Integrin α5/fibronectin1 and focal adhesion kinase are required for lens fiber morphogenesis in zebrafish

Julie M. Hayes[a,*], Andrea Hartsock[a,*], Brian S. Clark[b], Hugh R. L. Napier[b], Brian A. Link[b], and Jeffrey M. Gross[a,c]

[a]Section of Molecular Cell and Developmental Biology and Institute for Cell and Molecular Biology and [c]Institute for Neuroscience, University of Texas at Austin, Austin, TX 78722; [b]Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226-0509

ABSTRACT **Lens fiber formation and morphogenesis requires a precise orchestration of cell–extracellular matrix (ECM) and cell–cell adhesive changes in order for a lens epithelial cell to adopt a lens fiber fate, morphology, and migratory ability. The cell–ECM interactions that mediate these processes are largely unknown, and here we demonstrate that fibronectin1 (Fn1), an ECM component, and integrin α5, its cellular binding partner, are required in the zebrafish lens for fiber morphogenesis. Mutations compromising either of these proteins lead to cataracts, characterized by defects in fiber adhesion, elongation, and packing. Loss of integrin α5/Fn1 does not affect the fate or viability of lens epithelial cells, nor does it affect the expression of differentiation markers expressed in lens fibers, although nucleus degradation is compromised. Analysis of the intracellular mediators of integrin α5/Fn1 activity focal adhesion kinase (FAK) and integrin-linked kinase (ILK) reveals that FAK, but not ILK, is also required for lens fiber morphogenesis. These results support a model in which lens fiber cells use integrin α5 to migrate along a Fn-containing substrate on the apical side of the lens epithelium and on the posterior lens capsule, likely activating an intracellular signaling cascade mediated by FAK in order to orchestrate the cytoskeletal changes in lens fibers that facilitate elongation, migration, and compaction.**

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INTRODUCTION

Because it is composed of only two principal cell types—lens epithelial cells at the anterior of the lens and lens fibers centrally and posteriorly—the lens is an ideal tissue in which to study how interactions between cells and their extracellular matrix (ECM) facilitate cell type–specific differentiation and morphogenesis during development (Wederell and de Iongh, 2006; Zelenka, 2004; Walker and Menko, 2009). Lens fibers are generated from proliferative lens epithelial cells in a subequatorial region of the lens called the transition zone, and here they initiate fiber differentiation and morphogenesis (Soules and Link, 2005; Greiling and Clark, 2009). During lens fiber morphogenesis, newly formed lens fibers elongate and migrate both anteriorly and posteriorly on their path to the midline of the lens, at which point they adhere to a fiber from the other side of the lens to generate the anterior and posterior lens sutures. Anterior elongation and migration involves an interaction between the apical side of the lens fiber and the apical side of the lens epithelium, which serves as a substratum, whereas posterior elongation and migration involves basal elongation of the fiber and adhesion between the basal end of the lens fiber and the lens capsule (Zelenka, 2004). As new fibers are generated at the transition zone, older fibers become compacted as successive rounds of differentiation stack newly generated fibers on top of these older fiber layers, displacing them into the central lens as the lens continues to grow.

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*These authors contributed equally to this work.

Address correspondence to: Jeffrey Gross (jmgross@austin.utexas.edu).

Abbreviations used: ECM, extracellular matrix; FAK, focal adhesion kinase; Fn, fibronectin; FRNK, focal adhesion kinase–related nonkinase; ILK, integrin-linked kinase.

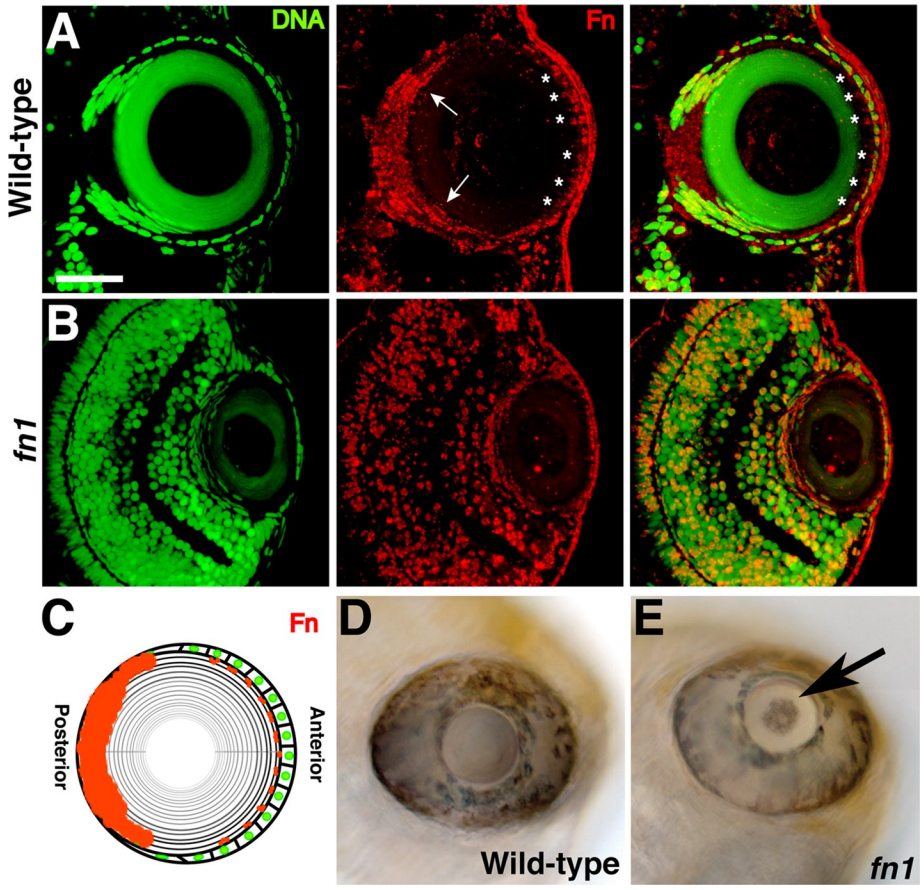


FIGURE 1: Fn is expressed in the lens, and *fn1* mutants possess cataracts. (A) Fn expression at 4 dpf in wild-type embryo. Fn is detected in the cornea, in puncta on the apical side of the lens epithelium (asterisks), and at the posterior of the lens in lens fibers (arrows). (B) *fn1* mutants retain Fn1 staining in the cornea, under the lens epithelium, and in posterior fibers, but levels are reduced. Shown is a *fn1* mutant with the highest level of Fn staining detected. Levels detected in mutants vary from that shown to almost none. (C) Cartoon depicting regions of the lens in which Fn is distributed. Fn in red and nuclei in green for all images. (D) Brightfield image of 4-dpf wild-type eye showing transparent lens. (E) *fn1* mutants possess structural defects in their lenses and obvious cataracts. Dorsal is up, anterior to the right in D and E. Scale bar, 50 μm.

Cell–ECM interactions facilitate a variety of morphogenetic events throughout development, and it is not surprising that where examined, both cell–ECM adhesion molecules and ECM components are expressed in the lens and are required for normal lens development (Zelenka, 2004; Wederell and de Iongh, 2006; Walker and Menko, 2009; Huang *et al.*, 2011). For example, laminins are required for the formation and maintenance of the lens capsule (Willem *et al.*, 2002; Lee and Gross, 2007), as are perlecan (Rossi *et al.*, 2003) and nidogen-1/entactin-1 (Dong *et al.*, 2002), two other components of the basement membrane. Integrin α3 and α6 are laminin-binding integrins, and mice deficient in both of these proteins display defects in lens formation (De Arcangelis *et al.* 1999; Wederell and de Iongh, 2006). α6 is also believed to be involved in lens fiber differentiation (Walker *et al.*., 2002), in which it shows an interesting change in isoform expression, with lens epithelial cells expressing an α6B isoform and lens fibers expressing α6A (Walker and Menko, 1999). Conditional knockouts of integrin β1 in mouse show severe defects in lens formation (Samuelsson *et al.*, 2007; Simirskii *et al.*, 2007), and functional blockade of integrin β1 in chick compromises adhesion between lens fibers and the posterior lens capsule (Bassnett *et al.*, 1999).

Despite these studies, and numerous others using in vitro and cell culture systems, how cell–ECM interactions facilitate lens fiber morphogenesis in vivo remains uncertain. Indeed, one of the ECM molecules for which the least functional information is known in the lens is fibronectin (Fn). Fn is expressed in the embryonic rat and chick lens capsule (Kurkinen *et al.*, 1979; Parmigiani and McAvoy, 1984), weakly in the adult bovine lens capsule (Cammarata *et al.*, 1986), and in the posterior aspects of the adult mouse lens capsule (Duncan *et al.*, 2000). E16 rat lens epithelial cells cultured on a Fn substratum are able to use it for migration, although they lose this ability by embryonic day 19, suggesting a developmental switch in its use (Parmigiani and McAvoy, 1991). Similarly, rabbit lens epithelial cells are able to attach and spread when placed on Fn-coated slides (Zelenka, 2004). Integrin α5/β1 is the principal Fn receptor, and integrin α5 has been reported to be present in the chick (Menko *et al.*, 1998) and mouse lens (Barbour *et al.*, 2004; Wederell and de Iongh, 2006). Although both *Fn* (George *et al.*, 1993) and *Itga5* (Yang *et al.*, 1993) mouse knockouts are lethal, a recent study using a tamoxifen-inducible Cre to conditionally inactive *Fn1* at later stages of mouse development demonstrated that Fn1 is required for lens placode formation and inactivation (Huang *et al.*, 2011). Moreover, functional perturbation in chick embryos using injected RGD peptides suggests that cell–ECM interactions, possibly mediated by Fn, are required for normal lens morphogenesis in vivo (Svennevik and Linser, 1993).

With an interest in how cell–ECM interactions facilitate normal lens development and what role Fn might play in the process, we took advantage of a zebrafish line that possesses a mutation in *fn1* (Trinh and Stainier, 2004) and examined lens development in the absence of Fn1 function. *fn1* mutants possessed obvious cataracts and defects in lens fiber morphogenesis. Lens fiber defects were also present in *itga5* mutants, as well as in embryos deficient in the *ptk2.1* focal adhesion kinase (FAK). These studies demonstrate a critical role for integrin α5 and Fn1 during lens fiber morphogenesis and demonstrate that FAK activity may be a mediator of the integrin α5/Fn1 interaction during zebrafish lens fiber morphogenesis.

