The Tumor Suppressor Merlin Is Required for Cell Cycle Exit, Terminal Differentiation, and Cell Polarity in the Developing Murine Lens


PURPOSE. Neurofibromatosis type 2 (NF2) is an autosomal-dominant CNS tumor syndrome that affects 1:25,000 children and young adults. More than 50% of NF2 patients also develop posterior subcapsular cataracts (PSCs). The authors deleted Nf2 from the lens to determine its role in fiber cell differentiation.

METHODS. Nf2 was conditionally deleted from murine lenses using the LeCre transgene. Standard histology and immunohistochemical and immunofluorescent methods were used to analyze lens morphology and markers of cell cycle progression, differentiation, and cell junctions in wild-type and knockout lenses from embryonic day 10.5 through postnatal day 3.

RESULTS. Fiber cells lacking Nf2 did not fully exit the cell cycle and continued to express epithelial cell markers, such as FoxE3 and E-cadherin, despite expressing the fiber cell marker Prox1. Many fiber cells lost their elongated morphology. Markers of apical-basal polarity, such as ZO-1, were mislocalized along the lateral and basal membranes of fiber cells. The lens vesicle failed to separate from the surface ectoderm, and prospective lens and corneal epithelial cells formed a multilayered mass of cells at the surface of the eye. Herniation of this membrane caused the fiber mass to erupt through the cornea.

CONCLUSIONS. Nf2 is required for complete fiber cell terminal differentiation, maintenance of cell polarity, and separation of lens vesicle from corneal epithelium. Defects identified in fiber cell differentiation may explain the formation of PSCs in patients with NF2. The lens provides an assay system to identify pathways critical for fiber cell differentiation and to test therapies for the tumors that occur in patients with NF2. (Invest Ophthalmol Vis Sci. 2010;51:3611–3618) DOI:10.1167/iovs.09-4371

Neurofibromatosis type 2 (NF2) is an autosomal dominant disease that affects 1 in 25,000 children and young adults and is characterized by the predisposition to develop multiple types of nervous system tumors.1,2,3,4,5 Tumor development in NF2 patients follows the classic “two-hit” model for tumor suppressor genes. Children typically inherit one mutant Nf2 allele. Random inactivation of the remaining wild-type (WT) allele in one cell of a susceptible tissue is sufficient to induce tumorigenesis.4,5 Characteristic NF2 tumors include schwannomas, meningiomas, ependymomas, and astrocytomas.3 In addition to tumors, more than 50% of NF2 patients develop posterior subcapsular cataracts (PSCs), adding debilitating vision to the complications of the disease.

The product of the Nf2 gene, merlin, is a member of the band 4.1 superfamily of proteins, which often link the actin cytoskeleton to plasma membrane proteins. Merlin is required for contact-dependent growth arrest in cultured primary keratinocytes.4 The interaction and colocalization of merlin with the components of adherens junctions, such as N-cadherin and β-catenin, suggested that merlin may control junctional dynamics, though this has not been explicitly demonstrated. In vivo studies, in which floxed Nf2 was deleted with Nestin-Cre, suggested that merlin is required for the assembly, but not the persistence, of adherens junctions.5 Merlin may control cell growth by suppressing the downstream pathways that are activated by growth factor receptors. Under different conditions, merlin has been shown to alter signaling by the epidermal growth factor receptor6,7 Ras,8 phosphoinositide-3-kinase,9 and MAP kinase.10 PSCs are one of the three main types of age-related cataracts. Risk factors for PSC formation include diabetes, exposure to immunosuppressive steroids, and therapeutic radiation treatment.11,12 Although the cellular and molecular mechanisms of PSC formation are not well understood, it is thought that abnormal proliferation of epithelial cells or failure of the proper differentiation of fiber cells may be involved.13,14,15

Two previous studies reported that loss of Nf2 caused defects in lens development.15,16 However, these defects were not studied further. To better understand PSC formation in general and the PSCs that occur in NF2 patients, we examined the cellular and molecular consequences of conditional deletion of Nf2 from the developing lens.

