallingford for helpful discussions and comments on this manuscript. JMG thanks John E. Dowling for support and his encouragement of these studies.

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