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A Tamoxifen-inducible Cre Knock-in Mouse for Lens-specific Gene Manipulation

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Abstract

Mouse models are valuable tools in studying lens biology and biochemistry, and the Cre-loxP system is the most used technology for gene targeting in the lens. However, numerous genes are either indispensable in lens development and the conventional knockout method either prevents lens formation or causes simultaneous cataract formation, hindering the studies of their roles in lens structure, growth, metabolism, and cataractogenesis during lens aging. An inducible Cre-loxP mouse line is an excellent way to achieve such a purpose. We established a lens-specific Cre ERT2 knock-in mouse (LCEK), an inducible mouse model for lens-specific gene targeting in a spatiotemporal manner. LCEK mice were created by in-frame infusion of a P2A-CreER^{T2} at the C-terminus of the last coding exon of the gene alpha A crystallin (Cryaa). LCEK mice express tamoxifen-inducible Cre recombinase uniquely in the lens. Through ROSA$^{mT/mG}$ and two endogenous genes (Gclc and Rbpj) targeting, we found no Cre recombinase leakage in the lens epithelium, but 50-80% leakage was observed in the lens cortex and nucleus. Administration of tamoxifen almost completely abolished target gene expression in both lens epithelium and cortex but only mildly enhanced gene deletion in the lens nucleus. Notably, no overt leakage of Cre activity was detected in developing LCEK lens when bred with mice carrying loxP floxed genes that are essential for lens development. This newly generated LCEK line will be a powerful tool to target genes in the lens for gene functions study in lens aging, posterior capsule opacification (PCO), and other areas requiring precision gene targeting.

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Author contributions

XF conceived the research; ZW and CH acquired the data; LG and Augusta Transgenic and Genome Editing Core produced the knock-in mice; MZ provided critical material and ideas for animal characterization; XF supervised the research; ZW, CH, LG, and XF analyzed and interpreted the data; XF wrote the manuscript.

Conflict of Interest

ZW, CH, MZ, LG, and XF declare they have no conflict of interest.

Introduction

Genetically modified or engineered mouse models are commonly used to study gene functions, often serving as excellent models for human disease research. To avoid embryonic lethality from global gene knockout, conditional gene targeting is an alternative approach to achieving such a purpose. Against several conditional gene targeting technologies, Cre-loxP recombination is the most popular system widely used in creating mouse models (Meinke et al., 2016; Nagy, 2000; Sternberg and Hamilton, 1981). The system is relatively straightforward, allowing spatial gene targeting (Kim et al., 2018). When introducing more regulatory mechanisms, such as inducible gene deletion, one can precisely target specific cell types at a desirable time, a spatiotemporal gene targeting system. Cre fused with optimized human estrogen receptor ligand binding domain ($CreER^{T2}$) (Indra et al., 1999) and doxycycline-inducible gene activation (Tet-On) and inactivation (Tet-Off) (Gossen et al., 1995; Kistner et al., 1996) technologies are well used for inducible gene targeting.

Several Cre mouse lines have been established for lens-orientated research, and all are transgenic mice. Le-Cre (Ashery-Padan et al., 2000), LR-Cre (Kreslova et al., 2007), P0-3.9GFPCre (Rowan et al., 2008), and Pax6(lens)-Cre (Yoshimoto et al., 2005) lines use Pax6 P0 promotor with an ectodermal enhancer for their minigene constructs. Pax6 promotor-driven minigene triggers Cre recombinase expression around embryonic day 9.5 (E9.5) (Lam et al., 2019). MLR39-Cre (Zhao et al., 2004) and maA-Cre (Lakso et al., 1992) use a 366bp mouse alpha-crystallin (αA) promotor for their minigene constructs and drive Cre recombinase expression around E12.5 (Lam et al., 2019). MLRIO-Cre uses a 366bp mouse αA promotor with a 20bp Pax6 consensus binding site insert as a minigene promotor which drives Cre recombinase expression around E10.5 (Zhao et al., 2004). The aforementioned lines are relatively lens-specific. Nes-Cre with rat nestin promotor drives Cre recombinase expression at E14.5 in the lens but is expressed broadly in the central and peripheral nervous system as well as the ciliary body and retina (Tranche et al., 1999). Of these Cre lines, Le-Cre and MLRIO-Cre mice are commonly used in lens gene targeting. Lam and co-workers (Lam et al., 2019) performed a comprehensive comparative analysis of several of these Cre lines. However, no inducible lens-specific Cre mouse line is currently available for temporal manipulation of target genes.