Nf2 was required for the cessation of cell proliferation that normally accompanies the terminal differentiation of fiber cells, the full expression of markers of fiber cell differentiation, maintenance of cell apical-basal polarity, and successful separation of the lens vesicle from the surface ectoderm. Any of these defects in fiber cell terminal differentiation could contribute to the formation of PSCs in patients with NF2.

MATERIALS AND METHODS

Generation of Nf2 CKO and Genotyping

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the approval of the Animal Studies Committee of the Washington Univers-
sity School of Medicine. Mice homozygous for a floxed Nf2 allele (Nf2lox/lox), one of which also expressed a Cre-transgene (LeCre),14 were mated to generate offspring, approximately 50% of which were Cre-positive (conditional knockout, Nf2lox/lox; LeCre+/− X Nf2lox/lox; LeCre−/−). Cre-mediated deletion of the floxed allele removes exon 2, resulting in the production of a truncated, nonfunctional protein.15 Timed matings were set up, and noon of the day of identification of a vaginal plug was considered embryonic day (E) 0.5. For genotyping, mouse genomic DNA was extracted from toe tissue, and standard PCR was performed as described previously.15

Histology
Embryos or postnatal day (P) 3 mouse heads were fixed in 10% neutral-buffered formalin overnight at 4°C, dehydrated through a series of ethanol concentrations, embedded in paraffin, and sectioned at 4 μm. For morphologic studies, sections were stained with hematoxylin and cosin (Surgipath, Richmond, IL).

Immunohistochemistry
Embryos or P3 mouse heads were fixed in 10% neutral-buffered formalin overnight at 4°C, embedded in paraffin, and sectioned at 4 μm. For antibody staining, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was inactivated with 3% H2O2 in methanol for 30 minutes at room temperature (RT) for those samples that would be treated for horseradish peroxidase (HRP). Epitope retrieval was performed in 0.01 M citrate buffer (pH 6.0). Slides were incubated in blocking solution containing 20% inactivated normal serum for 1 hour at RT, followed by incubation in primary antibodies overnight at 4°C. Slides were incubated for 1 hour at RT with biotinylated secondary antibodies and were treated with the ABC-peroxidase reagent (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA), followed by treatment with diaminobenzidine and H2O2. The slides were washed with PBS and counterstained with hematoxylin. Slides were visualized on an upright fluorescence microscope (BX60; Olympus, Tokyo, Japan), and images were collected with a digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI). Primary antibodies used for immunohistochemical staining were rabbit anti-human p53 (8298; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution and mouse anti-human Ki67 (556003; BD PharMingen, San Diego, CA) at a 1:400 dilution.

BrdU and TUNEL Staining
Pregnant female mice were injected with 50 mg/kg of a mixture of 10 mM BrdU (Roche, Indianapolis, IN) and 1 mM 5-fluoro-5′-deoxyuridine (Sigma, St. Louis, MO) and were killed after 1 hour. A monoclonal anti-BrdU antibody (1:250) (Dako, Carpinteria, CA) was used with an ABC kit (Vectastain Elite Mouse IgG ABC kit; Vector Laboratories). Sections were counterstained with hematoxylin. Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphatase nick end-labeling (TUNEL) was performed with a fluorescein direct in situ apoptosis detection kit (ApopTag; Chemicon, Temecula, CA). The deparaffinized slides were treated with 3% H2O2 in methanol for 30 minutes, followed by proteinase K treatment (20 μg/mL) for 15 minutes. Slides were incubated with TdT enzyme in equilibration buffer for 1 hour at 37°C. The reaction was terminated with wash buffer, provided by the manufacturer, for 10 minutes at room temperature. Anti–digoxigenin-peroxidase conjugate was added for 30 minutes at room temperature, followed by diaminobenzidine and H2O2 treatment. Slides were counterstained with hematoxylin.

