Integrin-Linked Kinase Deletion in the Developing Lens Leads to Capsule Rupture, Impaired Fiber Migration and Non-Apoptotic Epithelial Cell Death

Laura Cammas,¹ Jordan Wolfe,¹ Sue-Yeon Choi,¹ Shoukat Dedhar,² and Hilary E. Beggs¹

PURPOSE. The lens is a powerful model system to study integrin-mediated cell-matrix interaction in an in vivo context, as it is surrounded by a true basement membrane, the lens capsule. To characterize better the function of integrin-linked kinase (ILK), we examined the phenotypic consequences of its deletion in the developing mouse lens.

METHODS. ILK was deleted from the embryonic lens either at the time of placode invagination using the *Le-Cre* line or after initial lens formation using the *Nestin-Cre* line.

RESULTS. Early deletion of ILK leads to defects in extracellular matrix deposition that result in lens capsule rupture at the lens vesicle stage (E13.5). If ILK was deleted at a later time-point after initial establishment of the lens capsule, rupture was prevented. Instead, ILK deletion resulted in secondary fiber migration defects and, most notably, in cell death of the anterior epithelium (E18.5 – P0). Remarkably, dying cells did not stain positively for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or activated-caspase 3, suggesting that they were dying from a non-apoptotic mechanism. Moreover, cross to a *Bax^{fl/fl}/Bak^{-/-}* mouse line that is resistant to most forms of apoptosis failed to promote cell survival in the ILK-deleted lens epithelium. Electron microscopy revealed the presence of numerous membranous vacuoles containing degrading cellular material.

CONCLUSIONS. Our study reveals a role for ILK in extracellular matrix organization, fiber migration, and cell survival. Furthermore, to our knowledge we show for the first time that ILK disruption results in non-apoptotic cell death in vivo. (*Invest Ophthalmol Vis Sci.* 2012;53:3067–3081) DOI:10.1167/iov.11-9128

The extracellular matrix (ECM) is an essential contributor to the cellular microenvironment, and interaction with the ECM impacts critically cellular identity, behavior, and survival.

From the ¹Department of Ophthalmology, University of California, San Francisco; and the ²Department of Cancer Genetics, British Columbia Cancer Research Centre of the British Columbia Cancer Agency, Vancouver, British Columbia, Canada.

Supported by NIH Grant EY017379 (HEB), a Departmental Core Grant for Vision Research (P30EY002162), and an unrestricted grant from Research to Prevent Blindness to the Department of Ophthalmology. HEB is a recipient of an RPB Career Development Award.

Submitted for publication November 21, 2011; revised March 19, 2012; accepted March 26, 2012.

Disclosure: L. Cammas, None; J. Wolfe, None; S.-Y. Choi, None; S. Dedhar, None; H.E. Beggs, None

Corresponding author: Hilary E. Beggs, Box 0730, 10 Koret Way K127, Department of Ophthalmology, University of California at San Francisco, San Francisco, CA 94143-0730; Telephone 415-476-0644; Fax 415-476-0336; beggsh@vision.ucsf.edu.

Perturbation of ECM integrity or communication with the cell leads to loss of tissue homeostasis, apoptosis, and disease.^{1–4} Cell interaction with the ECM is mediated by integrins that are responsible for physical cell attachment, but also link extracellular signals to intracellular pathways by recruiting adaptor (or scaffolding) proteins to their cytoplasmic tails.^{5,6} Exactly how those integrin signals are integrated within a cell to trigger the correct response remains unclear in many circumstances.

Integrin-linked kinase (ILK) is a key regulator of integrin signaling with serine/threonine kinase catalytic activity,^{7,8} and functions critically as an adaptor protein.⁹ It acts as a hub to link extracellular integrin signals to intracellular signaling pathways, and regulates many aspects of cell biology, such as survival, proliferation, migration, and differentiation. It is localized at the cell-matrix interface, where it binds the cytoplasmic tail of beta1, beta2, and beta3-integrins, and couples them to the actin cytoskeleton.¹⁰ ILK knock-out mice are embryonic lethal due to impaired epiblast polarization, and display abnormal F-actin accumulation at sites of integrin attachment to the basement membrane zone.¹¹ Tissue-specific deletion of ILK results in a wide range of pathological phenotypes.^{12–18} The precise mechanism of ILK function in vivo, however, still is unclear. Overall, ILK regulates cytoskeletal dynamics as ILK deletion leads to disorganization of the actin cytoskeleton in mouse,¹¹ *Drosophila melanogaster*,¹⁹ and *Caenorhabditis elegans*.²⁰

A hallmark of ILK deletion in the mouse is loss of basement membrane integrity, which displays fragmentation and/or detachment.^{13–15,17,21–25} Loss of cell adhesion to the ECM (or disrupted cell-ECM signaling) results in anoikis, which is an apoptotic form of cell death,^{26,27} and a protective mechanism against inappropriate survival and proliferation of detached epithelial cells. Alterations in ILK function can, indeed, lead to apoptosis,^{16,28} either attributed to loss of matrix adhesion,²⁹ or due to an impaired stress response.³⁰

To study the involvement of ILK signaling in matrix organization and cell survival, we deleted it in the mouse ocular lens. The lens has a unique architecture featuring a close relationship between the epithelium and a specialized basement membrane, the lens capsule.^{31,32} During development, the lens pit detaches from the surface ectoderm to form the lens vesicle. This process requires synchronized cellular rearrangements, and remodeling of the ectodermal basement membrane to form an epithelial vesicle surrounded by the highly organized lens capsule. Primary fiber cells elongate to fill the hollow lens vesicle. Subsequently, the anterior epithelium proliferates and differentiates into secondary fibers at the equatorial transition zone. The basal tips of the secondary fibers interact with the posterior capsule through a basal membrane complex, which has been described as containing integrin signaling and cytoskeletal components.³³ It is unclear exactly how the basal membrane complex controls

the adhesion and migration of the fiber cells along the lens capsule. The apical tips of the secondary fibers interface with the apical anterior epithelium, where they also migrate inwardly towards the lens suture. However, the mechanism regulating the unusual apical-apical epithelial cell interaction remains unknown.

The requirement for integrin signaling during lens development is illustrated by the overlapping expression of many integrins in the anterior epithelium, including beta1 and beta3-integrins.^{34,35} Specifically, beta1-integrins are localized at the basal surface of the anterior epithelium and in the basal membrane complex,³⁶ which all are areas where the lens cells contact the capsule, and mediate the attachment of fiber cells to the capsule.³³ Beta1-integrins also are detected at the apical-apical interaction between the epithelium and the fibers, where their role is unclear in the absence of matrix.³⁶ Moreover, maintenance of the lens epithelial phenotype is dependent on beta1-integrin, as conditional deletion of beta1-integrin results in apoptosis of the lens epithelium and degeneration of fiber cells.^{37,38} ILK is expressed in the adult lens,^{32,39} and adult mice with conditional ILK lens deletion are aphakic.³⁷ These studies, therefore, emphasize the requirement for ILK during lens development. However, its exact role still is poorly understood.

Following conditional ILK deletion in the developing mouse lens using two different Cre recombinases, we demonstrated that ILK is critical for the early stages of basement membrane establishment, and that ILK is a key molecule required for fiber migration and lens epithelial survival. A major unanticipated finding is that ILK deletion resulted in non-apoptotic cell death in the lens epithelium.

MATERIALS AND METHODS

Mouse Lines

All experimental procedures on animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and use Committee (IACUC) of the University of California.

Conditional deletion of ILK in the lens was accomplished by crossing mice containing a conditional floxed ILK allele¹² to the *Le-Cre*⁴⁰ and *Nestin-Cre*⁴¹ lines. *Le-Cre* induces recombination at the time of lens placode invagination. In the *Nestin-Cre* line, early lens development is allowed to proceed normally, and ILK is deleted in the lens only after the lens vesicle has detached from the surface ectoderm, and primary lens fiber cells have formed and elongated.^{42,43} Genetic rescue of apoptotic cell death was achieved by crossing the conditional ILK mutant mice to *Bax*; *Bak* double mutants^{44,45} to obtain *Nestin-Cre*; *ILK*^{f/f}; *Bax*^{f/f}; *Bak*^{-/-} triple mutants.