RESULTS

fn1 is required for embryonic lens development

Fn is localized within the zebrafish lens in puncta at the interface between the apical side of the lens epithelium and the apical ends of lens fibers, as well as in puncta between the outermost layers of lens fibers and in the posterior pole of the lens at the fiber–capsule and fiber–fiber interfaces (Figure 1, A and C). Fn1 is also strongly expressed in the cornea (Figure 1A) and throughout the retina (unpublished data). Several recessive mutations in the *fibronectin 1* (*fn1*) gene have been identified in zebrafish (Trinh and Stainier, 2004;

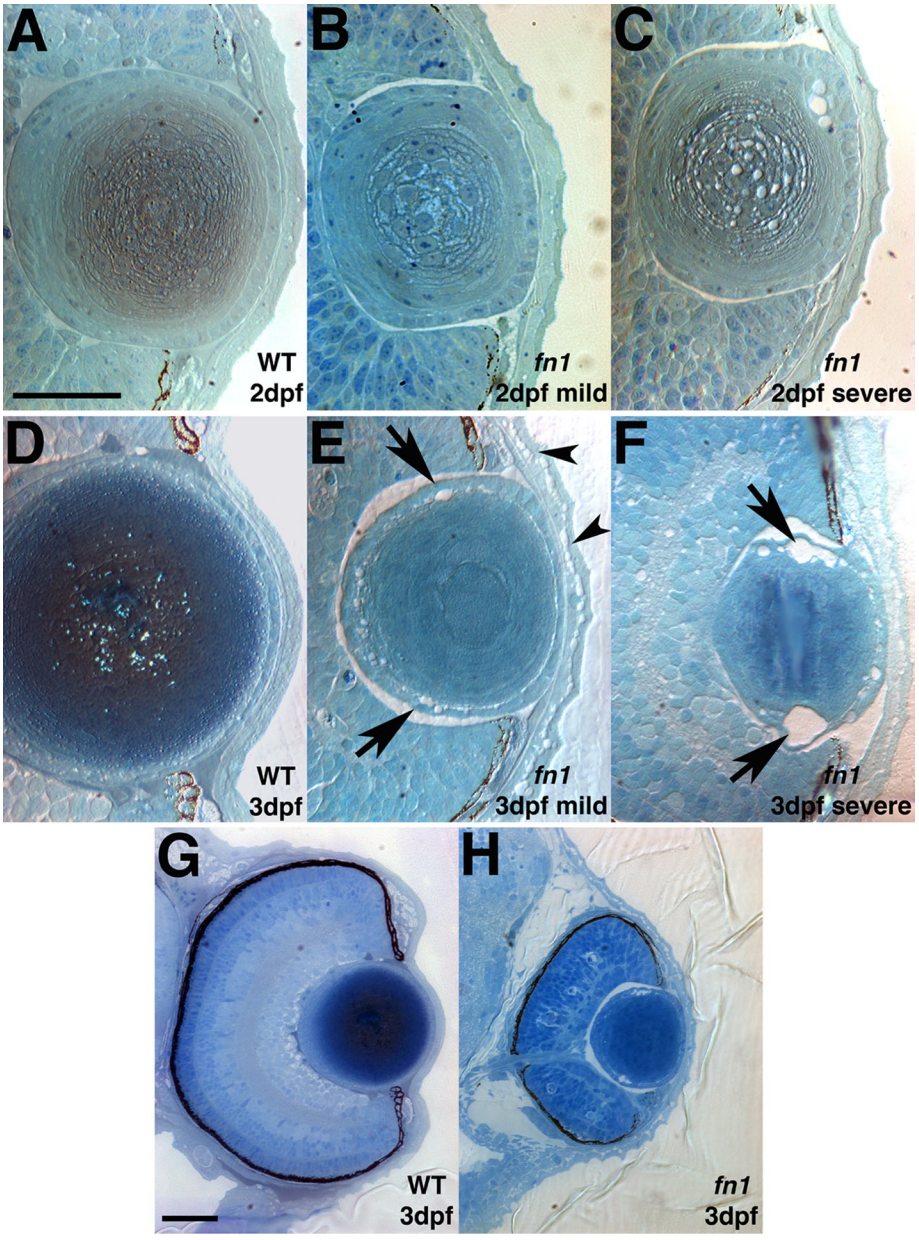


FIGURE 2: Histology of *fn1*-mutant lens and retina. (A) Wild-type (WT) lens at 2 dpf. (B) Mild and (C) severe *fn1*-mutant lenses. (B) Mild mutants possess abnormalities in the lens nucleus where fibers have not fully compacted and a slight gap between the lens and retina. (C) Severe mutants also possess gaps between the epithelium and lens fibers at the anterior of the lens. (D) WT lens at 3 dpf. (E) Mild and (F) severe *fn1* mutant lenses at 3 dpf. Both classes of mutants possess smaller lenses than WT, remain separated from the retina, and possess gaps within the cornea (arrowhead). In the lens, lens fibers are not tightly apposed to the lens epithelium at the anterior (asterisk) and possess gaps at the lens equator between lens epithelial cells and lens fibers (arrow) and at the posterior of the lens. (G) WT and (H) mild *fn1*-mutant retinas at 3 dpf. Mutants are microphthalmic and possess pyknotic nuclei in the retina, and other than ganglion cells, no morphologically obvious retinal neurons are detected. Mutants also show variable degrees of periocular swelling. Dorsal is up in all images. Scale bar, 50 um.

Koshida *et al.*, 2005), and here we use the *fn1*^{tl43c} (*natter*) allele, which possesses a premature stop codon at amino acid 81 of *fn1*, to examine the requirement for Fn1 during lens development (Trinh and Stainier, 2004). *fn1* mutants show an apparent reduction in the amount of Fn1 protein in the lens and cornea (Figure 1B). The amount of staining varied greatly among mutants, however, with some possessing almost no Fn1 (unpublished data) and others retaining some Fn1 in both lens and cornea (Figure 1B). This may reflect cross-reactivity of the antiserum with protein expressed from the other zebrafish Fn1 orthologue, *fn1b* (Sun *et al.*, 2005) and/or protein derived from maternal sources.

fn1 mutants are microphthalmic, and they possess cataracts (Figure 1, D and E). Histological examinations of mutant eyes revealed defects in lens morphology that were apparent by 2 d postfertilization (dpf; Figure 2, A–C). Mutants can be phenotypically grouped into mild and severe classes based on the degree to which their lenses were affected. Mild mutants displayed noncompacted primary lens fibers in the lens nucleus, although overall lens structure was relatively normal (Figure 2B). Severe mutants displayed a higher degree of noncompacted primary fibers, and they also showed defects at the anterior of the lens, in which the apical ends of elongating secondary lens fibers were not tightly apposed to the apical side of the lens epithelium (Figure 2, A and C). By 3 dpf, lens defects become more pronounced, with abnormalities detected in anterior, equatorial, and posterior regions of the lens, all regions in which Fn1 is distributed (Figure 2, D–F). Mutants can still be grouped into mild and severe classes; of importance, morphological defects were observed in anterior, equatorial, and posterior regions of the lens in mutants of both classes; however, they ranged in severity between these classes. In the anterior of the lens, the lens epithelium was present, but the apical ends of the lens fibers were not tightly apposed to the apical side of the lens epithelium, leaving a gap between these cell layers. At the lens equator, a similar defect was observed in which newly formed lens fibers were not apposed to the overlying epithelial layer, nor were they tightly apposed to the older fiber layers internal to them. Posteriorly, gaps were present between layers of lens fibers. In addition, in the *fn1*-mutant eye, the lens did not appear to be fully adhered to the retina, as in most mutants a gap between these two tissues was observed (Figure 2, G and H). Finally, *fn1* mutants also showed defects in retinal lamination and possessed regions of pyknotic nuclei, likely indicating elevated levels of cell death (Figure 2H), and cornea formation was compromised. The corneal epithelium appeared wavy or "scalloped" at 3 dpf, and obvious gaps were present between its two layers (Figure 2, E and F).

TEM analyses of the anterior and equatorial regions of the lens were performed to gain a more detailed view of the lens defects in *fn1* mutants (Figure 3A). In the anterior–central region of the wild-type lens, the lens capsule surrounds the lens and separates it from the cornea, which is composed of morphologically distinct

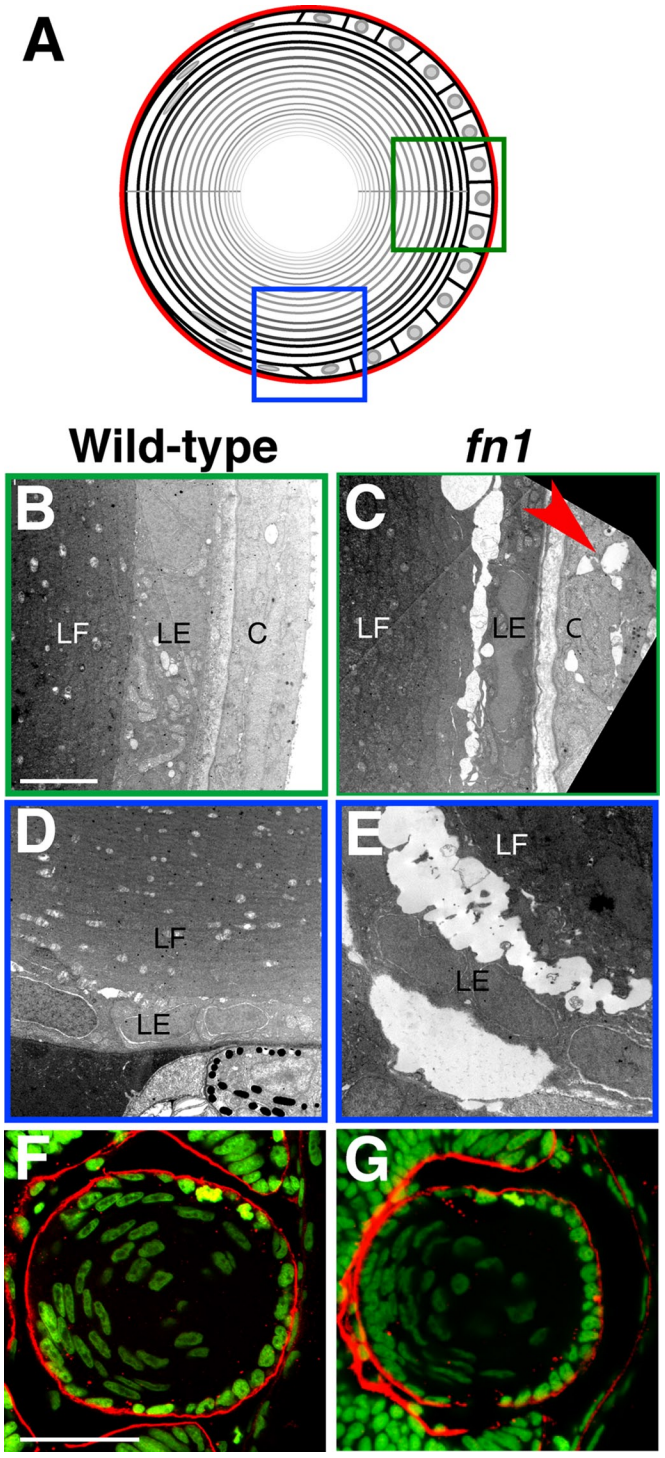


FIGURE 3: Ultrastructure of the *fn1* lens and analysis of the lens capsule. (A) Diagram of the lens indicating approximate regions where TEM images were obtained in B–E: the anterior-central lens epithelium (green square) and the lens equator (blue square). Lens capsule is colored red. (B) TEM images of the 3-dpf WT lens show lens fibers (*LF*) and a lens epithelium (*LE*) that is overlaid by a multilayered cornea (*C*) in which morphologically distinct corneal epithelium, stroma and endothelium are present. (C) *fn1*-mutant lenses possess gaps between the lens epithelium and lens fibers, as well as gaps between the two layers of the corneal epithelium (arrowhead). (D) Equatorially, in a WT lens, lens fibers are tightly apposed to the adjacent lens epithelial cells, whereas here, in *fn1* mutants, large gaps are observed between these two cell types, as well as between the lens and the retina. (F, G) Laminin-111 staining of the lens capsule in (F) wild-type embryo and (G) *fn1*-mutant embryo at 2 dpf. Laminin expression and lens capsule formation are unaffected in the mutant. Scale bar, 5 μm (B–E), 50 μm (F, G).