Immunofluorescence
Embryos were fixed as described. After rinsing in PBS, heads were dissected in half, embedded in 4% agarose, and allowed to set overnight at 4°C. Thick sections (120 μm) were cut using a tissue slicer (Electron Microscopy Sciences, Hatfield, PA). Sections containing the lens were blocked in 5% normal goat serum, 0.5% Triton X-100 for permeabilization, and 0.03% sodium azide for 1 hour at RT and were incubated with primary antibodies overnight at 4°C. After rinsing, sections were incubated with fluorescence-labeled secondary antibodies for 1 hour at RT and counterstained with DRAQ5 (Biotium Limited, Shepshed, Leicestershire, UK), a vital, fluorescent DNA dye. Sections were mounted in medium ( Vectashield; Vector Laboratories) mixed with PBS at a 1:1 ratio, mounted on glass coverslips, and visualized using a confocal microscope (LSM 510; Carl Zeiss, Inc., Thornwood, NY). Primary antibodies used for immunofluorescence in this study were rabbit anti-phospho-histone H3 (34H12; Cell Signaling, Danvers, MA) at 1:1000, rabbit anti–FoxE3 (a gift from Peter Carlsson) at 1:1000, mouse anti–Prox1 (MAB5652; Chemicon International) at 1:1000, rabbit anti–β-catenin (9587; Cell Signaling) at 1:1000, anti–E-cadherin (13–1900; Invitrogen, Carlsbad, CA) at 1:1000, and anti–ZO-1 (61–7300; Zymed Laboratories, San Francisco, CA) at 1:500. AlexaFluor-labeled phallolidin (Invitrogen) was used to stain for Factin at 1:1000. AlexaFluor-conjugated anti-mouse and anti-rabbit secondary antibodies (Invitrogen) were used at 1:1000.

RESULTS
To study the role of Nf2 in lens development, we used Cre recombinase to delete floxed alleles of Nf2. Transgenic Cre (LeCre) is first expressed on E9 in the lens placode, the surface ectoderm that will go on to form the lens.14

As described previously, the failure of lens vesicle separation and the resultant changes in cell organization caused many Nf2CKO lenses to herniate, leading to expulsion of lens fiber cells through a perforation in the cornea (Fig. 1B). By P3, Nf2cko lenses had mature lenses and corneas (Fig. 1C). When the lenses herniated, Nf2cko lenses were born with abnormally small lenses with serious structural defects (Figs. 1D, 1D′). Presumably because of the lack of normal-size lenses, these eyes were small and retinas were folded.

Nf2 Required for Cell Cycle Withdrawal
Lens fiber cells normally withdraw from the cell cycle as they initiate terminal differentiation. Nf2wt E11.5 embryos from females that were pulse-labeled with BrdU showed strong labeling in the anterior epithelial cells but not in elongating primary fiber cells (Fig. 2A). However, Nf2cko lenses had abundant BrdU staining in the nuclei of primary fiber cells (Fig. 2B). BrdU-labeled fiber cells were detected in Nf2cko lenses throughout subsequent development (not shown). We also stained lenses for Ki67, which labels cells that are actively cycling. Ki67 staining was restricted to the lens epithelium in Nf2wt lenses (Fig. 2C). However, numerous Ki67-positive cells were present in the posterior fiber cell region of Nf2cko lenses (Fig. 2D). Nf2cko lenses contained clusters of cells near the lens equator, many of which were BrdU-positive and displayed prominent nuclear accumulation of Ki67 (Figs. 2B, 2D, arrows). Nf2cko lenses also had phospho-histone H3-positive mitotic figures in the epithelium and in the posterior fiber region, indicating that DNA synthesis was followed by mitosis in at least some fiber cells (Fig. 2F). Mitotic cells were restricted to the epithelial layer of Nf2wt lenses (Fig. 2E).

Aberrant proliferation of cells in the process of terminal differentiation is often accompanied by increased apoptotic cell death. TUNEL-positive cells were rarely seen in Nf2wt lenses (Fig. 2G), whereas Nf2cko lenses showed a marked increase in TUNEL-positive cells in the lens vesicle (Fig. 2H) and, at later stages, in fiber cells (not shown). Increased apoptotic death in Nf2cko lenses was confirmed using immuno-
fluorescence labeling for cleaved (activated) caspase-3 (data not shown).