Histological Analysis and Immunofluorescence

Mouse embryo heads were fixed in 4% paraformaldehyde (PFA) and either dehydrated in an ascending alcohol to chloroform series followed by paraffin embedding or cryoprotected in 30% sucrose for embedding in Tissue-Tek OCT (Sakura Finetek, Torrance, CA). Coronal paraffin sections (7 μ m) were stained for eosin or PAS and hematoxylin staining using standard histological procedures. For immunofluorescence, coronal cryosections (10 μ m) were blocked with 10% normal goat serum in 0.3% TritonX-100 and incubated overnight at 4°C with antibodies directed against ILK (1:100, Cell Signaling 3862, Danvers, MA), Pax6 (1:200, Covance PRB-278P, Emeryville, CA), E-cadherin (1:100, Cell Signaling 4065), N-cadherin (1:100, BD Transduction 610920, San Jose, CA), ZO-1 (1:100, Zymed Lab 40-2300, San Francisco, CA or 1:200, Invitrogen 339100, Carlsbad, CA), α SMA (1:200, Sigma C6198, St. Louis, MO), collagen

IV (1:1000, Cosmo Bio Co LSL-LBL 1403, Carlsbad, CA), laminin (1:200, Sigma I9393), fibronectin (1:500, Sigma F7387), cleaved caspase-3 (1:200, Cell Signaling 9664), γ -crystallin (1:2000, gift from Sam Zigler, PhD), and Phospho Erk (1:200, Cell Signaling 9101). Alexa488-conjugated phalloidin was added at the same time as the secondary antibodies for F-actin visualization. Staining was visualized with Alexa488 or Alexa568 conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:500, Molecular Probes, Carlsbad, CA). To probe sections from the *Le-Cre* line with multiple antibodies, signal from the associated GFP transgene was quenched by sample processing.⁴⁰ For ILK and Phospho Erk staining, signal amplification was achieved using the TSA Plus kit (PerkinElmer NEL741, Waltham, MA). Nuclei were labeled with Topro-3 iodide (1:2000, Molecular Probes T3605), sections were mounted in Vectashield, and images obtained and analyzed using a Zeiss Pascal confocal microscope. Images were processed using Adobe Photoshop software and adjusted electronically for brightness and contrast.

ILK Western Blot

Mouse lenses (E17.5) from the same embryo were pooled and homogenized in RIPA buffer containing a protease and phosphatase inhibitor cocktail (Roche, San Francisco, CA), separated on a 4–15% acrylamide gel (Biorad, Hercules, CA) by SDS-PAGE, and transferred to a PVDF membrane. Membranes were blocked for 1 hour (5% milk powder, 0.1% Tween-20 in tris-buffered saline [TBS]) and incubated overnight with 1:1000 anti-ILK (Cell Signaling 3862) or 1:5000 HRP-conjugated anti-beta actin in 5% bovine serum albumin (BSA), 0.1% Tween 20 in TBS. Immunoblots were developed by enhanced chemiluminescence (ECL, Thermo Scientific, Waltham, MA).

Bromodeoxyuridine (BrdU) and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assays

Cell proliferation was determined with a BrdU incorporation assay (5-bromo-2'-deoxyuridine, Roche) to detect cells in the S-phase of the cell cycle. Timed pregnant females were injected with 100 mM BrdU per gram of body weight and were sacrificed 3 hours after injection. The isolated embryos were fixed with 4% PFA, cryoprotected in 30% sucrose, embedded in ornithine carbamoyltransferase (OCT) and cryosectioned (10 μ m). Sections were incubated overnight with anti-BrdU (1:200, BD Pharmingen 555,627, San Jose, CA) and visualized with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibodies (MOM kit, VectorLab PK-2200, Burlingame, CA), followed by color development with 3,3'-diaminobenzidine (DAB, Vector Lab SK-4100), and the sections were counterstained with hematoxylin. The number of BrdU-positive nuclei and the total number of epithelial cell nuclei per section were counted in a minimum of 3 sections per embryo ($n = 3$ –6 embryos per genotype). Results are expressed as mean $\pm$ SEM. Statistical analyses were performed with Student's *t*-tests using Microsoft Excel software. Data were considered to be significantly different for $P < 0.05$.

Apoptotic cell death in cryosections (10 μ m) of whole embedded embryonic heads was visualized by TUNEL staining using an InSitu Cell Death Detection kit (Roche, TMR red) according to the manufacturer's instructions. Positive control staining was confirmed in other tissue areas with known developmental cell death (mesenchyme, tongue, and otic epithelium) of the mouse coronal section.

Transmission Electron Microscopy

For electron microscopy, eyes were emersion-fixed in 2% PFA; 2.5% glutaraldehyde, and embedded in epoxy resin. Ultra-thin sections were viewed on an FEI Tecnai transmission electron microscope, and images captured using an SIA-L9C cooled CCD camera.

FIGURE 1. ILK is expressed ubiquitously in the embryonic lens, and becomes restricted to the anterior epithelium and differentiating secondary fibers during development. Immunostaining of ILK (green) and nuclei (blue: Topro-3 iodide) at various embryonic stages in control (A–H), *Le-Cre; ILK^{fl/fl}* (J) or *Nestin-Cre; ILK^{fl/fl}* (K–N) deleted lenses. Rectangles in C and L indicate magnified regions in D/E and M/N, respectively. (I) Western Blot of control and *Nestin-Cre; ILK^{fl/fl}* E17.5 whole lens extract showing effective ablation of ILK protein expression. CB, ciliary body; Co, cornea; Le, lens; ON, optic nerve; Ret, retina; TZ, transition zone; Va, vasculature. Scale bars are 50 μm.

RESULTS

ILK Protein Expression during Lens Development

The spatio-temporal expression of ILK was examined during mouse lens development (Fig. 1). At the lens vesicle stages (E11.5), ILK was detected in all cells of the lens (Fig. 1A), and remained strongly expressed in the anterior epithelium and elongating primary fibers until the lens vesicle was filled completely (E12.5, Fig. 1F). By E14.5, when secondary fibers begin to differentiate, ILK expression became restricted to the anterior epithelium and the newly forming fibers in the transition zone (Figs. 1B, 2G), where it remained expressed at E17.5. This is consistent with the expression observed in the equatorial region of the adult lens.³⁹ Additionally, ILK also was detected in the developing retina, ciliary body, and hyaloid vasculature.

To investigate the role of ILK in lens development, we crossed mice harboring the ILK floxed allele¹² with mice expressing one copy of either the *Le-Cre* line that drives recombination in the lens placode starting at E9.5,⁴⁰ or the *Nestin-Cre* line that drives recombination in the lens epithelium from E14.5.^{41,46,47} ILK deletion in the mutant lenses was confirmed by ILK immunostaining. Loss of ILK expression was

evident in *Le-Cre; ILK^{fl/fl}* lens at E11.5, whereas staining still was detected in the retina (Fig. 1J). *Le-Cre* is expressed in the surface ectoderm that gives rise to the lens and part of the prospective cornea. However, ILK is not expressed in the cornea at this time (Fig. 1A), so all phenotypes observed were a result of ILK function/deletion in the lens and not from interacting tissues. Similarly, ILK expression was reduced significantly at E14.5 in the *Nestin-Cre; ILK^{fl/fl}* lens (Fig. 1K), and was completely undetectable at E17.5 (Fig. 1L) in the anterior lens epithelium (Fig. 1M) and the differentiating fibers (Fig. 1N). In addition, Western Blot (Fig. 1I) showed effective ILK deletion in the E17.5 whole lens. The faint band observed likely is due to contamination with hyaloid vasculature (that is non-recombined and ILK-expressing) during dissection.

Early ILK Deletion Leads to Anterior Capsule Rupture and Lens Degeneration

Histological analysis of the *Le-Cre; ILK^{fl/fl}* lens (Fig. 2) revealed significant morphological defects at the time and site of lens vesicle detachment from the surface ectoderm. While the mutant ILK lens vesicle detached properly from the surface ectoderm, the anterior epithelial cells were disorganized

(E11.5, Figs. 2B, 2B'), and the capsule was discontinuous in this region (Fig. 2B', pink stain, arrowhead). Epithelial cell derangement and capsule abnormalities remained evident at E12.5 and E13.5 (Figs. 2D', 2F'), where the capsule was less organized as lamellae detached from the capsule (arrows). At the same time, elongation of the primary fibers and general structure of the posterior capsule looked relatively normal in the *Le-Cre; ILK^{fl/fl}* mutant lenses. In 50% of the E13.5 mutants and 100% of the E14.5 mutants, the anterior part of the lens capsule ruptured, with extrusion of the epithelial and fiber cell mass into the aqueous space (Figs. 2H, 2H'). Exiting of the fiber cells was observed further with the specific lens fiber marker γ -crystallin (Figs. 3G, 3H). Once ruptured, the lens subsequently degenerated and resulted in a microphthalmic eye (data not shown).