epithelium, stroma, and endothelium layers (Figure 3B; Zhao *et al.*, 2006). The lens epithelium overlays compacted lens fibers that are devoid of organelles. In *fn1* mutants, the lens epithelium is present, but there are obvious gaps between the epithelium and the underlying lens fibers. In the mutant cornea, the epithelium, stroma, and endothelium are all present, but there are gaps between the outer and inner layers of the corneal epithelium (Figure 3C). In the equatorial region of the lens, the lens capsule forms a boundary between the lens and the retina, and lens epithelial cells are tightly apposed to newly formed and migrating lens fibers, which migrate along the apical side of the epithelium (Figure 3D). In *fn1* mutants, lens fibers have separated from the lens epithelium, resulting in large gaps between the epithelium and fiber layers (Figure 3E), and, often, small gaps are also observed between layers of newly differentiated fibers (unpublished data). Moreover, as noted from the histology images, there are significant gaps between the lens and the retina in *fn1* mutants (Figure 3E). The lens capsule, a site of Fn deposition in other species (Parmigiani and McAvoy, 1984; Cammarata *et al.*, 1986; Duncan *et al.*, 2000), was present when higher-magnification transmission electron microscope (TEM) images were examined (unpublished data). Defects in lens capsule formation and maintenance in zebrafish (Lee and Gross, 2007) and mouse (Dong *et al.*, 2002; Rossi *et al.*, 2003) lead to severe lens malformations, and therefore to verify that the lens capsule was intact in *fn1* mutants, we examined laminin-111 expression. We detected no differences between wild-type and mutant embryos (Figure 3, F and G).

itga5 is required for embryonic lens development

Integrin α5/β1 serves as the principal fibronectin receptor, and although integrin α5 function has not been investigated in the lens, various perturbations to integrin β1 in chicks and mice affect lens development (Bassnett *et al.*, 1999; Simirskii *et al.*, 2007; Samuelsson *et al.*, 2007). We hypothesized that integrin α5 would be required in zebrafish for normal lens formation and predicted that loss of integrin α5 function would phenocopy the lens defects in *fn1* mutants. To test this prediction, we analyzed lens development in the *itga5* mutant *itga5*^{kt451}, which possesses a splice donor-site mutation in intron 4 of *itga5*, leading to an N-terminal truncation and an absence of the ligand-binding domain of the protein (Koshida *et al.*, 2005). Like *fn1* mutants, *itga5* mutants also possessed visible cataracts at 4 dpf (Figure 4, A and B), and histological examination revealed defects in retinal, corneal, and lens development that were indistinguishable from those in *fn1* mutants (Figure 4C,D). The severity of lens defects in *itga5* mutants also ranged from mild to severe (unpublished data). Ultrastructural analyses revealed corneal defects and fiber–epithelium and fiber–fiber defects in the lens (Figure 4, E and F), and lens capsule formation was also unaffected in *itga5* mutants (Figure 4G).

fn1 and *itga5* are required for lens fiber morphogenesis

Interaction between integrin α5 and a Fn-containing ECM enables cell migration in a number of developmental and pathological contexts (Lock *et al.*, 2008; Tsang *et al.*, 2010). Thus we reasoned that deficiencies in either of these proteins could result in an inability of lens fibers to properly migrate and undergo morphogenesis. TEM

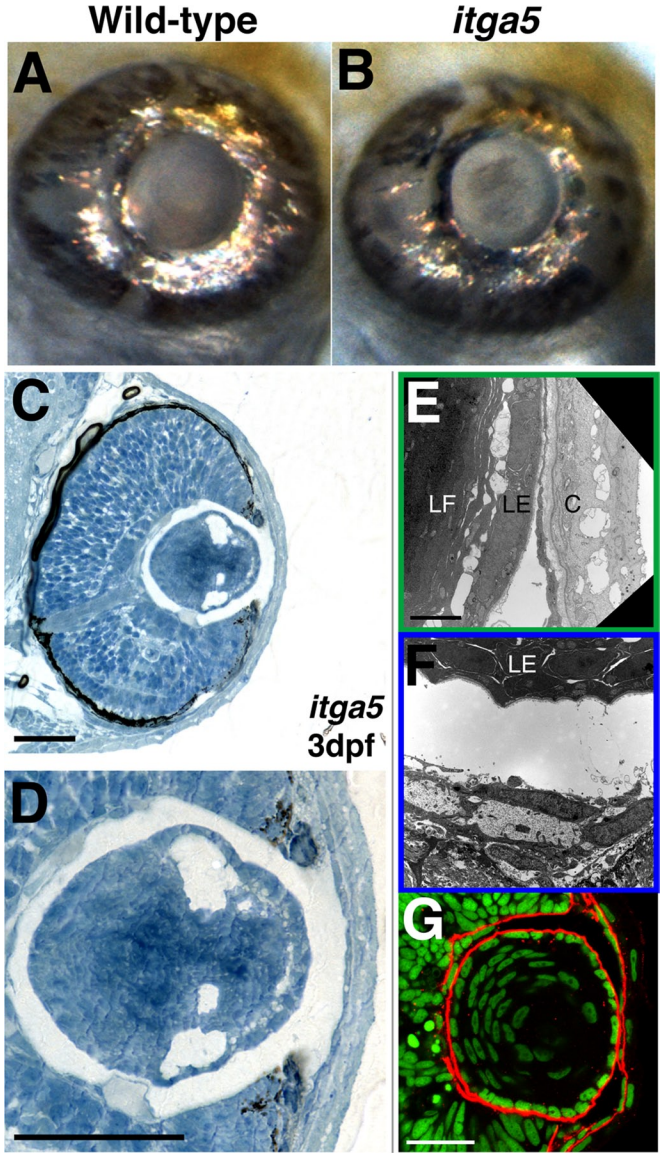


FIGURE 4: *itga5* is required for lens formation. (A) Brightfield image of 4-dpf WT eye and (B) *itga5* mutant with cataract. (C, D) Histological images of 3-dpf *itga5* mutant. (E, F) TEM images of (E) anterior and (F) equatorial regions of the lens. *itga5* mutants are microphthalmic, the lens remains separated from the retina, and they possess gaps within the cornea. Gaps are present between the lens epithelium and lens fibers, evident in both histological and TEM images. (G) Laminin-111 expression, and thus the lens capsule, is normal in *itga5* mutants. Scale bar, 50 μm (C, D, G), 5μm (E, F). C, cornea; LE, lens epithelium; LF, lens fiber.

data from mutant lenses support this prediction, and to further analyze lens fiber morphology and organization in wild-type, *fn1*, and *itga5* mutants, we examined F-actin organization via Alexa 488–phalloidin labeling (Figure 5).

At 2 dpf, wild-type lens fibers have initiated morphogenesis, and secondary fibers at the lens cortex begin to become organized into concentric rings surrounding the core of differentiating primary lens fibers, with these secondary fibers flattening as new fiber layers are added on top of them (Figure 5A; Greiling and Clark, 2009). By 3 dpf, primary fibers have compacted to a degree such that they are no longer stained by phalloidin, whereas the more recently generated secondary fibers continue to surround the primary fibers, compacting and adhering at the midline to fibers from the opposite side of the lens to form anterior and posterior sutures (Figure 5B). This trend continues through 4 dpf, when cortical secondary fibers are well organized and elongated around a central lens nucleus, with both anterior and posterior sutures evident (Figure 5C and unpublished data). At all time points, F-actin also appears to be enriched at the apical and basal ends of the lens fibers, regions to which the fibers adhere to the epithelium and capsule, respectively (Figure 5, A–C). F-actin is also enriched on the basal side of the lens epithelium, where the epithelium adheres to the lens capsule. This enrichment likely represents actin stress fibers that form as a result of the interaction between lens epithelial cells and the ECM of the lens capsule, and these have been observed in the chick central lens epithelium (Weber and Menko, 2006).

In contrast to the pattern of F-actin organization and fiber morphology in the wild-type lens, *fn1* and *itga5* mutants possess a number of defects in lens fiber morphogenesis (Figure 5, D–I). Figure 5 shows F-actin organization in a severe *fn1* mutant and a mild *itga5* mutant to show the range of phenotypes present in both mutant lenses. At 2 dpf, lens fibers in both mutants are mildly affected, with some disorganization of the secondary fibers that surround the lens nucleus (Figure 5, D and G). At 3 and 4 dpf, these defects become more pronounced, and secondary fibers are highly disorganized, are not properly compacted, and in many cases appear to have either not fully elongated to the midline of the lens or not adhered to fibers from the opposite side to form the lens sutures (Figure 5, E, F, H, and I). In addition, the primary fibers of the lens nucleus/central lens are also disorganized to the extent that they can still be stained with phalloidin, suggesting that they have not fully compacted into a transparent core of fibers. Whereas F-actin accumulation in the apical and basal ends of the fibers is not substantially affected in the mild mutants (Figure 5, G–I), in those more severely affected, substantially less F-actin is observed in each of these regions (Figure 5, D–F). Of note, basal F-actin accumulation in the lens epithelium is present, even in the severe mutants (Figure 5, E and F), suggesting that this accumulation results from adhesion of the epithelial cells to a non-Fn substrate in the lens capsule.