**Incomplete Terminal Differentiation of Nf2\textsuperscript{CKO} Fiber Cells**

In normal lens development, cells at the lens equator withdraw from the cell cycle, move posteriorly, and then elongate to form fiber cells. Withdrawal of differentiating lens cells from the cell cycle requires the cyclin-dependent kinase inhibitor p57KIP2.\textsuperscript{16} Fiber cell differentiation is also accompanied by the nuclear localization of p57KIP2, beginning near the equatorial region of the lens and extending throughout the posterior fiber cells (Fig. 3A). In Nf2\textsuperscript{WT} lenses, p57KIP2 was present in the nuclei of many fiber cells. However, even when overstained, some cells in the fiber region displayed weak p57KIP2 staining, or lacked detectable p57KIP2 (Fig. 3B, arrowheads). Lack of p57KIP2 expression was especially noticeable in the clusters of cells that consistently accumulated near the equator of Nf2\textsuperscript{CKO} lenses (Fig. 3B, arrow).

In Nf2\textsuperscript{WT} lenses, staining for Prox1, a marker of fiber cell differentiation, was absent in epithelial cells, appeared at the equator, and was strong in all fiber cell nuclei (Figs. 3C, 3C'). In Nf2\textsuperscript{CKO} lenses, all cells in the fiber zone stained for Prox1, though staining intensity varied more than it did in Nf2\textsuperscript{WT} lenses (Figs. 3D, 3D'). In Nf2\textsuperscript{WT} lenses, staining for FoxE3 was strong in the nuclei of lens epithelial cells but was lost near the equator, where epithelial cells differentiate into fiber cells (Fig. 3C'). In Nf2\textsuperscript{CKO} lenses, peripheral lens epithelial cells stained for FoxE3. However, expression was retained in many cells in the posterior fiber region. Many of the cell clusters that contained Ki67- and BrdU-positive cells also had many FoxE3-positive cells (Fig. 3D'), suggesting that the cells in these clusters retain the characteristics of lens epithelial cells. Conversely, in Nf2\textsuperscript{CKO} lenses, many of the nuclei of cells near the central region of the epithelium, where the lens failed to separate from the prospective corneal epithelium, had low or undetectable levels of FoxE3.

Occasional double-labeled cells were also seen at the boundary between epithelial and fiber cells in Nf2\textsuperscript{WT} lenses (Fig. 3C'). However, a number of cells...
The expression in \( Nf2^{CKO} \) lenses of another fiber-specific transcription factor, c-Maf, differed from that observed for Prox1. As expected, c-Maf expression was strong and localized to the nuclei of all \( Nf2^{WT} \) fiber cells, beginning at the bow region (Fig. 3E). In \( Nf2^{CKO} \) lenses, c-Maf was correctly expressed in many fiber cells. However, a number of fiber cells either showed weak staining or lacked c-Maf staining altogether (Fig. 3F, arrow and arrowheads). Even when the images were contrast enhanced to create the appearance of overstaining, these \( Nf2^{CKO} \) fiber cells showed no detectable c-Maf staining.

MIP, also known as aquaporin 0, is exclusively expressed in lens fiber cells and constitutes approximately 60% of total membrane protein in the fibers, making it a good marker for mature, terminally differentiated fiber cells. \(^{21,22}\) \( Nf2^{CKO} \) lenses showed strong staining for MIP that was restricted to the fiber cell layer (Fig. 3G). Consistent with data observed for FoxE3 and Prox1, MIP was detected in most cells in the fiber region of \( Nf2^{CKO} \) lenses, though staining was generally low or undetectable in the cell clusters near the lens equator (Fig. 3H).

**Defects in Cell Adhesion and Loss of Apical-Basal Polarity**

At E12.5, filamentous actin localized at the apical ends of lens epithelial and fiber cells and along the lateral membranes of elongated fiber cells in \( Nf2^{WT} \) lenses (Fig. 4A). Although there were no discernible differences in the amount of filamentous actin between \( Nf2^{WT} \) and \( Nf2^{CKO} \) lenses, F-actin was also more concentrated at the apical ends of fibers cells in the knockout lenses (Fig. 4B).