Capsule Organization Is Impaired in the ILK Deficient Lens

Since capsule defects were observed at the time of lens vesicle formation, we examined the deposition and organization of individual ECM components at E11.5-E13.5 in control and *Le-Cre; ILK^{fl/fl}* mutant lenses (Figs. 3A-N). The lenses also were examined for the presence of apoptotic cell death (Figs. 3A-F).

Separation of the lens pit from the surface ectoderm involves localized apoptosis of epithelial cells at the site of detachment followed by epithelial fusion.^{48,49} In the detaching epithelium, normal apoptosis was detected in controls and mutants at E11.5 and E12.5 as shown by positive TUNEL staining profiles (Figs. 3A-D, long arrows), indicating that separation occurs normally. In the fibers, however, no apoptotic cells should be detected as shown in the control lens (Figs. 3A, 3C, 3E). In the *Le-Cre; ILK^{fl/fl}* mutant, some additional abnormal apoptotic cells were detected in the center of the lens vesicle (Figs. 3B, 3D, 3F, short arrows), presumably due to extrusion of the fibers cells following capsule rupture.

Remodeling of the lens capsule ECM is necessary as the epithelium detaches from the surface ectoderm. The existing basal lamina breaks down and a new one is synthesized to join the fusing edges of the capsule. We examined the distribution of collagen IV and laminin, which are major components of the lens capsule.³¹ In contrast to the regular, compact appearance in the controls, the collagen IV staining was irregular in the *Le-Cre; ILK^{fl/fl}* lenses (Figs. 3B, 3D) and showed improper fusion of the capsule edges at E12.5 (Fig. 3D). Discontinuities (Fig. 3D, arrowheads) and regions of diffuse staining were observed in 100% of the E12.5 and 85% of the E13.5 lenses examined (see Supplementary Fig. 1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9128/-/DCSupplemental>). This diffuse signal likely was due to detachment of lamellae from the capsule and a lack of compaction rather than an increase in extracellular matrix synthesis. At E13.5, the collagen IV staining became more discontinuous in the mutant lens and the capsule was ruptured (Fig. 3F, arrowheads). Laminin

FIGURE 2. ILK is necessary for early lens development and capsule integrity. Sections from control and *Le-Cre; ILK^{fl/fl}* mutant lenses at embryonic stages E11.5, E12.5, E13.5, and E14.5 were analyzed with a combination of PAS staining to visualize the lens capsule basement membrane (pink: A-F), and hematoxylin and eosin staining (G, H) as indicated. Disorganization of the lens epithelium and altered organization of the anterior lens capsule beginning at E11.5 are evidenced at the site of vesicle detachment (arrowheads), where detached lamellae (arrows) are observed in the mutant. The lens ruptures by E14.5. Enlarged pictures show the anterior epithelium (A'-F') or exiting of primary fibers (G', H') of the corresponding lens. Scale bars are 50 μ m.

FIGURE 3. Capsular defects at the lens vesicle stage lead to anterior rupture of the *Le-Cre; ILK^{fl/fl}* lens. Collagen IV (green: A–F) staining reveals progressive anterior capsular defects in the mutant (B, D, F). After vesicle detachment, collagen IV staining is continuous in the control lenses (C, E), whereas in the mutants arrowheads (D, F) point at the sites of capsule rupture. TUNEL (red: A–F) staining shows apoptotic cells. Long arrows show normal apoptosis in the ALE located at the site of vesicle detachment in control (A, C) and mutant (B, D). Short arrows in B, D, and F evidence abnormal apoptosis in the fibers. Laminin (I, J and enlargements), another major component of the capsule, shows the same defects as collagen IV, notably capsule discontinuities and lamellae detachment (arrowheads). Fibronectin is an early component of basement membranes. Its expression (K, L and enlargements) is deficient at the tips of the fusing matrix (arrowheads) at E11.5, and remains diffuse and discontinuous at E13.5 (M, N and enlargements, arrowheads). Enlarged pictures on top of I–N show the anterior epithelium as shown by the rectangle in I, K, and M. γ -Crystallin (G, H), which specifically stains fiber cells, shows extrusion of fibers at E14.5. All cells within the lens are positive for γ -crystallin. Blue staining is Topro-3 iodide for nuclei. Scale bars are 50 μ m.

organization in the anterior capsule of the *Le-Cre; ILK^{fl/fl}* mutant lenses also was impaired (Figs. 3I–J); however, not as severely as collagen IV. Capsule discontinuities and lamellae detachment (Fig. 3J) were observed. Therefore, collagen IV and laminin were expressed following ILK deletion, but discontinuously and diffusely, which is indicative of a disorganized basement membrane. Since development of a mature basement membrane depends on the proper formation of a

provisional fibronectin scaffolding,^{50,51} we investigated fibronectin expression at the earliest stages of lens capsule formation. In the E11.5 control lens, fibronectin was detected at the edges of the fusing capsule (Fig. 3K and enlargement), but showed improper diffuse staining in the ILK mutant (Fig. 3L and enlargement). At E13.5, fibronectin remained diffusely expressed and failed to form a continuous basement membrane (Fig. 3N and enlargement, arrowheads).

FIGURE 4. The ILK-deleted epithelium displays loss of organization, stress and impaired molecular apical organization. No convincing signs of EMT are detected following ILK deletion, as PAX6 (A, B), N-cadherin (C, D), and E-cadherin (E, F) expression are unchanged in the ILK-deleted epithelium. However, the stress marker aSMA is upregulated in the epithelium (I, J), and E-cadherin staining reveals epithelial disorganization following ILK deletion (G, H). The epithelium-fiber interface is slightly disorganized, as shown by the perturbed expression of the apical marker ZO-1 (K, L), which is discontinuous (arrow). All samples are E13.5. Blue staining is Topro-3 iodide for nuclei. Scale bars are 50 μ m.

The ILK-deleted Lens Epithelium Displays aSMA Independent of EMT

Next, we explored whether epithelial molecular defects could explain the morphological disorganization observed in the ILK-deleted anterior epithelium.

Disruption of integrin signaling in the lens triggers some features of EMT and loss of epithelial identity.³⁸ Therefore, we investigated the expression of markers of EMT, E- and N-cadherin that are deregulated during EMT,⁵² and PAX6, which

is essential for maintenance of the epithelial phenotype in the lens.⁵³ Unexpectedly, E-cadherin, N-cadherin, and PAX6 expression levels were not changed significantly in the *Le-Cre; ILK^{fl/fl}* anterior epithelium (Fig. 4 compare A, C, E to B, D, F). However, because E-cadherin stains the cell membranes, it evidenced the disorganization of the epithelium layer at the cell-cell interaction level (Figs. 4G, 4H).

Another common marker for detection of EMT and cellular stress is aSMA.⁵⁴ In the control lens epithelium, its expression was very weak, but showed a strong upregulation in the *Le-Cre; ILK^{fl/fl}* mutant (Fig. 4 compare I and J). In the lens, the tight junction protein ZO-1 is expressed regionally at the apical side of the epithelium (Fig. 4K). In the *Le-Cre; ILK^{fl/fl}* mutant its expression showed minor interruptions (Fig. 4L, arrow). N-cadherin also is expressed at the apical-apical epithelial-fiber interactions as shown by the sharp green staining in the control (Fig. 4C, arrows), whereas in the mutant its expression was not as defined and intense (Fig. 4D).

Therefore, ILK deletion in the lens did not cause true EMT, but resulted in upregulation of aSMA and moderately disturbed expression of ZO-1, most likely indicating that those cells are under stress or undergoing an intermediate stage of EMT.

ILK Is Involved in Fiber Migration and Epithelial Cell Survival

To analyze further the function of ILK in the developing epithelium, we deleted ILK in the lens after the establishment of the capsule by crossing the *ILK^{fl/fl}* mice with mice harboring the *Nestin-Cre* transgene,⁴¹ which drives recombination in the lens epithelium from E14.5.^{42,43} As shown in Figures 1L–N, *Nestin-Cre* reduces significantly the levels of ILK expression at E14.5, and ILK protein is absent completely in later stages.

Surprisingly, ILK ablation by the *Nestin-Cre* line led to progressive loss of the central epithelium (Fig. 5). This central cell loss rapidly extended to the peripheral epithelium and eventually resulted in complete lens degeneration. Disorganization of the anterior epithelium was evident at E17.5 (Fig. 5C), where 45% of the embryos ($n = 11$) exhibited multilayers of unevenly spaced cells. In 100% ($n = 3$) of E18.5 lenses, the epithelial cell nuclei were spread out but their cytoplasm still was detectable (Fig. 5G, arrows), indicating that fewer but larger epithelial cells are present and have expanded over regions of cell loss. Finally, at P0 ($n = 5$) large gaps of missing cells were observed in 60% of the embryos (not shown), while total loss of the anterior epithelium was observed in 40% of the embryos (Figs. 5M, 5N, arrows), some of which already displayed degenerating lenses (Fig. 5N, inset).