Cell–ECM interactions mediated by integrins modulate cell differentiation in a number of developmental contexts (De Arcangelis and Georges-Labouesse, 2000); indeed, integrin α6 is required for lens fiber differentiation in vitro (Walker *et al.*, 2002). Thus the defects in lens fiber morphogenesis in *fn1* and *itga5* mutants could reflect an underlying inability of fiber cells to properly undergo terminal differentiation. To test this possibility, aquaporin 0 (Aq0; Shiels and Bassnett, 1996; Shiels *et al.*, 2001) expression was analyzed in wild-type embryos at 2, 3, and 4 dpf (Figure 6, A–C) and compared with that in *fn1*- and *itga5*-mutant lenses (Figure 6, D–I). In the 2-dpf wild-type lens, Aq0 is localized throughout the lens, in both differentiating primary and secondary fibers (Figure 6A). As lens development proceeds to 3 and 4 dpf, Aq0 continues to be detected in the newly formed secondary fibers at the lens cortex, but expression is no longer detected in the dense fiber matrix in the lens nucleus or in the secondary fibers immediately surrounding it (Figure 6, B and C). In *fn1* and *itga5* mutants, Aq0 is detected at all time points in lens fibers, indicating that lens fiber differentiation progresses normally in the mutant lens (Figure 6, D–I). However, like F-actin, Aq0 distribution also reveals the abnormal fiber morphology, with mutant fibers located in the center of the lens remaining noncompacted and stained with the Aq0 antibody. Mutant fibers also expressed crystallin βB1 (unpublished data; Harding *et al.*, 2008).

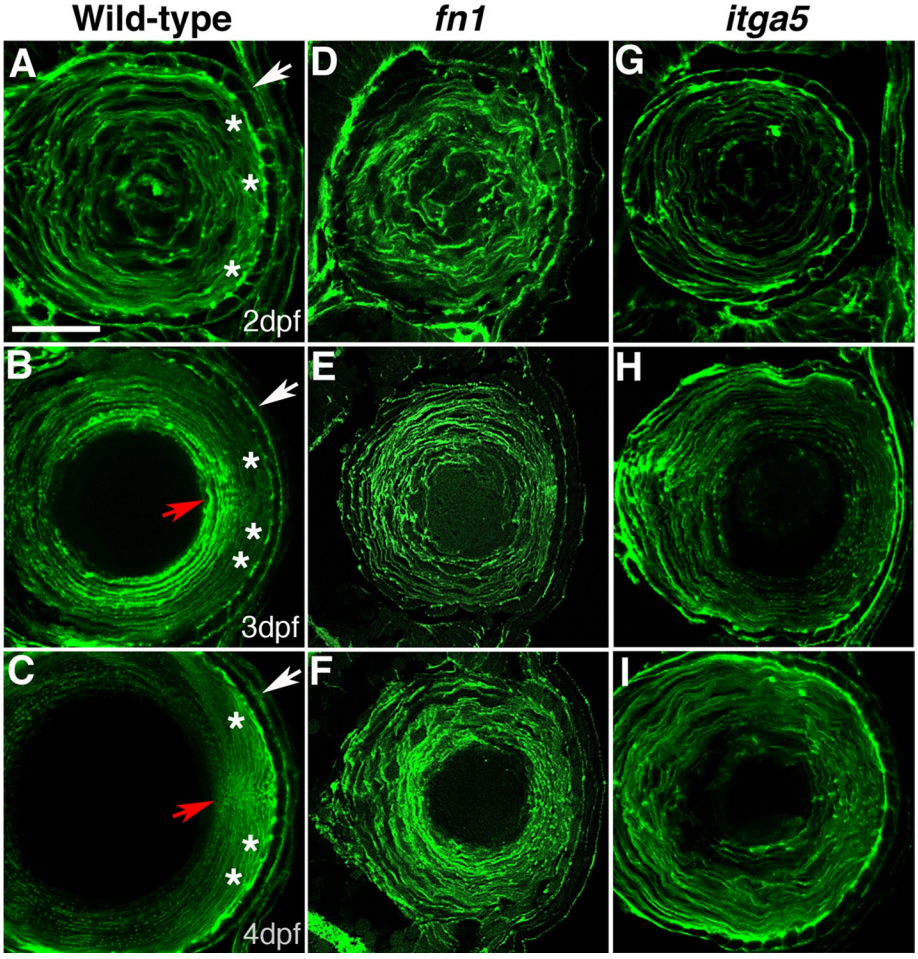


FIGURE 5: *fn1* and *itga5* mutants possess defects in lens fiber morphogenesis. Transverse sections stained with Alexa 488–phalloidin to show F-actin organization in lens fibers. (A–C) Wild-type, (D–F) severe *fn1* mutant, and (G–I) mild *itga5* mutant embryos at (A, D, G) 2 dpf, (B, E, H) 3 dpf, and (C, F, I) 4 dpf. (A–C) F-actin is enriched in wild-type lens fibers at their apical (asterisks) and basal ends and is observed throughout the fibers generating an image composed of rings of concentric secondary fiber shells that span the circumference of the lens. At 3 and 4 dpf, lens sutures are evident (red arrow), where fibers from opposite sides of the lens adhere to one another. F-actin is also enriched on the basal side of the lens epithelium (white arrow). (D–I) In both *fn1* and *itga5* mutants, lens fiber elongation and organization are disrupted. Lens sutures are absent in severe mutants (E, F) and disrupted in mild mutants (H, I). (D–F) Apical and basal F-actin puncta in lens fibers are also absent in severe mutants, but basal accumulation within the lens epithelium appears unaffected. Lens fibers have also not fully compacted, and fibers remain visible in the central lens of both mutants. Scale bar, 50 μm.

Lens fiber differentiation also involves the degradation of the nucleus, mitochondria, and endoplasmic reticulum to generate fiber transparency (Bassnett, 2009). Indeed, in 3-dpf wild-type lenses, lens fiber nuclei can only be detected in the newly generated fibers at the posterior and lateral regions of the lens (Figure 6J). On average, 3-dpf wild-type lenses possess 20.75 ± 1.97 nuclei/section in the central lens (Figure 6L). Mutant lenses, however, possess nuclei throughout the central lens (Figure 6, K and L; 31.6 ± 1.03 in *fn1* mutants, $p < 0.01$; and 33.8 ± 4.13 in *itga5* mutants, $p < 0.01$), indicating that nucleus degradation is perturbed in *fn1* and *itga5* mutants.

Lens epithelial cell fate is maintained in *fn1* and *itga5* mutants

Conditional knockout of integrin β1 in the mouse lens resulted in ectopic expression of β- and γ-crystallins in the lens epithelium, and this correlated with a down-regulation of Pax6 and an up-regulation of cMaf and Prox1, suggesting that in the absence of integrin β1 function, lens epithelial cells ectopically initiate differentiation (Simirskii *et al.*, 2007). In *fn1*- and *itga5*-mutant lenses, both Aq0 and crystallin βB1 expression were normal, and no ectopic expression was detected in the lens epithelium (Figure 6, A–I, and unpublished data). Using two different Pax6 antibodies, we were unable to detect any expression in the wild-type zebrafish lens epithelium at 3 or 4 dpf, despite strong expression in the neural retina (unpublished data). Therefore, to determine whether the lens epithelial fate was maintained in the absence of Fn1 and integrin α5 function, we performed in situ hybridizations for *foxe3*, a gene dependent on Pax6 for expression in the lens epithelium and one whose function is required for normal proliferation there (Dimanlig *et al.*, 2001; Medina-Martinez *et al.*, 2005; Shi *et al.*, 2006). In the 2- and 4-dpf wild-type lens, *foxe3* is distributed to the anterior-lateral regions of the lens epithelium, likely in the proliferative epithelial cells. *foxe3* is distributed in a similar region of both *fn1*- and *itga5*-mutant lenses (Figure 7, A–F). Moreover, epithelial cells remain proliferative in both *fn1*- and *itga5*-mutant lenses at 2, 3, and 4 dpf, as marked by bromodeoxyuridine (BrdU) incorporation (Figure 7, G–L, and unpublished data), although both mutant epithelia show reduced numbers of proliferative epithelial cells at all time points. Proliferating cell nuclear antigen (PCNA) also marks proliferative lens epithelial cells at 4 dpf (Figure 7M), and expression is detected in both *fn1*- and *itga5*-mutant lens epithelia (Figure 7, N and O). In addition, no terminal deoxynucleotidyl transferase dUTP nick end labeling–positive cells were detected in either mutant lens, indicating that cell survival was not compromised in either mutant (unpublished data).

FAK is required for lens fiber morphogenesis

Downstream of integrin α5/Fn interactions, focal adhesion kinase (FAK) is activated, where it mediates numerous intracellular events (Parsons, 2003), and in the lens these may include lens fiber migration and differentiation (Bassnett *et al.*, 1999; Kokkinos *et al.*, 2007). The zebrafish genome possesses two genes encoding paralogous FAK proteins (Corsi *et al.*, 2006)—*ptk2.1* (*fak*; Henry *et al.*, 2001) and *ptk2.2* (Crawford *et al.*, 2003); *ptk2.2* is strongly expressed in the zebrafish embryo at the shield stage (i.e., 6 h postfertilization [hpf]) and it is maintained throughout embryonic development, whereas *ptk2.1* becomes expressed at later stages (Crawford *et al.*, 2003). To determine whether FAK activity is required for normal lens formation in zebrafish, we focused on *ptk2.1* and designed morpholino antisense oligos targeting either the *ptk2.1* translation start site (*ptk2.1*-ATGMO; Supplemental Figure S1) or the *ptk2.1* intron 5/exon 6 junction (*ptk2.1*-SPMO; Figure 8A) and injected these into