In \( Nf2^{WT} \) lenses, E-cadherin was strictly localized to the lens epithelium, where it demarcated cell-cell junctions (Figs. 4A, 4C). Like FoxE3, E-cadherin staining was lost at the lens equator as fiber cells terminally differentiated (Fig 4A, arrows). In contrast, \( Nf2^{CKO} \) lenses showed aberrant localization of E-cadherin (Figs. 4B, 4D). Although E-cadherin labeling was present in the epithelium, it was also prominent in cells throughout the posterior fiber region of \( Nf2^{CKO} \) lenses (Figs 4B, 4D, arrows). Many of these cells also displayed a rounded morphology rather than their characteristic elongated shape. In some areas in \( Nf2^{CKO} \) lenses, F-actin was colocalized with the E-cadherin that was retained in the posterior fiber zone (Fig. 4B). The subcellular localization of \( \beta \)-catenin and N-cadherin was similar in \( Nf2^{WT} \) and \( Nf2^{CKO} \) lenses, though the abnormal cell morphology of cells in the \( Nf2^{CKO} \) lenses was associated with altered distribution of these proteins (Figs. 3C–F). To test whether loss of merlin function altered adherens junction formation, we performed double labeling for \( \beta \)-catenin and E-cadherin. In \( Nf2^{WT} \) lenses, \( \beta \)-catenin labeled cell junctions throughout the entire lens and colocalized with E-cadherin in the epithelium (Fig. 4C). Even though E-cadherin expression was retained in the fiber zone of \( Nf2^{CKO} \) lenses, it

**Figure 3.** Most \( Nf2^{CKO} \) fiber cells express fiber-specific differentiation markers, but many coexpress epithelium-specific proteins. (A) At E13.5, high levels of p57KIP2 were present in nuclei at the lens equator and throughout the posterior fiber region of \( Nf2^{WT} \) lenses. (B) In \( Nf2^{CKO} \) lenses, many cells had weak expression or lacked detectable p57KIP2 (arrow and arrowheads). (C) In E12.5 \( Nf2^{WT} \) lenses, antibodies to Prox1 labeled the nuclei of cells near the equator and throughout the posterior fiber region. (D) As in WT lenses, Prox1 staining was first detected in nuclei at the equator of \( Nf2^{CKO} \) lenses. Careful examination revealed that all cells in the fiber region of the knockout lenses expressed Prox1, though levels were low in some cells. (C’) FoxE3 staining was present in the nuclei of all \( Nf2^{WT} \) epithelial cells. Notably, several nuclei near the lens equator were stained with both antibodies. (D’) Antibodies to FoxE3 stained many cells in the posterior fiber compartment of these lenses. Therefore, all fiber cells in \( Nf2^{CKO} \) lenses that expressed FoxE3 were also Prox1-positive. (E) In \( Nf2^{WT} \) lenses, c-Maf is strongly expressed in the nuclei of all elongating fiber cells. (F) In \( Nf2^{CKO} \) lenses, c-Maf is also localized to the nuclei of many fiber cells. However, there are many fiber cells that display either weak labeling or that lack positive labeling for c-Maf (arrowheads). This is especially evident in the clusters of cells near the equatorial region (arrow). Even when the images were contrast enhanced to give the impression of overstaining, the fiber cells in \( Nf2^{CKO} \) lenses appeared unstained (arrow and arrowheads). (G, H) At E12.5, antibody to MIP stained fiber cells in \( Nf2^{WT} \) and \( Nf2^{CKO} \) lenses. However, not all \( Nf2^{CKO} \) cells in the fiber compartment were stained. Cells in the epithelioid clusters near the equator had little or no detectable MIP staining. Scale bars: 25 µm (A, B, E, F), 50 µm (C, C’, D, D’), 20 µm (G, H).
expression also extended along the lateral membranes of the epithelium, as would be expected (arrow). F-actin showed prominent actin staining throughout the lens. Higher levels of F-actin were especially visible at the apical ends of both epithelial and fiber cells. E-cadherin expression labeled cell–cell junctions in the epithelial layer but was absent from differentiated fiber cells (arrow). Factin colocalized with E-cadherin at the apical ends of epithelial cells. (B) F-actin was also more prominent at the apical ends of elongating fibers cells in \( N_{f2}^{CKO} \) lenses. E-cadherin was typically seen along the lateral membranes and at the basal ends of \( N_{f2}^{CKO} \) fiber cells. The image shown here is an extreme case of apical F-actin in WT lenses. E-cadherin expression was retained in many fiber cells. Although not completely colocalized, there were areas in the fiber region of CKO lenses in which F-actin colocalized with E-cadherin (arrows). (C) In \( N_{f2}^{WT} \) lenses, \( \beta \)-catenin labeled cellular junctions throughout the lens and specifically colocalized with E-cadherin in the epithelium. (D) In \( N_{f2}^{CKO} \) lenses, E-cadherin that was retained in the posterior fiber zone was completely colocalized with \( \beta \)-catenin and in the epithelium, as would be expected (arrows). (E, F) There were no notable differences in the amount and organization of F-actin, \( \beta \)-catenin, or N-cadherin between \( N_{f2}^{WT} \) and \( N_{f2}^{CKO} \) lenses. Scale bars: 50 \( \mu \)m (A, B, E, F); 20 \( \mu \)m (C, D).