Along with the loss of epithelial cells, a striking difference in fiber cell migration was observed in the *Nestin-Cre; ILK^{fl/fl}* lenses. Normally, secondary fibers differentiate from the epithelial cells at the transition zone and elongate, contributing to the rounded shape of the lens. The apical tips of the fiber cells migrate along the apical side of the epithelium. When they reach the suture, they detach and link to tips of their symmetrical opposite⁵⁵ (Figs. 5B', 5F', arrows). The anterior end feet of the ILK-mutant secondary fibers consistently failed to migrate along the apical side of the epithelium (Fig. 5C', arrows) and piled up at the transition zone, creating a bowed morphology at E17.5 (100% of $n = 11$). (The bowed morphology also can be seen in Fig. 6R). Anterior fiber migration failure was observed further with phalloidin staining to visualize F-actin at E18.5 (Figs. 5I, 5I' and 5J, 5J'). As a result, the lens core was pushed-up against the epithelium (Figs. 5D, 5H). Areas of degeneration and vacuoles also were detected in the posterior part of the ILK-mutant lens (Figs. 5H, 5N). Finally, the lenses degenerated in newborns and were completely absent from adult mutant animals (data not shown). The basal

FIGURE 5. Central and progressive anterior epithelial cell loss and fiber migration defects are evidenced when ILK is deleted after the lens has formed. Hematoxylin and eosin staining of E17.5 (A–D), E18.5 (E–H), and P0 (K–N) control and mutant lenses as indicated. The major defects are loss of epithelial cells leading to an acellular anterior lens (*short arrows*) and failure of anterior fiber migration (*long arrows* indicate fiber direction). Phalloidin staining for F-actin (I–J) shows defective migration of the anterior fibers (*long arrows*). The posterior tip of the fibers migrate (O–T, *long arrows*), but at E17.5 fiber-fiber interaction seems abnormal (P, *dotted line*). Rectangles in A indicate magnified regions shown in B, B', and O. Rectangle in I indicates magnified region shown in P. Inset in N shows very severe phenotype of a mutant, showing phenotype heterogeneity. Scale bars are 50 μ m.

FIGURE 6. The ILK-deleted lens epithelium shows profound disorganization and impaired signaling. (A, B) E-cadherin shows epithelial disorganization. (C, D) Pax6 expression is unaltered. (E, F) aSMA is dramatically upregulated. (G–L) The ZO-1 domain of expression is reduced at the TZ (J, K), and expression is impaired at the apical side of the anterior epithelium (G, H). (M–R) Phospho-ERK expression is down-regulated in the anterior epithelium (M, N). All samples are E16.5. Rectangles in I and O indicate magnified regions in G, J and M, P. Scale bars are 50 μ m.

tips of the fibers were able to migrate along the posterior capsule at E17.5 (Figs. 5O, 5P, arrows), but the posterior lens displayed an abnormal fiber-fiber interaction zone at the suture (Fig. 5P, dotted line). At later stages, posterior migration appeared slightly disrupted; however, the presence of numerous vacuoles made it difficult to analyze the basal tip of the fibers in detail (Figs. 5Q–T).

ILK Deletion Does Not Lead to Lens Capsule Discontinuity in Nestin-Cre Mutants

Since capsular defects were observed in the *Le-Cre; ILK^{fl/fl}* mutant animals, we investigated capsule integrity in the *Nestin-Cre; ILK^{fl/fl}* line to determine if the loss of anterior cells possibly was related to a ruptured or absent lens capsule. The capsule was, indeed, present and continuous, displaying no holes in the *Nestin-Cre* ILK mutant (PAS staining, Figs. 7B, 7D), although sometimes thin and uneven in regions. The major ECM components of the lens capsule, collagen IV (Figs. 7E, 7F) and laminin (Figs. 7G, 7H) were expressed normally even in regions of epithelial cell loss. Since fibronectin is an early component of the basement membrane, its expression was limited at E17.5 (Figs. 7I, 7J) in the control and mutant lenses.

Anterior Epithelial Cells Are Disorganized and Lose Their Epithelial Characteristics

To understand better the anterior epithelial cell loss observed in the *Nestin-Cre; ILK^{fl/fl}* mutant lens, molecular analysis of the epithelium was performed at E16.5 just before the beginning of cell loss (Fig. 6) and showed the same type of phenotype as in the *Le-Cre; ILK^{fl/fl}*, notably a strong up-regulation of aSMA (Figs. 6E, 6F) indicative of cell stress. Misexpression of ZO-1 was observed in the anterior lens epithelium (ALE, Fig. 6H) along with

FIGURE 7. The capsule remains intact in the *Nestin-Cre; ILK^{fl/fl}* lens. Anterior epithelium (hematoxylin in A–D and Topro-3 iodide in E–J) showing capsule staining of control and *Nestin-Cre* ILK mutants. (A–D) PAS staining shows a thin but unruptured capsule (pink). (E–H) Collagen IV and laminin are expressed correctly even in regions of nuclear spacing (arrows). (I–J) At E17.5 fibronectin expression is nearly undetectable. Scale bars are 50 μ m.

conserved expression of PAX6 (Figs. 7C, 7D). Moreover, ZO-1 was not detected at the transition zone (Fig. 6K), which is consistent with the incapacity of fibers to migrate beyond this point in the mutants (Figs. 5C', 5G', 5J', 5M'). However, the epithelial organization of the lens was much more disturbed in the *Nestin-Cre; ILK^{fl/fl}* than in the *Le-Cre; ILK^{fl/fl}* as revealed by E-cadherin staining (Figs. 6A, 6B). In the control epithelium, the cells formed a monolayer and E-cadherin displayed a sharp, vertical staining, revealing the normal cuboidal shape of an epithelial cell (Fig. 6A). In the *Nestin-Cre; ILK^{fl/fl}* mutant epithelium, the nuclei were multilayered, the E-cadherin staining was diffuse, and the cells displayed a perturbed morphology showing the loss of architecture of the lens epithelium.

As the most abundant mitogen-activated protein kinase (MAPK) in the lens epithelium,⁵⁶ ERKs are implicated in all aspects of lens epithelial biology, including fibroblast growth factor (FGF)-induced cell proliferation and differentiation,⁵⁷ and apoptosis.⁵⁸ It also has been shown that beta1-integrin deletion leads to decreased activation of ERK and cell death of fetal islet cells.⁵⁹ Staining for phospho-ERK (Figs. 6M, 6R) revealed a dramatic reduction in ERK activation in the ILK-mutant epithelium, indicative of a generally disturbed function of the epithelium.

FIGURE 8. Proliferation and apoptosis are unchanged in the *Nestin-cre; ILK^{fl/y}* lens. BrdU incorporation assay was used to detect S-phase cells at E14.5, E16.5, and E17.5. (A, B) E17.5, BrdU-positive cells are in black and nuclei in purple. (C) Percentage of BrdU-positive cells in the anterior lens epithelium at different developmental stages. TUNEL assay (H–M) was used to detect apoptotic cell death in the E17.5 and P0 lenses as indicated. L and M are the same as J and K overlaid with staining for γ -crystallin. H'–M' show enlargements of the anterior epithelium from H–M. The circles highlight the high background in the lens core due to normal denucleation of the fibers that follows an apoptotic-like mechanism. In the P0 mutant (K') the circle indicates positive nuclei that were denucleating fibers pushed towards the anterior part of the lens and should not be mistaken with epithelial nuclei, as demonstrated by γ -crystallin staining that is specific of fiber cells (M, M'). Occasional apoptotic cells were detected (arrows) without significant difference between controls and mutants. Immunostaining of cleaved-caspase 3 in the E17.5 (D, E) and P0 (F, G) lenses showed no positive cells in either the controls or mutants. The inset in E shows positive cells, detected as internal positive controls from the same sections as the lenses. Scale bars are 50 μ m.

Anterior Epithelial Cells Die through a Non-Apoptotic Mechanism

Loss of the anterior epithelial cells could be explained by changes in proliferation or apoptosis. Whereas in the adult lens, cell proliferation is restricted mostly to the germinative zone in which epithelial cells differentiate into secondary fibers, at embryonic stages a large proportion of the developing anterior epithelium is proliferating rapidly.⁵⁵ Since ILK deletion

can lead to reduced proliferation in the brain,¹⁷ it seemed possible that reduced levels of proliferation could impact the number of epithelial cells in the ILK mutant lens. However, a BrdU incorporation assay revealed that proliferation was unaltered in the ILK mutants regardless of stage (Figs. 8A–C), suggesting that loss of the anterior epithelium was not due to exhaustion of the pool of epithelial cells following differentiation into fiber cells.