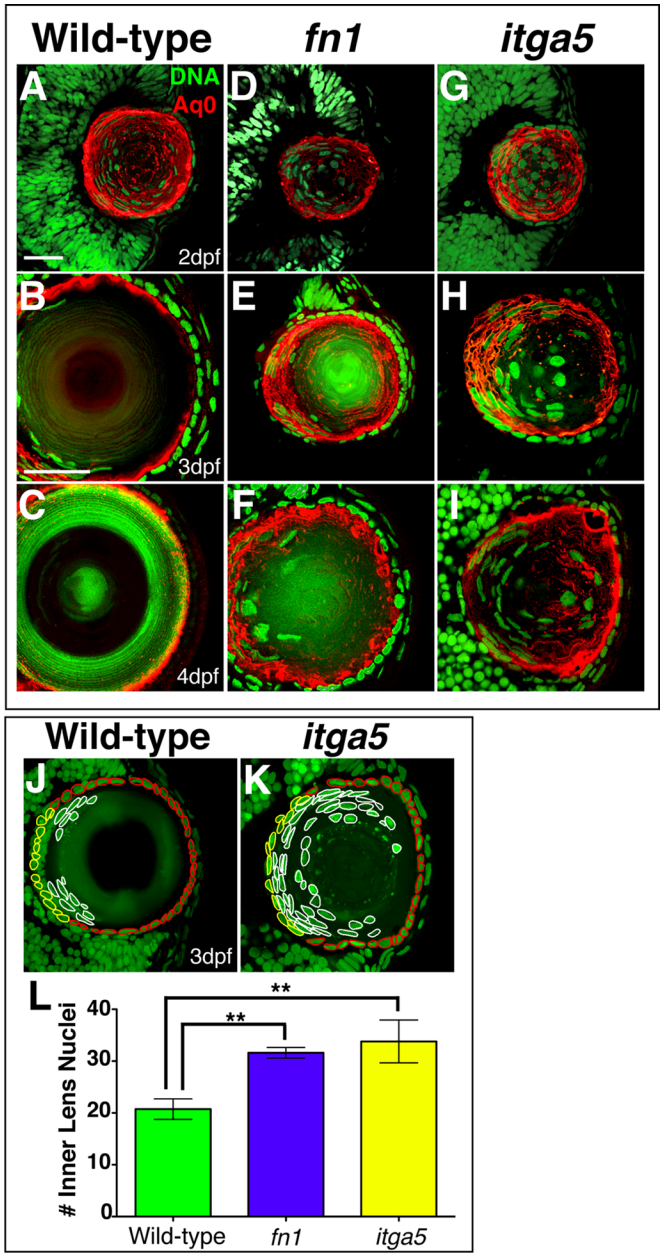
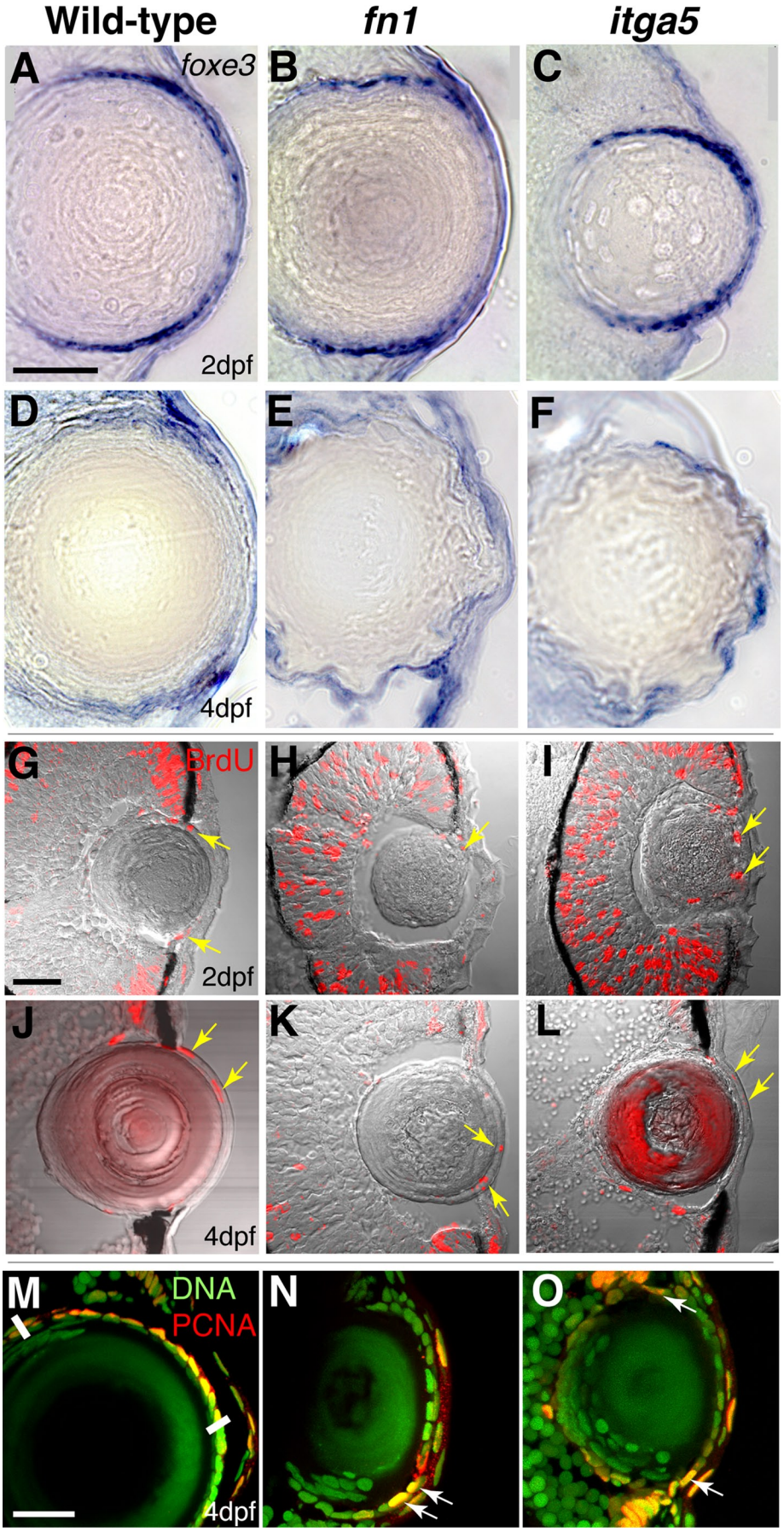


FIGURE 6: Lens fibers initiate differentiation in *fn1* and *itga5* mutants. Aq0 is expressed by differentiating lens fibers in (A–C) wild-type, (D–F) *fn1* mutant, and (G–I) *itga5* mutant lenses at (A, D, G) 2 dpf, (B, E, H) 3 dpf, and (C, F, I) 4 dpf. Nucleus degradation occurs in lens fibers to generate transparency. (J, K) Schematic of how inner-lens nuclei were counted at 3 dpf. Inner-lens nuclei (white) were counted, whereas lens epithelial nuclei (red) and those in the most-posterior lens fibers (yellow) were omitted from counts. (L) Quantification of nucleus counts ($n = 4$–5 embryos per condition). Both mutants possess elevated numbers of inner-lens nuclei. Error bars represent SEM; $^{**}p < 0.01$. Scale bar, 50 μm.

one-cell-stage embryos. Both morpholinos gave similar phenotypes; due to the ability to quantify the efficacy of the *ptk2.1*-SPMO, all subsequent experiments were performed with this morpholino. *ptk2.1*-SPMO injections at 6 and 4.5 ng/embryo resulted in profound developmental and ocular defects (unpublished data). Decreasing the amount of *ptk2.1*-SPMO injected (3 ng/embryo) resulted in defects in lens development (Figure 8), but overall embryonic development was largely normal in these "morphants." *ptk2.1*-SPMO morphants possess altered splicing in a subset of *ptk2.1* transcripts (Figure 8B), resulting in the removal of exon 6 and thereby leading to a frame shift and premature stop codon (Figure 8C). Although injection of higher doses of *ptk2.1*-SPMO resulted in more severe lens defects, these embryos also possessed such pronounced abnormalities in the retina and outside of the eye that it was difficult to ascribe direct roles for ptk2.1/FAK in the lens, and thus they were not analyzed further.

Histology from 3dpf *ptk2.1*-SPMO morphant eyes revealed lens defects identical to those in the mild class of *fn1* and *itga5* mutants. When compared with mismatch control (*ptk2.1*-MM)-injected embryos, *ptk2.1*-SPMO morphants possessed visible gaps between the apical ends of lens fibers and the lens epithelium (Figure 8, D, E, H, and I). Similarly, there were also gaps between the lens epithelium and lens fibers at the lens equator and between the layers of newly formed fibers at the posterior of the lens. The F-actin distribution was perturbed in *ptk2.1*-SPMO morphants, revealing defects in lens fiber morphogenesis (Figure 8, F and J). AqO was expressed normally in *ptk2.1*-deficient lens fibers, and, moreover, no Aq0 was detected in the lens epithelium (Figure 8, G and K), suggesting that, as in *fn1* and *itga5* mutants, ptk2.1/FAK was not required for maintenance of the lens epithelial cell fate.

Taken together, these data support a model in which integrin α5/Fn1 interactions are required for lens fiber morphogenesis, and ptk2.1/FAK might mediate this process. Moreover, these data suggest that FAK activity is required cell autonomously in lens fibers to mediate their morphogenesis during lens development. To test this prediction, we analyzed lens fiber phenotypes in lens fibers expressing a naturally occurring, dominant-negative FAK protein: focal adhesion kinase–related nonkinase (FRNK; Schaller *et al.*, 1993; Richardson and Parsons, 1996; Sieg *et al.*, 1999). Experimentally, lens fiber mosaics were created by injecting embryos derived from a *cryaa*:Gal4VP16 transgenic driver line with either Tol2-*UAS*:mCherry (control) or Tol2-*UAS*:FRNK-GFP cDNAs, along with *tol2* mRNA (Figure 9A). Embryos were grown to 60 hpf, and those with lenses that possessed fewer than 10 fluorescent fibers were identified and then fixed and sectioned for confocal imaging. With the use of central lens sections, quantification of the circumferential location of mCherry-expressing ($n = 88$) or FRNK-green fluorescent protein (GFP)–expressing ($n = 63$) fibers within the lens revealed that, whereas mCherry-expressing fibers were predominantly localized to later-born, secondary fiber layers in the outer region of the lens (67% of fibers), FRNK-GFP–expressing fibers were detected less frequently in the outer region of the lens (28.3% of fibers); instead, FRNK-GFP–expressing fibers accumulated at the posterior/transition zone of the lens (32.6% FRNK-GFP vs. 11.4% mCherry; Figure 9B). In addition, whereas mCherry-expressing fibers occasionally adopted an "amorphous" appearance, looking almost fibroblast like (4.5%), the incidence of such amorphous fibers was substantially higher upon expression of FRNK-GFP (15.2%; Figure 9B). Quantification of the polarity of fiber extension and the degree of extension along the anterior–posterior axis of the lens also revealed defects in FRNK-GFP–expressing lens fibers (Figure 9C and Supplemental Figure S2). 63.2% of mCherry-expressing fibers extended fully along the anterior–posterior axis of the lens, whereas only 36% of those expressing FRNK-GFP were fully extended (Figure 9C). The decrease in FRNK-GFP–expressing fibers fully elongated along their anterior–posterior axis occurred concomitantly with an increase in the number of FRNK-GFP–expressing fibers whose basal ends did not contact the posterior of the lens. Although the apical ends of these fibers reached the anterior pole of the lens, their posterior ends were



located internally within the lens (24% FRNK-GFP– vs. 8.8% mCherry-expressing fibers; Figure 9C). Taken together, these data support a model in which FAK activity is required cell autonomously in lens fibers to mediate migration and extension during lens fiber morphogenesis.