The lens is located between the corneal and retina, with the primary function of focusing light on the retina. The lens has a unique structure, a monolayer of the cuboidal epithelium at the anterior surface, differentiating fiber cells along the equator, the bulk of tightly-packed mature fiber cells at the cortical layer, and the fetal nucleus at the core. The entire lens is wrapped by the basement membrane, also known as the lens capsule. (Danysh and Duncan, 2009; McAvoy et al., 1999). The mature lens maintains its transparency for several decades before increasing the risk of developing cataracts in most people (Asbell et al., 2005; Lou, 2003). Cataracts affect vision acuity and severe cataracts can cause blindness (Khairallah et al., 2015). Cataract surgery by replacing an opaque lens with an intraocular lens (IOL) is the only treatment currently for people with cataracts. However, posterior capsule opacification (PCO) is a serious and the most common complication after surgery and has drawn increasing interest in this research field (Shihan et al., 2021; Walker et al., 2007; Wei et al., 2017; Wei et al., 2022; Wormstone et al., 2021). Animal models, especially

mouse models, are extremely valuable tools and continue playing a crucial role in studying lens biology and biochemistry.

In the present study, we established a lens-specific Cre ER^{T2} knock-in (LECK) mouse line for lens-specific gene targeting in a spatiotemporal manner. We characterized this inducible Cre line using both fluorescence-based reporter mice and mice carrying loxP floxed endogenous genes.

Material and Methods

Reagents -

All chemicals used were of analytical reagent grade. Milli-Q water was used for the preparation of standards and reagents. Tamoxifen (J63509.03) was purchased from Thermo Fisher (Waltham, MA, USA). Sunflower seed oil (S1929) was purchased from Spectrum Chemical (New Brunswick, NJ). All other chemicals were obtained from Sigma-Aldrich and Thermo Fisher Scientific. Antibodies used in this study are listed in Table 1.

Animals -

All animal experiments were performed in accordance with procedures approved by the Augusta University Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed under a diurnal lighting condition and allowed free access to food and water. Gclc loxP floxed mice were created by our group (Fan et al., 2012). Rbpj loxP floxed mice (stock #034200), and Gt(ROSA)26$Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J mice (Muzumdar et al., 2007) (Stock, 007576, named as mT/mG mice in this study) were purchased from Jackson Laboratory.

LCEK mice (C57BL/6-Cryaa<2A-Cre-ERT2>/J) production -

To generate the *Cryaa-2A-CreER^{T2}* knock-in allele, we used the CRISPR-Cas9 genome editing method and single guide RNA (sgRNA), which targeted sequences 5’-TCTGCACCCTCGTCCTGAGCTGG -3’ (with protospacer adjacent motif (PAM) site TGG), to insert a 2.1 kb P2A-$CreER^{T2}$ gene cassette as an in in-frame fusion to the C-terminus of Cryaa. The sgRNA, double-stranded 2A-$CreER^{T2}$ repair template (synthesized by Genewiz, Supplemental Figure SI), and Cas 9 protein (Alt-R®S.p. Cas9 Nuclease V3 from IDT) were co-injected into C57BL/6J zygotes. Founder mice were obtained and confirmed by PCR genotyping and Sanger sequencing. The external primer set to verify the targeted allele was 5’- CCGTCTGCCT-TCCAATGTGGACCAGT -3’ and 5’ -CCCTCCGACCAGC-TGGTGTTGAGCCT -3’, with PCR amplicons of 395 bp for *Cryaa* wild type allele and 2,449 bp for *Cryaa-2A-CreER^{T2}* knock-in allele. The correct targeted founders were bred with C57BL/6J mice, and PCR genotyping and Sanger sequencing were used to determine the germline transmission. PCR conditions: 95°C 30 sec, 58°C 30 sec, 68°C 30 sec, for 30 cycles. PCR products were resolved with 1.5% agarose gel electrophoresis and with GelRed (41008, Biotium, Fremont, CA).

Tamoxifen induction –

10 mg/ml of tamoxifen in the sunflower seed oil was freshly prepared every day before injection. Mice were injected intraperitoneally with 75mg/kg body weight of tamoxifen for 5 consecutive days. The Cre-loxP recombination efficiency was determined at 4 weeks after tamoxifen induction.