FIGURE 9. The ILK-deleted anterior epithelium dies independently from the pro-apoptotic proteins *Bax* and *Bak*. The ILK lens deletion was introduced in *Bax*; *Bak* mutant mice that display apoptosis resistance. PAS staining of E18.5 (A–D) and P0 (E–H) show that the anterior epithelium of triple mutant lenses is absent (B, F). Anterior epithelium cell death due to ILK deletion is not rescued by the *Bax*; *Bak* mutation. Scale bars are 50 μ m.

Therefore, apoptosis was explored as the favored mechanism to explain the loss of anterior epithelial cells following ILK deletion. We hypothesized that a significant number of epithelial cells would need to undergo apoptosis to explain the ALE loss. Moreover, as the cell loss was progressive over a few days of embryonic development, apoptosis likely would be detected at various stages. Therefore, TUNEL staining was performed before the cell loss phenotype was observed at E16.5 ($n = 3$ mutants), when cell loss was dramatically increasing at E17.5 ($n = 4$ mutants) and E18.5 ($n = 7$ mutants), and when the epithelium was nearly lost at P0 ($n = 5$ mutants). Surprisingly, anterior epithelial cells were TUNEL-negative in controls and mutants at E16.5, E17.5, and E18.5 (not shown and Figs. 8H, 8I). Only occasional positive cells were detected (arrows) with no significant difference between controls and mutants. In the lens core at early developmental stages, fibers normally displayed high TUNEL background (asterisks). This likely is due to the active denucleation process occurring in the lens fiber cells at this time, resulting in dispersion of degrading DNA and TUNEL-positive nuclei.⁶⁰ Negative TUNEL staining was not due to technical issues, as developmental apoptosis was detected strongly in other tissues present in the embryonic section, such as the tongue⁶¹ (E16.5–E17.5, not shown), corneal epithelium and eyelids (E18.5–P0, not shown). At P0 when major gaps were observed in the epithelium, only occasional apoptotic cells were detected (Figs. 8K, 8K', arrows) with no increased incidence near the gaps. In the

gaps, some TUNEL-positive nuclei were detected (Fig. 8K', asterisk). However, these nuclei also co-stained with fiber cell marker γ -crystallin,⁶² indicating that TUNEL-positive profiles belonged to denucleating fibers and not epithelial cells (Figs. 8L, 8M). Therefore, cell loss in the anterior epithelium of *Nestin-Cre*; *ILK*^{fl/fl} lenses was not a consequence of elevated apoptosis as detected by TUNEL staining.

To corroborate this result, E17.5 and P0 sections were stained for cleaved caspase-3, which is the key executioner caspase that ultimately will lead to the cleavage of cellular proteins during apoptosis.⁶³ Neither control nor *Nestin-Cre*; *ILK*^{fl/fl} lenses displayed caspase-3 staining (Figs. 8D–G) reinforcing the finding that cell loss in the anterior epithelium was not due to apoptosis. The inset in Figure 8E shows a positive control of cleaved caspase-3 staining in cells outside of the lens, but on the same tissue section.

To confirm further that ILK-deleted cells do not die from an apoptotic mechanism, the ILK lens deletion was introduced in *Bax*/*Bak* knock-out mice that are resistant to apoptosis.^{44,45} If the anterior epithelium was dying from a caspase-dependent mechanism, we would expect apoptosis to be blocked in these mice and some cells to be left in the central lens epithelium. However, the anterior cell loss phenotype was not rescued in the triple *ILK*; *Bax*; *Bak* mutants signifying that the anterior lens epithelium died independently from the pro-apoptotic members of Bcl-2 family (Fig. 9).

Therefore, ILK-deleted ALE cells died displaying none of the usual structural or molecular features of apoptosis. To characterize better the type of cell death involved, the ultrastructure of the dying cells was examined at P0. Transmission electron microscopy allows a clear detection of apoptotic cells by visualization of rounding-up and shrinkage of the cells, nuclear fragmentation, membrane blebbing, and presence of apoptotic bodies.⁶⁴ However, none of these features could be distinguished in the mutant cells (Fig. 10). Whereas the controls showed a typical epithelial organization (Fig. 10A), the mutant ALE was disorganized but displayed no apoptotic bodies or any features of apoptosis (Figs. 10B, 10C). Enlargements (Figs. 10D–F) show that the mutant nuclei displayed no difference from the controls, being irregularly shaped with evenly spread chromatin. No nuclear fragmentation and no rounding or shrinkage were observed in any mutants ($n = 3$). Collectively, these observations clearly eliminate the possibility of apoptosis in the ILK-deleted ALE.

Other major types of cell death are necrosis and autophagy. Necrosis is defined by cytoplasmic and organelle swelling, and rupture of the plasma membrane.⁶⁴ Swelling was not observed in the mutant ALE, neither at the cellular nor nuclear level (Fig. 10, compare D to E–F). However, whereas the control cells are outlined clearly by continuous cell membranes (Fig. 10E, arrowheads) the mutant cell membrane is less clear (Figs. 10F, 10G, arrowheads). Autophagy-associated cell death is characterized by massive vacuolization of the cytoplasm and accumulation of double-membraned autophagic vacuoles containing scattered material, typically remnants of membranes.^{64,65} Indeed, the mutant ALE was characterized by the presence of numerous vacuoles of all sizes (Figs. 10B, 10C, arrows) that never were detected in the control.

These vacuoles (Figs. 10G–I) were defined by a membrane (arrowheads) and contained degrading cellular material, including membranes, which is reminiscent of autophagic vacuoles.⁶⁴

In summary, these observations confirmed that apoptosis does not occur in the mutant lens, and suggested that death by either necrosis or an impaired autophagy pathway is possible. These results clearly involved ILK in cell survival in the ALE and point to an unexpected role for ILK in non-apoptotic cell death.

FIGURE 10. The ILK-deleted dying epithelium shows no morphological features of apoptosis but numerous membranous vesicles containing digested material. Transmission electron microscopy was used to visualize the ultrastructure of the ALE in P0 controls and mutants. (A) Control epithelium showing the capsule, regular pattern of epithelial cells, and underlying fibers. (B–C) Dying ALE from two different mutants showing a disturbed epithelium and appearance of numerous vacuoles (arrows), but no apoptotic bodies. (D) Enlargement of a control ALE cell delineated by a sharp cell membrane (arrowheads). (E–F) Enlargement of mutant ALE cells with vacuoles and fading of the cell membrane (arrowheads). (G–I) Enlargement of vacuoles from the dying mutant epithelium. Vacuoles are delineated by membranes (arrowheads) and contain degrading cellular material. N, nucleus; Cap, capsule; arrows, vacuoles; arrowheads, membrane. Scale bars are 2 μm.

DISCUSSION

In an effort to understand the importance of integrin-mediated signaling during lens development, we investigated the phenotypic consequences of ILK deletion at different stages of lens development. We showed that ILK has an important role in organization of the lens capsular basement membrane, fiber migration and epithelial cell survival.

ILK Is Required for Early Assembly and Organization of the Lens Capsule ECM

The ECM is a major component of the cellular microenvironment that drives cellular changes during development, aging, and disease. It can be organized into a laminar basement membrane, which serves as a boundary between tissues and a platform for cell growth, differentiation, and survival.^{1,3} ILK is

required for basement membrane assembly in the brain^{17,22} and in the kidney glomerulus;¹⁵ however, the underlying mechanism remains unknown. Our study has enabled us to refine our understanding of ILK signaling in matrix organization *in vivo*, by examining the formation and maintenance of the lens capsule, one of the thickest basement membranes in the body.

Separation of the lens vesicle from the surface ectoderm occurs between E10.5 and E11.5, and is associated with destruction, re-synthesis, and rearrangement of the basal lamina of the prospective lens.⁶⁶ In the *Le-Cre; ILK^{fl/fl}* mutant lens, the epithelium separated normally from the surface ectoderm via apoptosis, but the capsule showed signs of weakness followed by rupture at later stages. This suggests that the matrix breakdown occurred, but the re-synthesis and/or rearrangement of the new basal lamina required to fuse with the old one was impaired. In contrast, the capsule of the

Nestin-Cre; ILK^{fl/fl} mutant was affected only mildly with slight signs of unevenness but no rupture. The *Nestin-Cre* transgene is active only after the lens has generated the embryonic capsule, and has switched from robust matrix synthesis to maintenance of the existing basement membrane. The comparison of the capsule phenotypes observed with the early *Le-Cre* and late *Nestin-Cre* lines, therefore, highlights the requirement for ILK during ECM early assembly rather than during basement membrane maintenance.