Integrin-linked kinase mutation does not affect lens fiber morphogenesis in zebrafish

Integrin-linked kinase (ILK) interacts with the cytoplasmic region of integrin-β1 (Hannigan *et al.*, 1996) and is activated downstream of integrin–ECM interaction, where it mediates a number of intracellular events in adherent cells, including cytoskeletal polymerization and rearrangement (Dedhar, 2000; Sakai *et al.*, 2003; Wu, 2005). ILK is expressed in the mouse (Wederell and de Iongh, 2006; Weaver *et al.*, 2007) and zebrafish lens (Postel *et al.*, 2008), and therefore to determine whether ILK was required for lens development in zebrafish, we analyzed the *lost-contact* mutant, which possess a nonsense mutation in *ilk* (ilk^{Y319X}) that leads to nonsense-mediated RNA degradation and is a null allele (Knoll *et al.*, 2007). Histological analysis of *ilk* mutants revealed that although they were mildly microphthalmic at 3 dpf (Figure 10, A and B) and 4 dpf (Figure 10, E and F), overall lens structure appeared normal. Examination of lens fiber morphology via F-actin staining revealed an essentially wild-type pattern, with secondary fibers elongated and well organized around the core of primary and older secondary fibers (Figure 10, C, D, G, and H).

FIGURE 7: Lens epithelial identity is maintained in *fn1* and *itga5* mutants. (A, D) *foxe3* is expressed in the lateral regions of the lens epithelium at (A) 2 dpf and (D) 4 dpf. Expression is retained in (B, E) *fn1* mutants and (C, F) *itga5* mutants. Lens epithelial cells remain proliferative, and BrdU incorporation assays reveal the location of proliferative cells within the epithelium at (G–I) 2dpf and (J–L) 4 dpf. (G, J) In the wild-type lens proliferative cells are detected in the lateral regions of the lens epithelium at all time points (yellow arrows). Proliferative epithelial cells are also detected in (H, K) *fn1* mutants and (I, L) *itga5* mutants. (M–O) PCNA also marks proliferative epithelial cells. At 4 dpf in wild-type embryos, PCNA-expressing cells are detected along the lateral regions of the lens epithelium (dorsal epithelium marked with white brackets). In (N) *fn1* mutants and (O) *itga5* mutants, PCNA-expressing epithelial cells are maintained (white arrows). Scale bar, 50 μm.

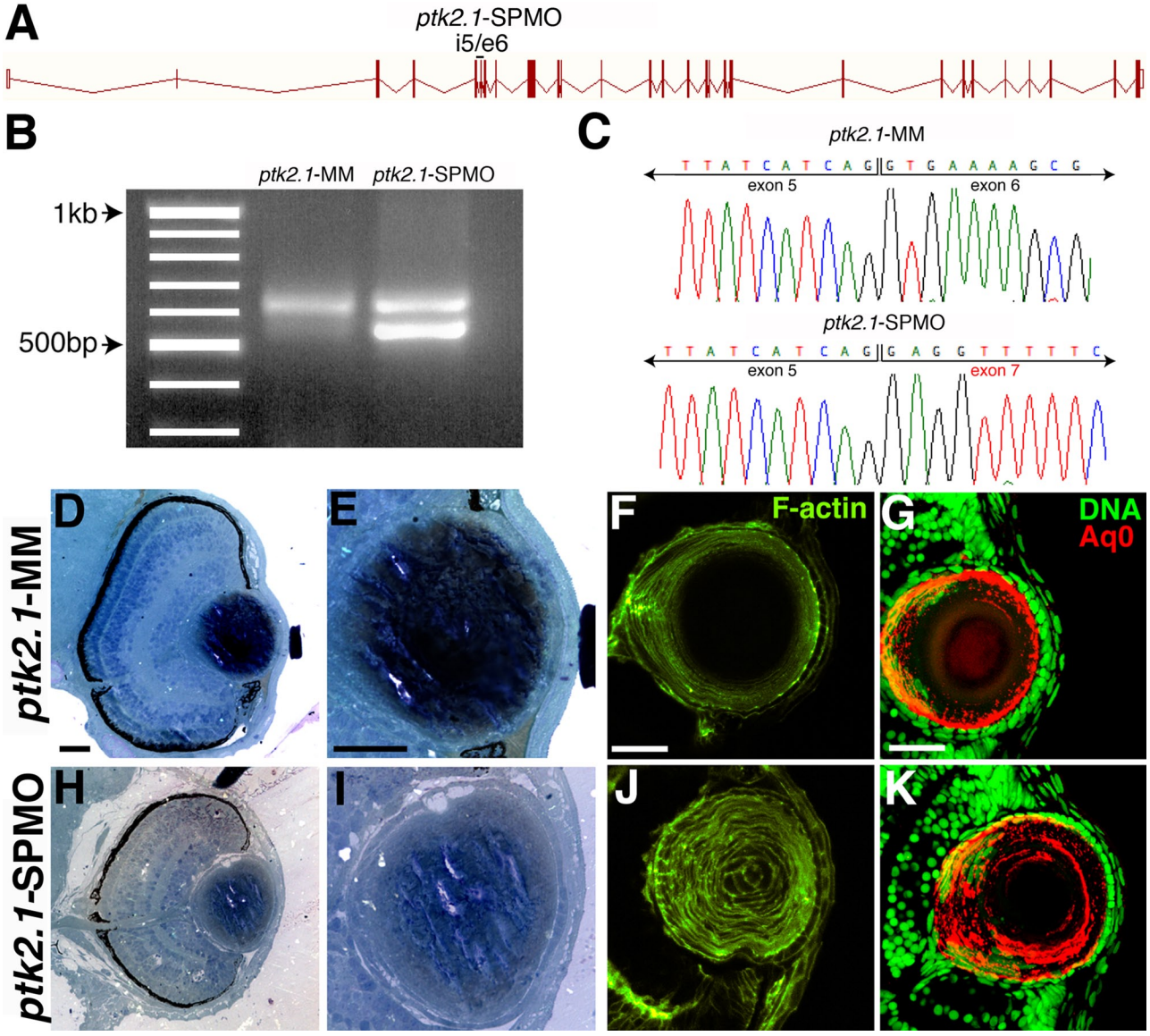


FIGURE 8: Loss of *ptk2.1* (FAK) function compromises lens fiber morphogenesis and phenocopies lens defects in *fn1* and *itga5* mutants. (A) Schematic of *ptk2.1* gene and location of *ptk2.1*-SPMO at the intron 5/exon 6 junction. (B) Reverse transcription PCR of *ptk2.1*-MM– and *ptk2.1*-SPMO–injected embryos demonstrating altered splicing by *ptk2.1*-MO, which removes the 80–base pair exon 6 (DNA ladder superimposed on image). (C) Sequence traces from PCR products derived from *ptk2.1*-MM– and *ptk2.1*-SPMO–injected embryos confirming removal of exon 6 in *ptk2.1*-SPM– injected splice-altered transcripts. (D, E) Histology from 3dpf *ptk2.1*-MM–injected control embryos. (F) F-actin staining and (G) Aq0 expression. (H, I) Histology from *ptk2.1*-SPMO–injected embryo. Loss of ptk2.1 (FAK) function results in gaps between lens fibers and the lens epithelium both anteriorly (asterisks) and equatorially (arrow). (J) F-actin distribution is compromised, and lens sutures are absent. (K) Aq0 is expressed, indicating that fiber differentiation has initiated. Scale bar, 50 μm.

DISCUSSION

By analyzing lens formation in *fn1*, *itga5*, and *ilk* mutants and in *ptk2.1* (FAK) morphants, we demonstrated critical in vivo roles for Fn1, integrin α5, and FAK during vertebrate lens development. Previous studies identified a role for Fn1 during lens placode formation and invagination (Huang *et al.*, 2011), and although later roles for these proteins during lens development were suggested from several studies (Parmigiani and McAvoy, 1991; Svennevik and Linser, 1993; Zelenka, 2004), our results provide the first in vivo demonstration of their requirement during postplacode stages of lens development. When combined with results from studies of cultured lens epithelial cells, these data highlight critical roles for Fn1-dependent cell–ECM interactions during vertebrate lens formation. Our results support a model in which integrin α5/Fn1 interactions are required for lens fiber morphogenesis and ptk2.1/FAK, but not ILK, may mediate this process in a cell-autonomous manner. Fn1, integrin α5, and ptk2.1/FAK are dispensable for some aspects of fiber differentiation (Aq0 and crystallin βB1 expression), but they are required for others (nucleus degradation). Finally, integrin α5 and Fn1 are not required for cell survival