Slit-Lamp and darkfield microscopy analysis -

The lens morphology and transparency/opacity were monitored by a slit-lamp (SL-D4, Topcon, Livermore, CA) with 40x magnification under sedation by isoflurane (46066-755-04, Aspen Veterinary Resources, Liberty, MO). The mydriatic eye drop (1.0 % tropicamide ophthalmic solution, NDC 1748-101-12, Akorn, Lake Forest, IL) was topically applied 5 minutes before recording.

For lens darkfield imaging, isolated mice lenses were immersed in HBSS buffer in a 35 mm petri-dish. A darkfield image was recorded by a Leica M80 microscope (Leica, Buffalo Grove, IL).

Lens epithelium dissection and staining -

The lens epithelium dissection was performed as previously described (Huangfu et al., 2021). In brief, to keep the anterior capsule intact, a tiny nick was made at the posterior side of the lens, and then 4 to 6 flaps were peeled from the posterior pore to the equator of the lens with a fine ophthalmic tweezer. The inside fiber mass was removed, and then the whole anterior lens capsule was pinned on the 35 mm dish for immediate fixation. Lens capsules were fixed with freshly made 4% paraformaldehyde containing 1 μg/ml Hoechst 33342 at room temperature for 15 minutes, and the capsule whole mount was subject to image capture.

Confocal microscopy -

Immunofluorescent images were taken with a Leica STELLARIS confocal microscope and were analyzed with LAS X software.

Lens capsular cell count –

For lens epithelium EGFP and tdTomato positive cell count, epithelia from mT/mG and LCEK$^{+/-}$/mT/mG (without tamoxifen induction) mice were used to define the confocal instrument background as all positive tdTomato signals and all negative EGFP signal. Lens epithelium confocal images were then recorded for cell counting purposes. From the center of each lens epithelium confocal image, a same-size square was placed, and five sites (four points and one center) were selected as the counting site. One hundred cells in each site were counted (green plus red cells), and all five sites (500 cells) were pooled for statistical analysis.

Immunohlot -

Immunoblot assay was followed with previously described procedures(Wei et al., 2021a). Briefly, tissues were homogenized in a cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM

NaCl, 1% TritonX-100) or 8M urea buffer containing proteinase inhibitors cocktail (P8340, Millipore Sigma, St. Louis, MO) and 1 mM PMSF (10837091001, Millipore Sigma). Protein concentration was measured by bicinchoninic protein acid (BCA) assay (23227, Thermo Fisher). An equal amount of protein was used for SDS-PAGE gel electrophoresis separation before being transferred to the PVDF membrane (0.45 μm pore size). ECL reagent was used for signal detection with X-ray film, and signal intensity was semi-quantified by band densitometry using ImageJ software (version 1.53s, NIH).

Statistical analysis -

Statistical analysis was performed by the unpaired two-tailed Student's t-test, One-Way ANOVA with Bartlett's test, and Two-Way ANOVA with Sidak's multiple comparison test. Only a *p*-value below 0.05 was considered to be significant. All experiments were repeated twice, and the representative result was shown.

Results

Generation of LCEK knock-in mice.

Alpha-A crystallin (αA) is the most prevalent protein in the mammalian eye lens and is often considered a lens-specific gene (Horwitz, 2003; Sprague-Piercy et al., 2021; Takemoto and Boyle, 1998). Of note, studies found small-scale αA expression in non-lens tissues, such as the retina, pancreas, thymus, and spleen (Deng et al., 2010; Kato et al., 1991; Srinivasan et al., 1992; Xi et al., 2003). To develop a lens-specific inducible Cre mouse line, we generated the LCEK inducible knock-in allele by in-frame infusion of a P2A-CreERT2 sequence at the C-terminus of the last coding exon of the gene Cryaa (Fig. 1A). The highly efficient P2A self-cleavage peptide can facilitate both αA crystallin and $CreER^{T2}$ expression from single transcript due to ribosomal skipping during protein translation (Chng et al., 2015). $CreER^{T2}$ is Cre recombinase fused to a mutant human estrogen ligand-binding domain that requires the presence of tamoxifen for activity. Thus, upon binding to the synthetic ligands 4-hydroxytamoxifen and tamoxifen, $CreER^{T2}$ can translocate to the nuclear compartment to initiate the Cre-lox recombination (Indra et al., 1999).