ECM assembly into an organized capsular basement membrane during development requires the generation of an initial fibronectin scaffold that allows subsequent binding and organization of other matrix constituents, such as collagen IV and laminin into a mature basement membrane.^{1,31} We showed in the *Le-Cre; ILK^{fl/fl}* mutant lens that collagen IV and laminin are expressed but are organized poorly, as seen by diffuse staining and lamellae detachment. Moreover, fibronectin expression was faint and diffuse, suggesting that impaired formation of the fibronectin scaffold in the ILK-mutant lenses inhibited further maturation of the capsular basement membrane. Our results are consistent with in vitro reports showing that ILK is required for fibronectin matrix deposition and fibrillogenesis.^{1,31,67–69} In accordance with these previous in vitro studies, we propose that ILK has a critical role in the assembly of the fibronectin scaffold in vivo.

Is ILK Required for Apical-Apical Cell Interaction?

The posterior end of the secondary fibers migrates along the capsule, then detaches and forms the posterior suture by connecting to the posterior end of the opposite fibers. Adhesion, migration, and detachment of the fiber cells from the capsule is mediated by the basal membrane complex (BMC).³³ Beta1-integrin is required for attachment of the BMC to the lens capsule,³³ suggesting that ILK also might be involved in this process. The ILK-deleted lens showed a slightly defective migratory pattern of the posterior fibers; however, the defect was mild, suggesting that ILK does not have a major role in this process. Other beta1-integrin binding partners, such as the focal-adhesion kinase (FAK), which is present in the BMC,³³ could be involved in posterior fiber migration.

In contrast, migration of the anterior tips of secondary lens fiber cells was completely defective in the *Nestin-Cre; ILK*-deleted lens. Normally, the apical tips of the fibers migrate along the apical side of the anterior epithelium in a peripheral to central direction, eventually detaching to form the anterior suture.⁵⁵ However, the cell and molecular pathways driving this unusual apical-apical interaction are not understood. In absence of ILK, the apical tips of the fibers could not migrate along the anterior epithelium and, instead, accumulated at the transition zone, where impaired expression of ZO-1 was observed. Integrins and ILK usually mediate cell-matrix interactions rather than cell-cell interactions.^{10,69,70} However, we show that in addition to the capsule-epithelial interface, ILK also is expressed all along the epithelial-fiber cell interface, including strong expression in the transition zone where the apical end of the new fibers are located (Figs. 1D, 1E) and where their migration is impaired in the mutant lens. Interestingly, ILK has been shown to be involved in early formation of cell-cell contacts in primary keratinocytes.⁷¹ Our results suggested the possibility that ILK regulates adhesion and/or migration at this unusual apical-apical cellular interface.

Interestingly, the same fiber migration defect is observed when the Rho GTPase-dependent signaling pathways are disturbed in the lens either by over-expression of a negative regulator,^{72,73} disruption of the upstream non-canonical Wnt/PCP pathway,⁷⁴ or following deletion of an adapter protein linked to this pathway.⁶⁸ In a manner similar to Rho GTPases,

ILK has a pivotal role in regulating actin cytoskeletal dynamics and cell adhesive interactions,⁷² events presumed to enable migration of the fibers along the anterior epithelium.⁷⁵ Studies of ILK deletion in vascular smooth muscle cells suggest that ILK regulates cell contractility and myosin light chain phosphorylation via the Rho pathway.⁷⁶ A potential mechanism that remains to be explored is whether ILK could regulate apical-apical adhesion through a Rho-family dependent mechanism.

Overlapping and Distinct Roles of Beta1-Integrin and ILK in Lens Development

ILK is known as an important regulator of integrin signaling and binds to beta1-integrin,⁷⁰ which is expressed in the lens epithelium.³⁹ Consequently, it was expected that deletion of ILK would generate a similar phenotype to the deletion of beta1-integrin³⁸ in the developing lens. Indeed, both mutants show cell loss in the anterior epithelium, beginning during late embryogenesis, which results in absence of epithelium at birth, followed by rapid lens degeneration. However, some significant differences are apparent.

Unlike ILK, beta1-integrin deletion impairs Pax6 and E-cadherin expression, indicating that beta1-integrin is essential to maintain the lens epithelial phenotype and is involved at least partially in EMT.³⁸ While the epithelial cells from ILK mutant lenses are disorganized and stain for α SMA, we do not have strong evidence that they are undergoing true EMT. Moreover, whereas ILK and beta1-integrin³⁶ are detected at the epithelial-fiber cell interface, no anterior fiber migration defects have been reported in the beta1-integrin mutant lens, suggesting that ILK function in apical-apical interactions also is independent of beta1-integrin. Finally, the modalities of epithelial cell death differ between the two mutants. The beta1-integrin deleted lens epithelium showed TUNEL-positive cells as early as E16.5 and caspase-3-positive staining in the newborn lenses, whereas we were unable to see any signs of increased apoptosis in the ILK-deleted lens epithelium. Overall, the partially overlapping phenotypes suggest that integrins and ILK display some distinct and some overlapping functions. This is possible, as beta1-integrin may connect with other adapters proteins, like FAK, and ILK may bind to other beta integrin subunits. Furthermore, ILK has been shown to signal downstream of growth factors,⁷⁷ and possess some cell-matrix independent functions in the cell nucleus and at cell-cell junctions.⁷⁸

ILK Deletion in the Lens Results in Non-Apoptotic Cell Death of the Anterior Epithelium

ILK deletion resulted in progressive loss of the central anterior lens epithelium leading to a lens devoid of epithelium, which degenerated rapidly. Three major reasons could explain cell disappearance. Anterior epithelial cells could migrate away from the lens resulting in gaps in the epithelium. However, this possibility is unlikely, since the lens capsule remains unruptured in the *Nestin-Cre; ILK^{fl/fl}* mutant and no epithelial cells are observed in the interior of the lens. Decreased proliferation also could lead to exhaustion of the pool of epithelial cells that differentiate into secondary fibers and explain cell loss. However, proliferation was not disturbed following ILK deletion as shown by BrdU incorporation studies. Therefore, cell death was the most probable mechanism to explain cell loss of the ALE.

Apoptosis is the most common cell death pathway encountered following genetic manipulation in the lens epithelium,^{38,62,66} and it has been shown previously that integrin signaling is required for cell survival in the developing

lens, as beta1-integrin deleted lens epithelium undergoes apoptosis.³⁸ Moreover, ILK has been shown to be involved in the regulation of anoikis, which is an apoptotic form of cell death induced by loss of cell-matrix attachment.²⁶ When cells are grown in an anchorage independent manner, overexpression of ILK leads to anoikis resistance, whereas expression of a dominant negative form of ILK induces apoptosis.^{29,69,79} A clear expectation would have been for the *Nestin-Cre; ILK^{fl/fl}* lens epithelial cells to die through the same mechanism. However, none of the morphological features of apoptosis could be detected at any stage in the *Nestin-Cre; ILK^{fl/fl}* anterior epithelium. TUNEL showed no DNA fragmentation, and electron microscopy showed no rounding-up of the cells, no reduction of the cellular and nuclear volume, no nuclear fragmentation, no membrane blebbing, and no apoptotic bodies,⁶⁴ pointing to a role for ILK in another cell death process than anoikis/apoptosis.

To characterize better the molecular features of ILK deletion-related cell death, the key executioner activated-caspase-3 was analyzed but showed no staining. The role of *Bax* and *Bak*, which are pro-apoptotic members of the Bcl-2 family, also was investigated by crossing the ILK-deleted mice to *Bax/Bak* deleted mice that are resistant to apoptosis.^{44,45} The rationale was that if the ILK-deleted cells died from apoptotic mechanisms, cell death would be prevented in the triple mutant. However, cell death was not rescued, eliminating *Bax* and *Bak* involvement. In *Bax/Bak* deficient mice, the cell-intrinsic apoptosis pathway is inhibited; however, in some cells, such as thymocytes, the extrinsic pathway can initiate apoptosis in the absence of *Bax* and *Bak*.^{44,45} However, the intrinsic and extrinsic apoptotic pathways converge to activation of caspase-3 and DNA fragmentation,⁶³ which could not be detected in the ILK-deleted lens epithelium. Taken together, these data indicate that the cells die from a non-apoptotic, *Bax*, *Bak* and caspase-3-independent mechanism.