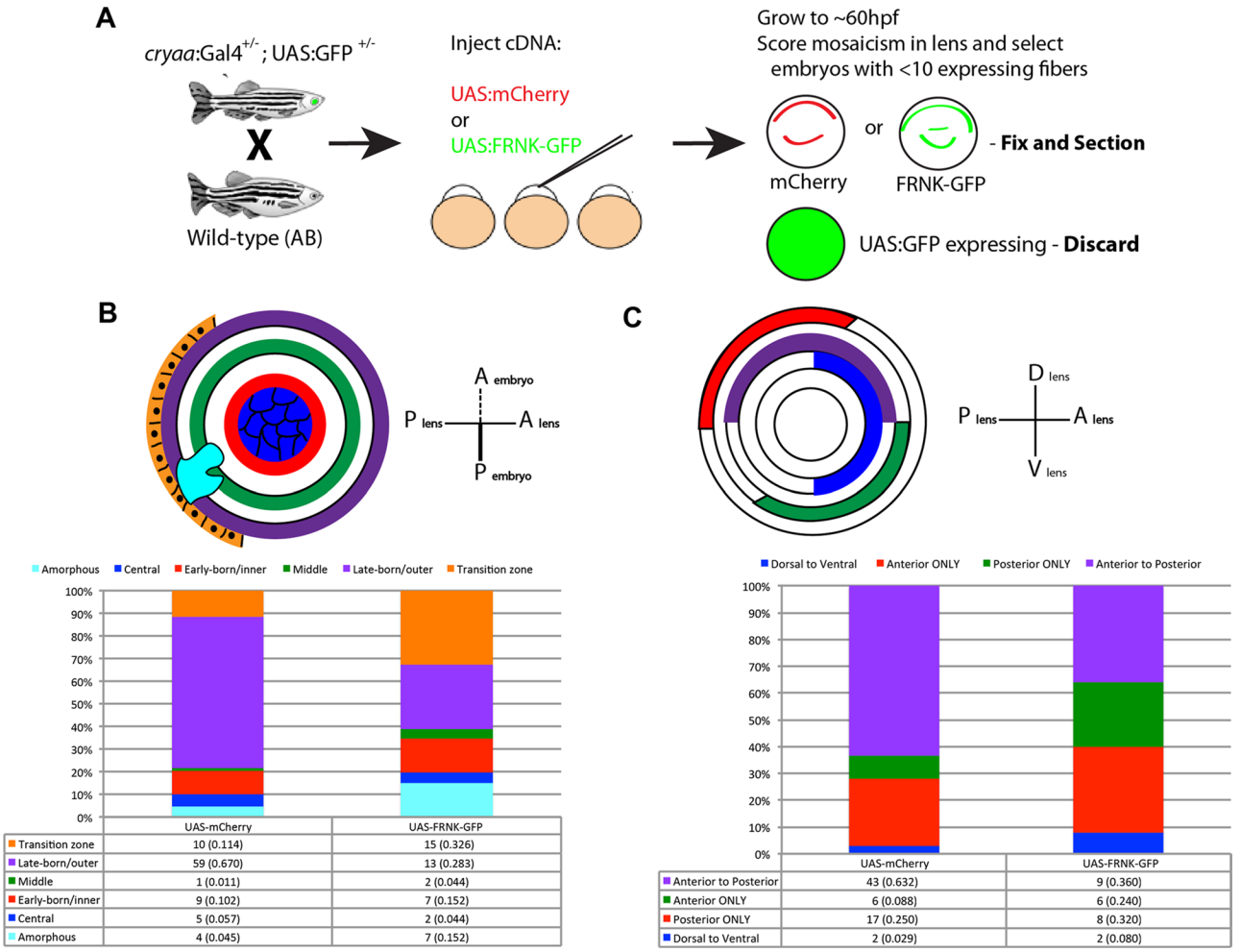


FIGURE 9: FAK activity is required cell autonomously for lens fiber morphogenesis. (A) Schematic of experiment. *cryaa*:Gal4$^{+/-}$;*UAS*-GFP$^{+/-}$ males were outcrossed to AB females. One-cell-stage embryos were injected with either Tol2-*UAS*-FRNK-GFP or Tol2-*UAS*-mCherry cDNA and *tol2* mRNA. Embryos possessing <10 lens fibers positive for FRNK-GFP or mCherry were fixed at 60 hpf, cryosectioned, and imaged. (B) Schematic of rubric used to quantify circumferential position within the lens. Circumferential zones within the lens are color coded (central, blue; early born/inner, red; middle, green; late born/outer, purple), as are posterior lens/transition zone, orange; and amorphous cells, aqua. Frequencies of mCherry and GFP-FRNK positions are graphed, and raw data are presented in table form. (C) Schematic of rubric used to quantify fiber polarity and degree of extension with the lens. Polarity/degree of extension are color coded (purple, full anterior to posterior extension; red, posterior attached; green, anterior attached; blue, dorsal/ventral polarity). Circumferential position on schematic for each fiber category is arbitrary and not representative of location of all fibers of that category. Frequencies of mCherry and GFP-FRNK polarity and degree of extension are graphed, and raw data are presented in table form.

in the lens epithelium or to maintain lens epithelial cell fates; however, they do influence proliferation there.

Integrin α5, Fn1, and ptk2.1/FAK in lens fiber morphogenesis

Fn1- and integrin α5–deficient lens fibers were disorganized, not properly compacted into tightly apposed fiber layers, and not fully elongated along the hemisphere of the lens to form the anterior and posterior lens sutures. Fn was expressed along the apical side of the lens epithelium and at the posterior of the lens, possibly in the lens capsule (Figure 1). When combined with these functional data, a model emerges in which integrin α5, expressed by elongating/migrating lens fibers, interacts with Fn1 along the apical side of the lens epithelium (anteriorly) and the lens capsule (posteriorly) to facilitate fiber morphogenesis. In chick lens explants, the addition of function-blocking antibodies targeting integrin β1 results in defects in adhesion between the basal membrane complex of lens fibers (Bassnett *et al.*, 1999). These data suggest that lens fiber cells may require β1-containing integrin heterodimers during their elongation and migration posteriorly to the center of the lens, and our results indicate that this migration is integrin α5 and Fn1 dependent.

Previous studies also demonstrated that FAK is expressed in the basal membrane complex of lens fibers in the chick eye (Bassnett *et al.*, 1999) and in the embryonic and postnatal rat eye (Kokkinos *et al.*, 2007). Moreover, active FAK (FAK Y^{397}) is also expressed in differentiating lens fibers in the rat (Kokkinos *et al.*, 2007) and in the

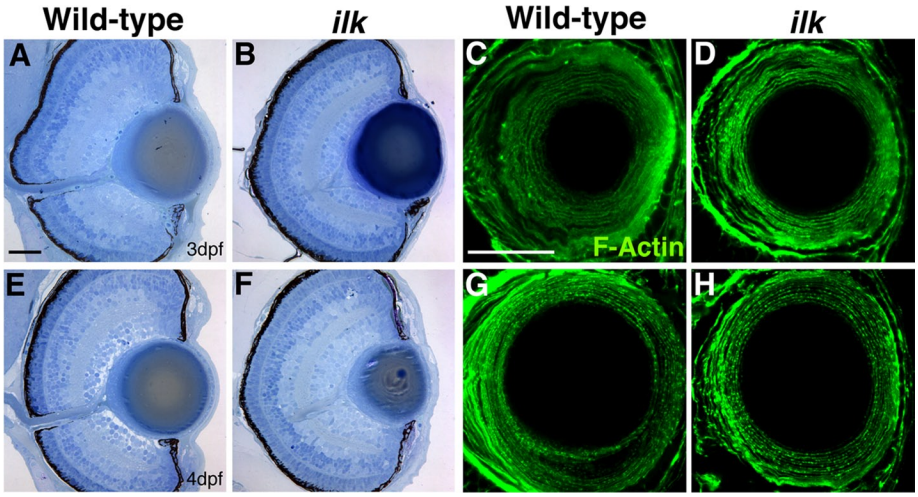


FIGURE 10: *ilk* mutation does not affect lens fiber morphogenesis or lens formation. Histology from (A, E) wild-type and (B, F) *ilk* mutant embryos at (A, B) 3 dpf and (E, F) 4 dpf. No defects are observed in the *ilk*-mutant lens at either time point. F-actin staining at (C, D) 3 dpf and (G, H) 4 dpf also did not reveal any defects in lens fiber morphogenesis or organization. Scale bar, 50 μm.

zebrafish cornea and lens (Semina *et al.*, 2006). Our data demonstrate that ptk2.1/FAK is required for lens fiber morphogenesis in zebrafish and suggest that integrin α5/Fn1 binding may lead to FAK activation in lens fibers, thereby triggering the cytoskeletal rearrangements necessary for fiber elongation and migration. Unfortunately, we have been unable to determine the expression of FAK Y^{397} in *itga5* and *fn1* mutants because antisera cross-reacting with the zebrafish protein is no longer available, and testing several commercially available polyclonal antibodies against FAK Y^{397} did not result in reproducible immunostaining.

Single-fiber analyses using FRNK-GFP to disrupt FAK activity revealed that FAK is required cell autonomously for normal lens fiber morphogenesis. mCherry-expressing control fibers that contacted the anterior pole only were rarely detected (8%), whereas this phenotype was observed in 24% of the FRNK-GFP–expressing fibers, and this was concomitant with a decrease in the level of fully elongated anterior–posterior fibers (63% of mCherry controls vs. 36% of FRNK-GFP fibers). These data suggest that fibers lacking FAK activity may lose their integrity and detach from the posterior capsule or posterior pole as they migrate anteriorly along the lens. These fibers ultimately reach the anterior pole, but they have lost their posterior contact during this migration. Moreover, these data also correlated with a decrease in the number of lens fibers found in the outer layers of the lens (68% of mCherry controls vs. 28% of FRNK-GFP–expressing fibers) and an increase in those found at the posterior/transition zone of the lens (11% of mCherry controls vs. 33% of FRNK-GFP–expressing fibers). This observation suggests that morphogenetic defects in FRNK-GFP–expressing cells may become more pronounced in the outer lens because fibers are required to travel a longer distance around the lens to reach the anterior pole. In the absence of FAK activity, these fibers are either unable to fully traverse the lens (posterior accumulation) or detach from the posterior pole during their migration (anterior-pole-only fibers).

ILK, a second intracellular signaling component activated downstream of integrin activation, appears to be dispensable in zebrafish for lens fiber morphogenesis because *ilk* mutants did not possess any detectable defects in lens formation. Taken together, the expression of Fn in the zebrafish lens and the similarities in phenotype between *fn1* and *itga5* mutants and *ptk2.1* (FAK) morphants, strongly support a model in which an integrin α5/Fn1 → FAK pathway is required for lens fiber morphogenesis in the zebrafish lens. Defects in lens fiber morphogenesis can result in cataracts (Kuszak *et al.*, 2004; Rao and Maddala, 2006; Wederell and de Iongh, 2006), and thus it is likely that the cataracts observed in *fn1* and *itga5* mutants reflect underlying defects in lens fiber morphogenesis in both mutants. Moreover, lens phenotypes in *fn1* and *itga5* mutants and ptk2.1/FAK morphants resemble ocular defects associated with posterior lenticonus in human patients, highlighting the critical role played by cell–ECM interactions during lens development and maintenance in preventing ocular disease.

Despite defects in lens fiber elongation and migration, lens fiber differentiation was only partially affected by loss of integrin α5 and Fn1. Marker gene expression was normal in *fn1*- and *itga5*-mutant lenses, indicating that fiber differentiation initiated properly; however, fibers did not efficiently degrade their nuclei, and both mutants possessed increased numbers of nuclei in the central region of their lenses. The molecular mechanisms underlying nuclear degradation have not been well studied, although it is known to be a DNase II–like acid DNase (DLAD)–dependent process in mice (Nishimoto *et al.*, 2003; Nakahara *et al.*, 2007). Our data suggest that nuclear degradation may be cued in differentiating lens fibers during their migration to the center of the lens and that this "signal" requires integrin α5 and Fn1.