Given the importance of αA crystallin in the lens, we determined the expression levels of αA crystallin in the LCEK mice at 9, 20, and 28 weeks compared with the wild-type (WT) mice lenses. As shown in Fig. 1B, LCEK heterozygous mice slightly reduced αA crystallin protein expression, and it was statistically significant. Both αA native form and alternative splicing mediated insertion form (King and Piatigorsky, 1983) showed reduced protein levels compared with WT lenses (Fig. 1C).

Because αA is the most abundant protein in the lens, we measured the lens's wet weight at 9, 20, and 28 weeks. As shown in Fig. 1D, a slightly but statistically significantly reduced lens wet weight was observed in the heterozygous LCEK mice compared to WT. However, we did not observe any abnormal lens phenotypes in heterozygous LCEK mice from the Slit lamp examination (Fig. 2A), lens dark-field imaging (Fig. 2B), and the hematoxylin and eosin (H&E) staining of Paraffin-embedded lens sections (Fig. 2C). We did see the homozygous LCEK mice developed cataract at one month of their age (Fig. S2). However,

we did not see a remarkable elevation in the severity of the opacity in 5-month-old mice compared with 1-month-old mice (Fig. S2).

Assessment of tamoxifen dependent and independent Cre-lox recombination.

To assess the Cre recombinase expression and inducibility, we bred LCEK mice with mT/mG mice (see full description in the method section). mT/mG mice are genetically engineered reporter mice carrying membrane-targeted tdTomato (mT) flanked by loxP sites followed by an EGFP expression cassette. Tissues without Cre-loxP recombination only express tdTomato, and tissues with Cre-loxP recombination express EGFP (Fig. 3A). One-month-old mT/mG and LCEK$^{+/-}$/mT/mG mice were administered with tamoxifen for five consecutive days at a 75 mg/kg body weight dosage per day. As shown in Fig. 3B and C, tamoxifen only induced EGFP expression in the lens of LCEK$^{+/-}$/mT/mG mice but not in that of mT/mG control mice.

Given the unique characteristics of the gradual denucleation process during lens fiber cell differentiation, the nuclear integrity declines, and the nuclear membrane of fiber cells becomes more porous (Bassnett and Mataic, 1997; Mikako Oka and Takehana, 2015), which may cause Cre recombinase leakage. To determine the spatial nuclear translocation of Cre recombinase in the lens, we measured EGFP and tdTomato expression in the lens epithelium, cortex, and nucleus regions with and without tamoxifen induction. As illustrated in Fig. 3D, we did not detect EGFP expression in the lens epithelium without tamoxifen induction. Four weeks after tamoxifen induction of one-month-old mice, near unique EGFP expression was seen in the lens epithelia. On the contrary, we detected EGFP expression in the lens cortex and nucleus regions without tamoxifen induction. We found around 50% leakage in the lens cortex without tamoxifen induction (Fig. 3E and F). The tdTomato deletion can be achieved in the lens cortex at about 80% four weeks after tamoxifen induction (Fig. 3E and F). We also found around 50% leakage in the lens nucleus without tamoxifen induction. However, no further enhancement of EGFP expression was seen after tamoxifen induction (Fig. 3E &F).

LCEK mice for gene targeting at various ages.

One of the major purposes of developing an inducible lens Cre mice is for age-related lens function and disease studies. To check age-related changes and tamoxifen induction efficiency, we first monitored Cre recombinase leakage in the lens epithelia, cortex, and nucleus in 1- and 5-month-old LCEK$^{+/-}$/mT/mG reporter mice. As demonstrated in Fig. 4A–C, we did not observe detectable leakage of Cre recombinase in the lens epithelia at both ages (Fig. 4A). Of note, we used lens fiber homogenate as a positive control for tdTomato and EGFP. We found that fibers had much higher tdTomato expression and displayed multiple tdTomato bands with the antibody we used in this study, so a less amount of sample was loaded in the first lane compared to other lanes in Fig. 4A. In contrast, we only detected a single tdTomato band from lens epithelial homogenate. There was a mild increase in Cre recombinase leakage in the lens cortex at five months vs. one month (Fig. 4B and C). We did not find further leakage of Cre recombinase in the lens nucleus at their 5-month-old compared with 1-month-old mice (Fig. 4B and C).