Merlin Required for Lens Development

The lens placode invaginates and separates from the surface ectoderm to form the lens vesicle.\(^{25}\) Formation of the vesicle and its separation from the ectoderm is associated with the fusion and subsequent separation of cell layers, increased cell death, changes in cell-cell associations, maintenance of cell polarity, and the destruction and resynthesis of the basal laminae of the prospective lens and corneal epithelia (Fig. 6).\(^{26} - ^{29}\) In \( N_{f2}^{CKO} \) lenses, the margins of the lens pit fused, but the lens vesicle failed to complete the process of separating from the surface ectoderm (Figs. 2B, 2H). Corneal and lens cells in the zone of fusion lost their epithelial morphology and appeared multipolar and multilayered, with extensive extracellular space. In addition, Figure 3D shows that the basal lamina of the lens vesicle, which normally breaks down during separation of the vesicle from the ectoderm (Fig. 6), remained continuous with the basal lamina of the surface epithelial cells in the \( N_{f2}^{CKO} \) lenses. Thus, several aspects of cell behavior that normally accompany vesicle separation failed to occur in \( N_{f2}^{CKO} \) lenses.

DISCUSSION

Examination of developing lenses after conditional deletion of the \( N_{f2} \) gene revealed four major phenotypes: loss of cell still colocalized with \( \beta \)-catenin, suggesting that merlin is not required for adherens junction formation in the lens (Fig. 4D).

The tight junction protein ZO-1 has previously been shown to localize to the membranes of epithelial cells within the developing lens.\(^{25} - ^{24}\) In \( N_{f2}^{WT} \) lenses at E10.5, ZO-1 staining was restricted to the apical ends of cells in the invaginating lens pit (Fig. 5A). \( N_{f2}^{CKO} \) lenses also showed intense apical staining for ZO-1. However, in the knockout lenses, ZO-1 expression also extended along the lateral membranes of the posterior fiber zone was completely colocalized with E-cadherin (arrowheads).

Failure of Separation of the Lens Vesicle from the Surface Ectoderm

In \( N_{f2}^{CKO} \) lenses, the margins of the lens pit fused, but the lens vesicle failed to complete the process of separating from the surface ectoderm (Figs. 2B, 2H). Corneal and lens cells in the zone of fusion lost their epithelial morphology and appeared multipolar and multilayered, with extensive extracellular space. In addition, Figure 3D shows that the basal lamina of the lens vesicle, which normally breaks down during separation of the vesicle from the ectoderm (Fig. 6), remained continuous with the basal lamina of the surface epithelial cells in the \( N_{f2}^{CKO} \) lenses. Thus, several aspects of cell behavior that normally accompany vesicle separation failed to occur in \( N_{f2}^{CKO} \) lenses.
apical-basal polarity, failure of the lens vesicle to separate from the surface ectoderm, failure to properly exit the cell cycle during fiber cell differentiation, and incomplete terminal differentiation of fiber cells.

Conditional deletion of the floxed \( Nf2 \) construct used in this study causes in-frame deletion of exon 2, resulting in the expression of a nonfunctional protein. For this reason, it was not possible to use antibody staining to determine at what stage merlin was lost from the lens because antibodies specific to exon 2 were not available. The earliest phenotype detected, aberrant distribution of the tight junction protein ZO-1, was seen at E10.5, followed soon by failure of the lens vesicle to separate from the ectoderm. Because we could not determine when merlin was lost from the lens, it remains possible that defects occurred between E9 and E10.5 that were not detected in this study.