Can the death of the anterior epithelium result from loss of epithelial fiber contact and, therefore, be a secondary consequence of defective fiber migration? Several studies report a defect in migration of the anterior tip of the lens fibers in mice that is very similar to what we describe.^{72,73,80} However, this defective migration was not accompanied by massive cell death, suggesting that the ALE can survive without fiber interactions and that the cell death observed in the *Nestin-Cre; ILK^{fl/fl}* anterior lens epithelium is, indeed, a consequence of ILK deletion. However, it is notable that ILK deletion has been performed in various organs and generally is not linked to cell death or cell survival issues in vivo.^{17,22} Most of the studies that have shown ILK inhibition contributing to cell death have been performed in vitro.^{18,28,29,16} When cell death is detected as a result of ILK deletion in vivo, it is in a pathological context of hepatitis,¹⁶ where cells, therefore, are stressed. Apart from anoikis, an alternative cell death/survival pathway that involves regulation of ILK levels is resistance of cells to stress.^{30,81} In cultivated lens epithelial cells, inhibition of ILK binding and/or activity results in increased death following serum deprivation or exposure to tunicamycin.³⁰ Moreover, the authors indicate that their results point to an apoptotic mechanism, but do not formally exclude non-apoptotic mechanisms. Interestingly, in the ILK-deleted anterior epithelium, electron microscopy evidenced massive vacuolization of the cytoplasm, and accumulation of double-membraned vacuoles containing degenerating material that closely resemble autophagosomes,⁶⁴ and point to an autophagic cell death as defined by Kroemer et al.⁶⁴ Autophagy is induced as a means of cell survival during cell stress, such as nutrient deprivation or trophic factor withdrawal, by digesting and recycling non-necessary material, but can lead to cell death upon sustained exposure to stress.^{63,64} A possible, yet

untested, explanation in the ILK-deleted anterior epithelium is that the cells cannot benefit from the aqueous growth factors as the capsule, which is a reservoir for growth factors, and the interface between the aqueous and the epithelium is impaired, leading to elevation of autophagy as a mean to supply energy and, ultimately, to cell death as the stress factor (i.e., ILK deletion and capsule deficiency) is not withdrawn.

In summary, we show that ILK is required for organization of the lens capsule matrix, migration of the secondary fibers at the apical-apical interface, and cell survival, and that ILK deletion in late embryogenesis leads to non-apoptotic cell death. The significance and possible involvement of ILK in autophagy-associated cell death remains for future study. However, the lens may provide a powerful and unique model system to study the role of ILK in novel cell death pathways in a physiological context.

Acknowledgments

Sam Zigler, PhD, kindly provided the γ -crystallin antibody.

References

1. Singh P, Carraher C, Schwarzbauer JE. Assembly of fibronectin extracellular matrix. *Annu Rev Cell Dev Biol*. 2010;26:397–419.
2. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. *J Cell Sci*. 2010;123:4195–4200.
3. LeBleu VS, Macdonald B, Kalluri R. Structure and function of basement membranes. *Exp Biol Med (Maywood)*. 2007;232:1121–1129.
4. Hynes RO. The extracellular matrix: not just pretty fibrils. *Science*. 2009;326:1216–1219.
5. Cabodi S, Di Stefano P, Leal Mdel P, et al. Integrins and signal transduction. *Adv Exp Med Biol*. 2010;674:43–54.
6. Legate KR, Fassler R. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. *J Cell Sci*. 2009;122:187–198.
7. Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, et al. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. *Nature*. 1996;379:91–96.
8. Maydan M, McDonald PC, Sanghera J, et al. Integrin-linked kinase is a functional Mn2+-dependent protein kinase that regulates glycogen synthase kinase-3beta (GSK-3beta) phosphorylation. *PLoS One*. 2010;5:e12356.
9. Wickstrom SA, Lange A, Montanez E, Fassler R. The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase! *Embo J*. 2010;29:281–291.
10. McDonald PC, Fielding AB, Dedhar S. Integrin-linked kinase—essential roles in physiology and cancer biology. *J Cell Sci*. 2008;121:3121–3132.
11. Sakai T, Li S, Docheva D, et al. Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. *Genes Dev*. 2003;17:926–940.
12. Terpstra L, Prud'homme J, Arabian A, et al. Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes. *J Cell Biol*. 2003;162:139–148.
13. Lorenz K, Grashoff C, Torka R, et al. Integrin-linked kinase is required for epidermal and hair follicle morphogenesis. *J Cell Biol*. 2007;177:501–513.
14. Wang HV, Chang LW, Brixius K, et al. Integrin-linked kinase stabilizes myotendinous junctions and protects muscle from stress-induced damage. *J Cell Biol*. 2008;180:1037–1049.
15. Kanasaki K, Kanda Y, Palmsten K, et al. Integrin beta1-mediated matrix assembly and signaling are critical for the

- normal development and function of the kidney glomerulus. *Dev Biol.* 2008;313:584–593.
16. Gkretsi V, Mars WM, Bowen WC, et al. Loss of integrin linked kinase from mouse hepatocytes in vitro and in vivo results in apoptosis and hepatitis. *Hepatology.* 2007;45:1025–1034.
 17. Mills J, Niewmierzycka A, Oloumi A, et al. Critical role of integrin-linked kinase in granule cell precursor proliferation and cerebellar development. *J Neurosci.* 2006;26:830–840.
 18. Friedrich EB, Liu E, Sinha S, et al. Integrin-linked kinase regulates endothelial cell survival and vascular development. *Mol Cell Biol.* 2004;24:8134–8144.
 19. Zervas CG, Gregory SL, Brown NH. Drosophila integrin-linked kinase is required at sites of integrin adhesion to link the cytoskeleton to the plasma membrane. *J Cell Biol.* 2001;152:1007–1018.
 20. Mackinnon AC, Qadota H, Norman KR, Moerman DG, Williams BD. C. elegans PAT-4/ILK functions as an adaptor protein within integrin adhesion complexes. *Curr Biol.* 2002;12:787–797.
 21. Nakrieko KA, Welch I, Dupuis H, et al. Impaired hair follicle morphogenesis and polarized keratinocyte movement upon conditional inactivation of integrin-linked kinase in the epidermis. *Mol Biol Cell.* 2008;19:1462–1473.
 22. Niewmierzycka A, Mills J, St-Arnaud R, Dedhar S, Reichardt LF. Integrin-linked kinase deletion from mouse cortex results in cortical lamination defects resembling cobblestone lissencephaly. *J Neurosci.* 2005;25:7022–7031.
 23. Belvindrah R, Nalbant P, Ding S, Wu C, Bokoch GM, Muller U. Integrin-linked kinase regulates Bergmann glial differentiation during cerebellar development. *Mol Cell Neurosci.* 2006;33:109–125.
 24. Gheyara AL, Vallejo-Illarramendi A, Zang K, et al. Deletion of integrin-linked kinase from skeletal muscles of mice resembles muscular dystrophy due to alpha 7 beta 1-integrin deficiency. *Am J Pathol.* 2007;171:1966–1977.
 25. Postel R, Vakeel P, Topczewski J, Knoll R, Bakkers J. Zebrafish integrin-linked kinase is required in skeletal muscles for strengthening the integrin-ECM adhesion complex. *Dev Biol.* 2008;318:92–101.
 26. Chiarugi P, Giannoni E. Anoikis: a necessary death program for anchorage-dependent cells. *Biochem Pharmacol.* 2008;76:1352–1364.
 27. Gilmore AP. Anoikis. *Cell Death Differ.* 2005;12(suppl 2):1473–1477.
 28. Chen H, Huang XN, Yan W, et al. Role of the integrin-linked kinase/PINCH1/alpha-parvin complex in cardiac myocyte hypertrophy. *Lab Invest.* 2005;85:1342–1356.
 29. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. *Oncogene.* 2000;19:3811–3815.
 30. Weaver MS, Workman G, Sage EH. The copper binding domain of SPARC mediates cell survival in vitro via interaction with integrin beta1 and activation of integrin-linked kinase. *J Biol Chem.* 2008;283:22826–22837.
 31. Danysh BP, Duncan MK. The lens capsule. *Exp Eye Res.* 2009;88:151–164.
 32. Wederell ED, de Iongh RU. Extracellular matrix and integrin signaling in lens development and cataract. *Semin Cell Dev Biol.* 2006;17:759–776.
 33. Bassnett S, Missey H, Vucemilo I. Molecular architecture of the lens fiber cell basal membrane complex. *J Cell Sci.* 1999;112:2155–2165.
 34. Barbour W, Saika S, Miyamoto T, Ohkawa K, Utsunomiya H, Ohnishi Y. Expression patterns of beta1-related alpha integrin subunits in murine lens during embryonic development and wound healing. *Curr Eye Res.* 2004;29:1–10.
 35. Walker J, Menko AS. Integrins in lens development and disease. *Exp Eye Res.* 2009;88:216–225.
 36. Menko AS, Philip NJ. Beta 1 integrins in epithelial tissues: a unique distribution in the lens. *Exp Cell Res.* 1995;218:516–521.
 37. Samuelsson AR, Belvindrah R, Wu C, Muller U, Halfter W. Beta1-integrin signaling is essential for lens fiber survival. *Gene Regul Syst Bio.* 2007;1:177–189.
 38. Simirskii VN, Wang Y, Duncan MK. Conditional deletion of beta1-integrin from the developing lens leads to loss of the lens epithelial phenotype. *Dev Biol.* 2007;306:658–668.
 39. Weaver MS, Toida N, Sage EH. Expression of integrin-linked kinase in the murine lens is consistent with its role in epithelial-mesenchymal transition of lens epithelial cells in vitro. *Mol Vis.* 2007;13:707–718.
 40. Ashery-Padan R, Marquardt T, Zhou X, Gruss P. Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev.* 2000;14:2701–2711.
 41. Tronche F, Kellendonk C, Kretz O, et al. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet.* 1999;23:99–103.
 42. Cang Y, Zhang J, Nicholas SA, et al. Deletion of DDB1 in mouse brain and lens leads to p53-dependent elimination of proliferating cells. *Cell.* 2006;127:929–940.
 43. Yang J, Bian W, Gao X, Chen L, Jing N. Nestin expression during mouse eye and lens development. *Mech Dev.* 2000;94:287–291.
 44. Lindsten T, Ross AJ, King A, et al. The combined functions of proapoptotic Bcl-2 family members *bak* and *bax* are essential for normal development of multiple tissues. *Mol Cell.* 2000;6:1389–1399.
 45. Takeuchi O, Fisher J, Suh H, Harada H, Malynn BA, Korsmeyer SJ. Essential role of *BAX, BAK* in B cell homeostasis and prevention of autoimmune disease. *Proc Natl Acad Sci U S A.* 2005;102:11272–11277.
 46. Haigh JJ, Morelli PI, Gerhardt H, et al. Cortical and retinal defects caused by dosage-dependent reductions in VEGF-A paracrine signaling. *Dev Biol.* 2003;262:225–241.
 47. Sclafani AM, Skidmore JM, Ramaprakash H, Trumpp A, Gage PJ, Martin DM. Nestin-Cre mediated deletion of *Pitx2* in the mouse. *Genesis.* 2006;44:336–344.
 48. Zhang L, Yan Q, Liu JP, et al. Apoptosis: its functions and control in the ocular lens. *Curr Mol Med.* 2010;10:864–875.
 49. Mohamed YH, Amemiya T. Apoptosis and lens vesicle development. *Eur J Ophthalmol.* 2003;13:1–10.
 50. Sottile J, Hocking DC. Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. *Mol Biol Cell.* 2002;13:3546–3559.
 51. Velling T, Risteli J, Wennerberg K, Mosher DF, Johansson S. Polymerization of type I and III collagens is dependent on fibronectin and enhanced by integrins alpha 11beta 1 and alpha 2beta 1. *J Biol Chem.* 2002;277:37377–37381.
 52. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest.* 2009;119:1420–1428.
 53. Lovicu FJ, Steven P, Saika S, McAvoy JW. Aberrant lens fiber differentiation in anterior subcapsular cataract formation: a process dependent on reduced levels of Pax6. *Invest Ophthalmol Vis Sci.* 2004;45:1946–1953.
 54. de Iongh RU, Wederell E, Lovicu FJ, McAvoy JW. Transforming growth factor-beta-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. *Cells Tissues Organs.* 2005;179:43–55.
 55. McAvoy JW, Chamberlain CG, de Iongh RU, Hales AM, Lovicu FJ. Lens development. *Eye (Lond).* 1999;13:425–437.
 56. Li DW, Liu JP, Wang J, Mao YW, Hou LH. Expression and activity of the signaling molecules for mitogen-activated protein kinase pathways in human, bovine, and rat lenses. *Invest Ophthalmol Vis Sci.* 2003;44:5277–5286.