To test the efficiency of gene deletion in the lens epithelia at young and old age, we administrated tamoxifen in 1 and 8-month-old LCEK$^{+/-}$/mT/mG reporter mice. Four weeks after tamoxifen induction, we counted tdTomato and EGFP positive lens epithelial cells in the lens epithelia. As illustrated in Fig. 4D and E, 87.1% of lens epithelial cells were EGFP positive in 1-month-old mice lens epithelia. In comparison, 69.5% of EGFP positive lens epithelial cells were found in 8-month-old mice lens epithelia after tamoxifen induction (Fig. 4D and E).

Establishing tamoxifen-inducible lens-specific Gclc knockout mice.

To examine the lens gene targeting by LCEK mice, gamma-glutamylcysteine ligase, catalytic subunit (Gclc) gene- floxed mice were used to produce LCEK$^{+/-}$/Gclc$^{fl/fl}$ mice (Fig. 5A). One-month-old mice were injected with tamoxifen for gene targeting. We first determined the tissue specificity of Gclc deletion in LCEK$^{+/-}$/Gclc$^{fl/fl}$ mice. As shown in Fig. 5B, 4 weeks after tamoxifen induction, only lens Gclc was successfully deleted in the lens after tamoxifen induction, and no remarkable changes in GCLC expression in the corneal and retina. No gene deletion in the retina suggests that LCEK mice have no impact on non-lens tissues, at least in the retina where a low-scale αA is expressed. GCLC is the glutathione *de novo* synthesis enzyme, and lens Gclc deficient MLR10-Cre/Gclc$^{fl/fl}$ mice, also known as LEGSKO (Fan et al., 2012), had significantly reduced levels of glutathione (GSH). MLRIO-Cre is a transgenic mouse expressing Cre recombinase at E10.5 that is suitable for targeting floxed genes from the developmental stage (Zhao et al., 2004). We compared the lens GSH levels between 2-month-old LEGSKO and LCEK$^{+/-}$/Gclc$^{fl/fl}$ (4 weeks after tamoxifen) mice. We detected a similar concentration of lens GSH concentrations (Fig. 5C).

We further determined spatial targeting of Gclc in the lens by measuring the GCLC protein expression in the lens epithelium, cortex, and nucleus. As shown in Fig. 5B and E, there was almost no detectable GCLC protein in the lens epithelium (>98% efficiency). We did not observe Cre recombinase leakage in the lens epithelium (Fig. 5D and E). We did see around 70% Cre recombinase leakage in the lens cortex reflected by the lower GCLC protein levels (Fig. 5F&G), and tamoxifen induction resulted in a greater than 90% reduction of GCLC expression (Fig. 5F and G). Lens nucleus also had a significant leakage (~80%), and tamoxifen induction had a mild impact on GCLC protein expression (Fig. 5F and G). These results demonstrate that LCEK expresses a lens-specific Cre recombinase with similar Cre/loxP recombination efficiency as MLR10-Cre transgenic mice.

Absence of leaking Cre recombinase activity in developing LCEK lens.

Ideally, an inducible Cre mouse line can target genes even if they are indispensable in the developmental stage. Concerning Cre recombinase leakage of the LCEK knock-in mice, it is important to test if cortex and nuclear Cre leakage may affect lens development. RBPJ is a critical transcriptional effector of Notch signaling and plays an indispensable role in lens development. Lens-specific Rbpj deficient mice manifested with aphakia and microphthalmia (Le et al., 2012; Rowan et al., 2008). Therefore, Rbpj-floxed mouse is an excellent line to test the impact of LCEK mice on lens development. LCEK$^{+/-}$/Rbpj$^{fl/fl}$ mice were created, as shown in Fig. 6A. Excitingly, we did not see any noticeable abnormality

of lenses in LCEK$^{+/-}$/Rbpj$^{fl/fl}$ mice documented by both slit-lamp, lens darkfield images, and H&E staining (Fig. 6B–E, Fig. S3). Also, as expected, RBPJ was completely deleted 4 weeks after tamoxifen induction of 1-month-old mice (Fig. 6F). These results indicate a lack of leaking Cre recombinase activity in developing LCEK lens and demonstrate that the LCEK inducible Cre mouse line is a valuable tool to target genes that are indispensable during lens development.