**\( Nf2 \) Function: Role in Early Fiber Cell Terminal Differentiation**

Its unique spatial organization makes the lens a useful model in which to study the mechanisms that control the choice between continued cell proliferation and terminal differentiation.\(^{16,30-35}\) Although it is known that \( Nf2 \) is required to suppress excess cell growth in tumors, the mechanisms by which it regulates cell proliferation or quiescence are not understood.\(^{4,34-36}\) The lens may provide a useful in vivo model to shed light on how merlin normally inhibits cells from entering the cell cycle.

Targeted gene deletion in mice revealed that the cyclin-dependent kinase inhibitor \( Cdkn1c \) (\( p57\text{KIP2} \)) and the cell cycle regulator \( Rb \) are required for lens fiber cells to withdraw from the cell cycle.\(^{16,55}\) Deletion of the transcription factor \( Prox1 \) or receptors for the FGF or BMP families of growth factors decreased \( p57\text{KIP2} \) levels and increased cell proliferation in the lens fiber cell compartment.\(^{15,18,57}\) Deletion of \( c-Maf \) caused effects similar to those of the loss of \( Prox1 \), but \( p57\text{KIP2} \) expression was not altered.\(^{19,20}\) Given that the deletion of \( Nf2 \) resulted in increased cell proliferation and decreased expression of \( c-Maf \) and \( p57\text{KIP2} \) in the fiber cell compartment, it may be that merlin is required to link the activation of FGF or BMP receptors to the expression of these factors. In this case, the deletion of \( Nf2 \) would interrupt the normal differentiation program, permitting cell proliferation and the continued expression of epithelial cell markers, such as FoxE3 and E-cadherin. However, our data showed that, in the absence of \( Nf2 \), most of the cells in the fiber cell compartment elongated and expressed the fiber cell-specific protein MIP. Therefore, though merlin appears to be required for the suppression of lens epithelial characteristics during fiber cell formation, it is not required to activate all aspects of the fiber cell differentiation program.

Although the loss of merlin affects fiber cell terminal differentiation, it is unclear why the loss of merlin leads to the loss of \( c-Maf \) and \( p57\text{KIP2} \) in some cells without also preventing the expression of Prox1. Further studies are needed to determine where merlin fits in the signaling pathways that control fiber cell differentiation. Based on Ki67 staining, we can conclude that most cells in the posterior \( Nf2\text{CKO} \) lens are actively cycling. It seems reasonable to suggest that the inhibition of fiber terminal differentiation in the absence of merlin is a secondary effect resulting from a failure of \( Nf2\text{CKO} \) fiber cells to exit the cell cycle.

**\( Nf2 \) Required for Lens Cell Adhesion and Polarity**

Previous studies suggested that merlin functions in cell-cell adhesion by stabilizing newly formed adherens junctions.\(^3\) During the process of lens vesicle invagination, clusters of cells separated from the epithelial layer. These cells became trapped in the lens vesicle and persisted beneath the epithelium near the lens equator. In spite of their loss of association with the lens basement membrane and their rounded appearance, these cells retained several of the molecular characteristics of lens epithelial cells, such as FoxE3 and E-cadherin expression. Together with the redistribution of ZO-1 in \( Nf2\text{CKO} \) lenses, these results suggest defects in the maintenance of cell adhesion and apical-basal polarity.

Localization of the epithelial-specific cadherin E-cadherin was greatly altered in \( Nf2\text{CKO} \) lenses. Many fiber cells retained expression of E-cadherin, and many of these cells appeared to lose adhesion or normal polarity. It is unclear whether the loss of fiber cell polarity in \( Nf2\text{CKO} \) lenses simply reflected the failure to stop cell proliferation and silence the expression of epithelial-specific genes or whether ectopic E-cadherin expression caused defects in polarity by interfering with the function of other adhesion molecules in fiber cells.

