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

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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.

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.
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 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 Jongh, 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 (Svencevik 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 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 Jongh, 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 perlecans (Rossi et al., 2003) and nidogen-1/entactin-1 (Dong et al., 2007), and functional blockade of 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 Longh, 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 (Samuelson et al., 2007; Simirski 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).

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Itga5, Fn1, and FAK in lens development

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 dpf 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, 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
are observed between these two cell types, as well as between the adjacent lens epithelial cells, whereas here, in (D) Equatorially, in a WT lens, lens fibers are tightly apposed to the between the two layers of the corneal epithelium (arrowhead). Gaps between the lens epithelium and lens fibers, as well as gaps stroma and endothelium are present. (C) cornea (fibers (capsule is colored red. (B) TEM images of the 3-dpf WT lens show lens epithelium (green square) and the lens equator (blue square). Lens TEM images were obtained in B–E: the anterior-central lens capsule. (A) Diagram of the lens indicating approximate regions where 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 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; Simirski 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<sup>kit4</sup>, 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 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).
data from mutant lenses support this prediction, and to further ana-
lyze lens fiber morphology and organization in wild-type, fn1, and
itga5 mutants, we examined F-actin organization via Alexa 488–phal-
loidin labeling (Figure 5).

At 2 dpf, wild-type lens fibers have initiated morphogenesis, and
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 en-
riched 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 mor-
phology in the wild-type lens, fn1 and itga5 mutants possess a num-
ber 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 ei-
ther 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 dif-
ferentiation 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 correctly undergo
terminal differentiation. To test this possibility, aquaporin 0 (Aq0;
Shiels and Bassnett, 1996; Shiels et al., 2001) expression was ana-
alyzed 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 cor-
tex, but expression is no longer detected in the dense fiber matrix
in the lens nucleus or in the secondary fibers immediately sur-
rounding 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 ab-
normal fiber morphology, with mutant fibers located in the center
of the lens remaining uncompacted and stained with the Aq0
antibody. Mutant fibers also expressed crystallin βB1 (unpublished
data; Harding et al., 2008).

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.
ectopic expression of Conditional knockout of integrin and Lens epithelial cell fate is maintained in mutants.

cating that nucleus degradation is perturbed in mutants, the central lens (Figure 6L). Mutant lenses, however, possess nuclei age, 3-dpf wild-type lenses possess 20.75 ± 1.97 nuclei/section in the anterior-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 lens epithelium appears unaffected. Lens fibers have also not fully compacted, and basal accumulation within the lens epithelium appears unaffected. Lens 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 lens epithelium (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 Frox1, 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 ptk2.2 and designed morpholino antisense oligos targeting either the ptk2.2 translation start site (ptk2.2-ATGMO; Supplemental Figure S1) or the ptk2.2 intron 5/exon 6 junction (ptk2.2-SPMO; Figure 8A) and injected these into...
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. 

**FIGURE 6:** Lens fibers initiate differentiation in fn1 and itga5 mutants. AqO 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 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.

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 AqO 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 (ilkY319X) 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) 2 dpf 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.
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 in postplacode stages.
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 Y397) is also expressed in differentiating lens fibers in the rat (Kokkinos et al., 2007) and in the lens epithelium or to maintain lens epithelial cell fates; however, they do influence proliferation there.
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 Y397 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 Y397 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 Jongh, 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 longh, 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 (Semia 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 fn1143C, itga5c451, and ilk4607 (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) strain transgenic line was generated from microinjection of transposase mRNA and a Tol2 plasmid (Kawakami, 2004, 2005) containing an 881-base fragment of the zebrafish αA-crystallin promoter (Kunta 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): cryaa forward, 5′-GATAATGACTCTCCACACACGC-3′; cryaa reverse, 5′-AATTGTCAGACCTGTAACCTC-3′. Transgenic founders giving lens fiber-specific transgene expression were isolated from matings to the Tg(UAS:GFP)ca23 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 ptk2.1-1-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′-TaCAgCTGCACACATGAGATAT-3′)
- ptk2.1-SPMO (5′-TTCCACCTGGACACATAGAGATAT-3′)
- ptk2.1-ATGMO (5′-ATGGCTTTGGTGGGTGCTAACTGTC-3′)
- ptk2.1-SPMO efficacy was confirmed by PCR and sequencing using the following primers:
  - Forward primer (5′-GGAGCCAGTTAGCCACACCAAG-3′)
  - Reverse primer (5′-aagaatctcgcagaccaagc-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 situ were modified as follows: 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 β1 (1:100; Harding et al., 2008; kindly provided by David Hyde, University of Notre Dame, Notre Dame, IN); and anti-aquaporin-0 (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)16imad transgenic line. Specifically, cryaa:Gal416imad;UAS-GFP16imad 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 = 68 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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