Discussion

Genetically engineered mouse models are valuable tools and have achieved a great deal of understanding in almost every aspect of lens biology and biochemistry. In the present work, we created a lens-specific inducible Cre mouse (LCEK) by fusing CreER^{T2} to the C-terminus of the native αA crystallin gene separated by the P2A self-cleavage peptide. Heterozygous LCEK mice maintain a normal lens morphology without noticeable pathology in the C57BL/6 genetic background. The lens phenotypical change in different genetic backgrounds remains to be thoroughly tested. For example, Le-Cre mice demonstrate severe lens abnormalities in some genetic backgrounds(Dora et al., 2014). Nevertheless, LCEK mice shall always be used as a proper control for the gene targeted mice. There is no Cre recombinase leakage in the lens epithelium, but between 50-70% leakage can be seen in the lens cortex and 50-80% in the nucleus. Tamoxifen induction can achieve almost complete gene deletion in the lens epithelium and cortex. Notably, Cre recombinase leakage seems to have minimal impact on development of the lens indispensable genes, such as Rpbj. LCEK mice will likely be useful for the following applications.

Spatiotemporal gene targeting in lens epithelial cells

Lens epithelial cells continue proliferating and differentiating throughout life, which is responsible for the lifelong growth of the lens, though the rate substantially declines after around age 20 in humans (Augusteyn, 2010; McAvoy et al., 1999). Several groups have classified the lens epithelium into several zones, such as the central zone, germinative zone, and transition zone. (McAvoy, 1978; Sikic et al., 2017) Generally, epithelial cells sitting in the central epithelium are believed to be quiescent, while epithelial cells in the germinative zone are mitotically active. However, the quiescent lens epithelial cells can also rapidly convert to mitotically active cells to repair neighboring wounded cells (Wei et al., 2021b). The lens epithelium is essential for maintaining lens homeostasis and transparency by providing nutrients and regulating lens function through ion channels, transporters, and chaperone protein synthesis (Andley, 2008; Donaldson et al., 2001; Fan et al., 2017; Fan et al., 2006; Kiel, 2010; Merriman-Smith et al., 1999). For example, amino acids are delivered to the lens from the aqueous humor by epithelial cells and then delivered to the lens fibers (Reddy, 1973, 1979). A variety of genes and pathways closely regulate lens epithelial cell functions, and some of these genes are indispensable in lens development.

LCEK mouse line will be a powerful tool to study gene functions in a spatial and temporal manner, even the lens developmental indispensable genes. We did not detect Cre recombinase leakage in the lens epithelium of young and aged mice. Importantly, tamoxifen

administration can induce almost complete (>98%) gene deletion reflected by both Gclc and Rbpj mouse models.

The Cre recombinase leakage in the lens cortex and nucleus raised a major concern about the usefulness of LCEK knock-in mice if leakage impacts lens development. After reviewing various crucial lens developmental genes, we found that Notch signaling effector gene Rbpj is an indispensable gene for lens formation, and lens-specific Rbpj deficient mice display severe lens defects. Interestingly and excitingly, $LCEK^{+/-}/Rbpj^{fl/fl}$ mice have no noticeable lens abnormality compared to $LCEK^{+/-}$ and WT mice lenses. As expected, we were able to completely delete RBPJ protein expression after tamoxifen induction. We did not see an obvious lens phenotype 4 weeks after tamoxifen induction. However, full lens development in $LCEK^{+/-}/Rbpj^{fl/fl}$ mice may be due to Rbpj-mediated Notch signaling is primarily present in the lens epithelium during development. Genes that significantly impact fiber cells in the developmental stage may be affected. Also, the impact of Rbpj on postnatal lens function remains to be studied. Nevertheless, it is most likely that LCEK mice, when bred with mice carrying lens developmental indispensable loxP floxed genes, will avoid lens developmental defect. Rbpj-mediated Notch signaling is primarily Therefore, LCEK mice can be an effective Cre line for lens-specific gene targeting in both spatial and temporal manner.