**FIGURE 6.** Diagram of lens vesicle formation. (A) At E10, the lens pla-
code invaginates, forming the lens pit. The letters A and B represent cells located on either side of the lens pit. (B) At E10.5, the sides of the lens pit fuse. (C) By E11, the lens vesicle separates from the surface ectoderm that will become the corneal epithelium. This process involves the fusion of epithelia, the exchange of cell neighbors (some A cells now lie next to B cells) with concomitant destruction and reformation of cell–cell junctions, the destruction of part of the original basal lamina, and the production of a new basal lamina where the epithelia fuse and separate.
Failure of Separation of Lens Vesicle from the Surface Ectoderm: Functional Evidence of Defects in Epithelial Cell Adhesion and Polarity

In humans, an eye defect resulting from the failure of separation of the lens and prospective corneal epithelial layers is called Peters anomaly (OMIM 604229, http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=604229), a syndrome that may occur sporadically or in association with mutations in the transcription factors PAX6, PITX2, or FOXC1 or the enzyme CYP1B1. Peters anomaly also occurs in several mouse knockout models used to study lens development. In Peters anomaly, the lens epithelium remains continuous with the surface ectoderm, the future corneal epithelium. The persistence of this “corneal-lenticular stalk” prevents the migration and differentiation of the mesenchyme cells that would normally form the corneal stroma and endothelium, leading to severe defects in the central cornea.

A significant difference between the phenotype of Nf2CKO eyes and the typical appearance of Peters anomaly was the absence of an obvious corneal-lenticular stalk. In lenses lacking Nf2, cells that would normally form the corneal and lens epithelia lose their epithelial characteristics and form a membrane composed of several layers of loosely organized cells rather than a continuous epithelium connecting the corneal and lens epithelia. These cells appear to lose their apical-basal polarity and their cell-cell and cell-substrate adhesions. As in typical Peters anomaly, the basal lamina of Nf2CKO lenses, the future lens capsule, is continuous with the basal lamina of the surface ectoderm. During normal lens vesicle formation, the basal lamina must be disrupted and then reformed for the lens and future corneal epithelia to separate (Fig. 6). Evidence of more severe defects in cell organization in Nf2CKO eyes was the herniation that typically occurred in later gestation. Although expulsion of the lens contents through the cornea can occur in Peters anomaly, it is a rare complication of this syndrome. It was previously suggested that failure of cells lacking Nf2 to properly complete the invagination of the neural tube and lens resulted from the inability to reform cadherin-containing adherens junctional complexes. Our observations are consistent with defects in the reformation of cell junctions, but they also suggest more severe deficiencies in cell polarity, cell-substrate adhesion, and substrate remodeling.

Successful separation of the lens vesicle from the surface ectoderm depends on the function of FoxE3. FoxE3 expression decreased in the anterior cellular cells of Nf2CKO lenses, especially in the area of fusion between the lens and corneal epithelial cells (Fig. 3D). This suggests that reduced FoxE3 expression could have contributed to the failure of separation of the lens and cornea. However, the separation defect in Nf2CKO lenses appeared to be more severe than in FoxE3 null lenses, suggesting that additional aspects of merlin function contributed to the severe “fusion” phenotype of Nf2CKO lenses. The apparent dependence of FoxE3 expression on merlin function may warrant further analysis.

Significance of Studying Nf2 in Lens Development

The aberrant fiber cell differentiation or loss of polarity seen in Nf2CKO lenses could help explain the posterior subcapsular cataracts seen in NF2 patients. Because the NF2 phenotype requires the inactivation of both NF2 alleles, loss of merlin function would be expected in only a few cells in the lenses of NF2 patients. If these cells had increased rates of proliferation and aberrant differentiation, they could contribute to PSC formation. The early expression of the Cre transgene used in the present study complicated the analysis of this aspect of the phenotype because the lens separation phenotype often led to expulsion of the fiber cells from the eye. Use of a Cre transgene expressed later in development or induced in the adult eye could provide a more realistic model of the PSCs that develop in patients with NF2 and a genetic model in which to study the cell biology of PSC formation in general. Future studies of merlin function in the lens could identify pathways that might be targeted to provide therapies for the tumors that develop in patients with NF2.

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