Recent work in mouse has shown that FAK may also be required to anchor lens-derived filopodia to the retinal ECM and that these filopodial–retinal interactions mediate lens pit invagination in a Cdc42- and IRSp53-dependent manner (Chauhan *et al.*, 2009). Staining of zebrafish embryos with F-actin and Bodipy-ceramide did not reveal any filopodia spanning the gap between the developing lens and retina during the early stages of eye morphogenesis (J.H., unpublished observations). The zebrafish lens forms from a solid mass of cells delaminating from the lens placode (Soules and Link, 2005; Dahm *et al.*, 2007; Greiling and Clark, 2009), and therefore it is entirely possible that the mechanisms underlying its morphogenesis differ from those in the mouse. However, that the lens remained separated from the retina in most *fn1* and *itga5* mutants and also in *ptk2.1* (FAK) morphants indicates that integrin α5, Fn1, and FAK might also play some role in the coordinated morphogenesis of the lens and optic cup. Zebrafish embryos are endowed with maternal mRNA and proteins, and thus any early defects in lens formation in *fn1* and *itga5* mutants would likely be obscured by their presence. Similarly, any roles in lens placode formation/delamination or formation of the lens nucleus/primary fibers could also be mitigated by the presence of maternal stores. Future work combining maternal-zygotic mutants with embryological transplants will be useful to examine earlier roles for integrin α5 and Fn1 during lens formation.

Cell–ECM interactions and maintenance of the lens epithelium

Several studies implicated cell–ECM interactions in preventing premature differentiation of the lens epithelium, as well as in maintaining the overall viability of lens epithelial cells. As discussed earlier, integrin β1 plays a key role in maintaining the lens epithelium

(Simirskii *et al.*, 2007), and integrin β1 serves as a binding partner for several integrin α subunits, including α3, α5, and α6 (Takada *et al.*, 2007). Integrin α3/α6 double-knockout mice have an apparent loss of the lens epithelium, although there are no reports analyzing molecular marker expression for the lens epithelium and lens fibers in these mice (De Arcangelis *et al*. 1999; Wederell and de Iongh, 2006). α3β1 and α6β1 heterodimers are laminin receptors (Takada *et al.*, 2007), and laminin is a major component of the zebrafish lens capsule (Figure 3; Lee and Gross, 2007). That lens epithelial fates were maintained in *fn1* and *itga5* mutants and that these cells remained viable despite showing decreases in the number of proliferative cells indicate that integrin α5/Fn1 interactions are dispensable for maintenance of the lens epithelium in zebrafish. Basal actin accumulation, possibly in stress fibers, was observed in the chicken lens epithelial cells, likely resulting from adhesion between epithelial cells and the lens capsule (Weber and Menko, 2006). Moreover, pharmacological disassembly of actin fibers in primary lens cultures from quail embryos grown on a laminin substratum resulted in ectopic differentiation into lens fibers, concomitant with a decrease in proliferation, and these cells ultimately initiated apoptosis (Weber and Menko, 2006). Phalloidin staining in the zebrafish lens revealed F-actin puncta at the basal side of lens epithelial cells that may be stress fibers (Figure 5). Lens capsule formation was normal in *fn1* and *itga5* mutants, and basal actin accumulation was also retained, again indicating that adhesion between the lens epithelium and lens capsule was not grossly perturbed by loss of integrin α5/Fn1 interaction. Taken together, these data support a model in which laminin-dependent adhesion between the lens epithelium and lens capsule is necessary to maintain the lens epithelium, whereas fibronectin-dependent adhesion and FAK function during lens fiber morphogenesis. Zebrafish with mutations in the genes encoding laminin α1, β1, and γ1 have been identified (Semina *et al.*, 2006; Lee and Gross, 2007) and although all show severe defects in lens formation, no analyses of lens epithelium gene expression have been reported. It will therefore be of interest to analyze lens epithelium formation in these mutants and determine whether fibronectin- and laminin-dependent adhesive processes facilitate different aspects of lens development and maintenance in the vertebrate eye.

MATERIALS AND METHODS

Zebrafish maintenance

Zebrafish (*Danio rerio*) were maintained at 28.5°C on a 14-h-light/10-h-dark cycle. Embryos were obtained from the natural spawning of heterozygous carriers set up in pairwise crosses. Alleles used in these studies were *fn1*^{tl43c}, *itga5*^{kt451}, and *ilk*^{hu801} (Trinh and Stainier, 2004; Koshida *et al.*, 2005; Knoll *et al.*, 2007). Animals were treated in accordance with University of Texas at Austin Institutional Animal Care and Use Committee provisions.

Generation of Tg(*cryaa*:Gal4vp16) fish

The Tg(*cryaa*:Gal4vp16)^{mw46} transgenic line was generated from microinjection of *transposase* mRNA and a Tol2 plasmid (Kawakami, 2004, 2005) containing an 881–base pair (–881 to –1) fragment of the zebrafish αA-crystallin promoter (Kurita *et al.*, 2003) driving Gal4-vp16. The promoter sequence was isolated from wild-type zebrafish genomic DNA using the following sequence-specific primers with attB sites added to facilitate Gateway recombination (Invitrogen, Carlsbad, CA) into the Tol2 system (Kwan *et al.*, 2007): crystaa forward, 5′-CATAATGACTTCAAAACAGC-3′; crystaa reverse, 5′-AATGTCAGACCTGGTAACTC-3′. Transgenic founders giving lens fiber–specific transgene expression were isolated from matings to the Tg(*UAS*:GFP)^{kca33} line.

Morpholino injections

ptk2.1 splice-blocking (*ptk2.1*-SPMO), *ptk2.1* translation-blocking (*ptk2.1*-ATGMO), and *ptk2.1* mismatch (*ptk2.1*-MM) morpholinos were purchased from Gene Tools (Philomath, OR). *ptk2.1*-SPMO and *ptk2.1*-MM were injected at 3 ng/injection, and *ptk2.1*-ATGMO was injected at 4.25 ng/injection at the one-cell stage into wild-type AB embryos.

Morpholino sequences are as follows:

ptk2.1-MM (5′-TaCAgCTGCACACATtGAGAtATAT-3′)

ptk2.1-SPMO (5′-TTCACCTGGACACATAGAGAAATAT-3′)

ptk2.1-ATGMO (5′-ATGGCTTTGGTGGGTGCTAACTGTC-3′)

ptk2.1-SPMO efficacy was confirmed by PCR and sequencing using the following primers:

Forward primer (5′-GGACAGTTAGCACCCACCAAAG-3′)

Reverse primer (5′-aagaatctccgcagaccaacg-3′)

Riboprobes and in situ hybridization

Hybridizations using digoxigenin-labeled antisense RNA probes were performed essentially as described (Jowett and Lettice, 1994). Day 1 in situs were modified as followed: embryos were washed out of MeOH and into 1× phosphate-buffered saline with 0.1% Tween-20, incubated in hybridization buffer at 60°C for 1 h, and finally incubated in probe overnight at 60°C. *foxe3* cDNA was obtained from ZIRC (Eugene, OR).

Histology

Histology was performed as described in Nuckels and Gross (2007).

Transmission electron microscopy

TEM was performed as described in Lee and Gross (2007).

Immunohistochemistry

Immunohistochemistry was performed on cryosections as described in Uribe and Gross (2007), with the following exceptions: PCNA and BrdU cryosections were treated with 4 M HCl for 10 min at 37°C before blocking, and when staining for laminin-111, cryosections were treated with 0.5% SDS for 20 min at 37°C before blocking. The following antibodies and dilutions were used: anti-fibronectin (F3648, 1:500; Sigma-Aldrich, St. Louis, MO); anti–laminin-111 (L-9393, 1:200; Sigma-Aldrich); anti-BrdU (ab6326, 1:200; Abcam, Cambridge, MA); anti-PCNA (SC-7907, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA); anti–crystallin βB1 (1:100; Harding *et al.*, 2008; kindly provided by David Hyde, University of Notre Dame, Notre Dame, IN); and anti-aquaporin0 (ab3071, 1:500; Chemicon, Temecula, CA). Nuclei were counterstained with SYTOX Green (1:10,000; Molecular Probes, Eugene, OR). F-actin was stained with Alexa 488–phalloidin (1:50; Molecular Probes). Images were obtained on a Zeiss Pascal confocal microscope (Carl Zeiss, Jena, Germany), and all images are 1-μm optical sections.

BrdU assays

BrdU incorporation assays were performed as described (Ng *et al.*, 2009). Embryos were bathed in 10 mM BrdU for 4 h and fixed immediately thereafter.

Nuclei counts

Cryosections obtained from the center of the lens were stained with SYTOX Green (n = 4–5 embryos/condition). Nuclei in the inner

region of the lens (Figure 6, J and L) were counted and statistical significance determined using a two-parameter, unpaired *t* test (Prism; GraphPad Software, La Jolla, CA).

Mosaic FRNK expression

The *GAL4/UAS* system (Scheer and Campos-Ortega, 1999) was used to specifically direct expression of *UAS* promoter–driven constructs to the lens fibers cells using a Tg(*cryaa*:Gal4vp16)^{mw46} transgenic line. Specifically, *cryaa*:Gal4$^{+/-}$;*UAS*-GFP$^{+/-}$ males were outcrossed to AB females, and one-cell-stage embryos were injected with either 8 pg of Tol2-*UAS*-mCherry or 9.6 pg of Tol2-*UAS*-FRNK-GFP cDNA (kindly provided by Stephanie Woo, University of California, San Francisco, San Francisco, CA) and 33 pg of *tol2* mRNA. Embryos were incubated at 28.5°C for ~58 h. Embryos possessing <10 single lens fibers that expressed FRNK-GFP or mCherry were selected and fixed at 60 hpf in 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C and processed for cryosectioning as described. The 30-µm transverse cryosections were taken and imaged on a Zeiss Pascal laser scanning confocal microscope under a 63× objective at 1-µm *z*-intervals.

Single-lens-fiber analyses

Confocal images of lenses with fibers expressing Tol2-*UAS*:mCherry or Tol2-*UAS*:FRNK-GFP cDNAs were examined and single lens fibers scored to identify their circumferential location and/or their polarity and degree of extension within the lens. To determine circumferential location within the lens, although full lenses were encompassed within three to four 30-µm sections, only fibers located in the central lens were scored, to prevent curvature-induced skewing of position data ($n = 88$ for mCherry and $n = 63$ for FRNK-GFP). Lens fibers were quantified based on circumferential location as being central, early-born/inner, middle, late-born/outer, transition zone (posterior), or amorphous. To quantify polarity and degree of fiber extension, single fibers were scored if they were either contained within one single 30-µm *z*-stack, for those lenses that contained multiple expressing fibers, or if they were the only fiber expressing the construct in the lens, for those fibers spanning multiple sections ($n = 68$ for mCherry and $n = 25$ for FRNK-GFP). Polarity of extension (anterior/posterior or dorsal/ventral) and degree of extension (fully extended anterior–posterior, anterior only, posterior only) were then scored and quantified. FRNK-GFP–positive lens fiber data resulted from three biological replicates, with data quantified from seven embryos and 11 lenses. mCherry-positive lens fiber data resulted from three biological replicates, with data quantified from 11 embryos and 22 lenses.

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