57. Lovicu FJ, McAvoy JW, de Jongh RU. Understanding the role of growth factors in embryonic development: insights from the lens. *Philos Trans R Soc Lond B Biol Sci.* 2011;366:1204–1218.
58. Kuracha MR, Burgess D, Siefker E, et al. Spry1 and Spry2 are necessary for lens vesicle separation and corneal differentiation. *Invest Ophthalmol Vis Sci.* 2011;52:6887–6897.
59. Saleem S, Li J, Yee SP, Fellows GF, Goodyer CG, Wang R. Beta1 integrin/FAK/ERK signalling pathway is essential for human fetal islet cell differentiation and survival. *J Pathol.* 2009;219:182–192.
60. Bassnett S. On the mechanism of organelle degradation in the vertebrate lens. *Exp Eye Res.* 2009;88:133–139.
61. Nie X. Apoptosis, proliferation and gene expression patterns in mouse developing tongue. *Anat Embryol (Berl).* 2005;210:125–132.
62. Shaham O, Smith AN, Robinson ML, Taketo MM, Lang RA, Ashery-Padan R. Pax6 is essential for lens fiber cell differentiation. *Development.* 2009;136:2567–2578.
63. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. *N Engl J Med.* 2009;361:1570–1583.
64. Kroemer G, Galluzzi L, Vandenabeele P, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ.* 2009;16:3–11.
65. Boland B, Kumar A, Lee S, et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J Neurosci.* 2008;28:6926–6937.
66. Wiley LA, Dattilo LK, Kang KB, Giovannini M, Beebe DC. The tumor suppressor merlin is required for cell cycle exit, terminal differentiation, and cell polarity in the developing murine lens. *Invest Ophthalmol Vis Sci.* 2010;51:3611–3618.
67. Boulter E, Van Obberghen-Schilling E. Integrin-linked kinase and its partners: a modular platform regulating cell-matrix adhesion dynamics and cytoskeletal organization. *Eur J Cell Biol.* 2006;85:255–263.
68. Guo L, Wu C. Regulation of fibronectin matrix deposition and cell proliferation by the PINCH-ILK-CH-ILKBP complex. *Faseb J.* 2002;16:1298–1300.
69. Wu C, Keightley SY, Leung-Hagesteijn C, et al. Integrin-linked protein kinase regulates fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. *J Biol Chem.* 1998;273:528–536.
70. Legate KR, Montanez E, Kudlacek O, Fassler RILK. PINCH and parvin: the tIPP of integrin signalling. *Nat Rev Mol Cell Biol.* 2006;7:20–31.
71. Vespa A, D'Souza SJ, Dagnino L. A novel role for integrin-linked kinase in epithelial sheet morphogenesis. *Mol Biol Cell.* 2005;16:4084–4095.
72. Maddala R, Reneker LW, Pendurthi B, Rao PV. Rho GDP dissociation inhibitor-mediated disruption of Rho GTPase activity impairs lens fiber cell migration, elongation and survival. *Dev Biol.* 2008;315:217–231.
73. Grove M, Demyanenko G, Echarri A, et al. ABI2-deficient mice exhibit defective cell migration, aberrant dendritic spine morphogenesis, and deficits in learning and memory. *Mol Cell Biol.* 2004;24:10905–10922.
74. Chen Y, Stump RJ, Lovicu FJ, Shimono A, McAvoy JW. Wnt signaling is required for organization of the lens fiber cell cytoskeleton and development of lens three-dimensional architecture. *Dev Biol.* 2008;324:161–176.
75. Rao PV. The pulling, pushing and fusing of lens fibers: a role for Rho GTPases. *Cell Adh Migr.* 2008;2:170–173.
76. Kogata N, Tribe RM, Fässler R, Way M, Adams RH. Integrin-linked kinase controls vascular wall formation by negatively regulating Rho/ROCK-mediated vascular smooth muscle cell contraction. *Genes Dev.* 2009;23:2278–2283.
77. Dedhar S, Williams B, Hannigan G. Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. *Trends Cell Biol.* 1999;9:319–323.
78. Bottcher RT, Lange A, Fässler R. How ILK and kindlins cooperate to orchestrate integrin signaling. *Curr Opin Cell Biol.* 2009;21:670–675.
79. Persad S, Attwell S, Gray V, et al. Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. *Proc Natl Acad Sci U S A.* 2000;97:3207–3212.
80. Sugiyama Y, Akimoto K, Robinson ML, Ohno S, Quinlan RA. A cell polarity protein aPKC λ is required for eye lens formation and growth. *Dev Biol.* 2009;336:246–256.
81. Ohnishi M, Hasegawa G, Yamasaki M, et al. Integrin-linked kinase acts as a pro-survival factor against high glucose-associated osmotic stress in human mesangial cells. *Nephrol Dial Transplant.* 2006;21:1786–1793.