Spatiotemporal gene targeting in lens cortical fiber cells

Fiber cells are the dominant cell type (99%) in the lens and are tightly packed in a particular order (Bassnett et al., 2011; Wride, 2011). Fiber cell alignment is precisely regulated by a variety of genes and pathways. Perturbation of these genes often causes lens fiber defects and even cataract formation (Cheng et al., 2017; Maddala et al., 2011; Wang et al., 2016). Nascent lens fiber cells are continuously produced through differentiated lens epithelial cells at the lens equator, and these nascent fiber cells eventually transit to terminally differentiated and fully matured fiber cells through a complex process of elongation and cell organelle elimination (Audette et al., 2016; Brennan et al., 2021; Disatham et al., 2019), including fiber cell denucleation (Basu et al., 2014; Rowan et al., 2017; Wang et al., 2010). The gradual denucleation process likely impacts fiber cell nucleus integrity. Nuclear and chromatin degradation are well documented in the lens fiber cortex (Bassnett and Mataic, 1997; Counis et al., 1998), and a much higher mRNA degradation is also found in the fiber cell of the lens cortex than in the epithelium (Mikako Oka and Takehana, 2015). We speculate that the Cre recombinase leakage in the lens cortex and nucleus is likely caused by impaired nucleus integrity in the process of fiber cell differentiation. However, the exact mechanism still requires further exploration. Since the lens nucleus carries no cell organelles, including the nuclei, no Cre recombination can occur in the nucleus. It would be impossible to target genes in the adult lens nucleus. The gene deletion detected in the lens nucleus is likely derived from prenatal lens development.

LCEK mice, after breeding with mice carrying loxP floxed genes, suppress 50-70% target gene expression in the lens cortex and 50-80% in the lens nucleus without tamoxifen induction, while lens epithelium is intact. This type of mouse represents a lens fiber-specific

gene knocking down model. Upon tamoxifen induction, it can be converted to both lens epithelium and cortex gene deletion mouse models with reduced nuclear gene expression.

Gene targeting for lens aging and posterior capsule opacification studies

Understanding the lens aging process, including its growth, structural, physiological behavior, and biological function has always been an attractive and actively pursued research area. Simply because age-related cataract (ARC) is the major cause of impaired visual acuity and blindness, and aging is the primary risk factor for ARC (Wang et al., 2017). Several animal models were created over the years to simulate age-related cataractogenesis (Fan et al., 2012; Fan et al., 2006; Reddy et al., 2001; Wu et al., 2014). However, numerous genetically engineered mice manifested new newborn cataracts (Gong et al., 1997; Hegde et al., 2016; Shiels et al., 2001). Age-related biological and biochemical functions of many of these genes in the lens remain inconclusive. LCEK mice offer the window of opportunity needed to temporally target genes that are either indispensable to lens development or trigger simultaneous cataract formation. Of note, the efficiency of the tamoxifen-induced gene deletion is gradually reduced but remains at a high level. For instance, around 20% reduced Cre recombination was seen in 8 months old mice compared to 1-month-old mice. If we use the Cre recombination efficiency data we obtained from endogenous mouse models, then we estimate around 78% gene deletion in 8-month-old mice by tamoxifen induction.

Posterior capsule opacification (PCO) is a disease derived from lens epithelial cell abnormal proliferation and transdifferentiation, which ultimately block light due to accumulated fibrotic epithelial cells in the posterior capsule (Wormstone et al., 2021). PCO is a multifactorial disease resulting from various factors and signaling pathways (Taiyab et al., 2016; VanSlyke et al., 2018). Despite extensive research efforts, PCO remains a major challenge. Long-term prevention of PCO and coping with PCO formation require delineating the underlying mechanisms of PCO. Since the introduction of an *in vivo* mouse cataract surgery PCO model in 2005(Lois et al., 2005), there has been an increasing number of studies using mouse cataract surgery in PCO research (Kurihara et al., 2009; Wei et al., 2022; Zukin et al., 2018), including genetically engineered mouse models (Mamuya et al., 2014; Nam et al., 2021; Wei et al., 2017). LCEK mice can effectively delete target genes in the lens epithelium at a desirable age. The inducible Cre-loxP system offers a tool to study genes even if they are indispensable in lens development and early cataractogenesis.

In summary, we developed a tamoxifen-inducible Cre knock-in mouse model for lens-specific gene targeting in a spatiotemporal manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A lens-specific knock-in Cre line is created.
- It is an inducible Cre KI mouse line allowing spatiotemporal gene targeting in the lens.
- Cre line is highly effective in gene deletion upon tamoxifen induction.
- Without tamoxifen induction, the Cre line does not affect lens development.

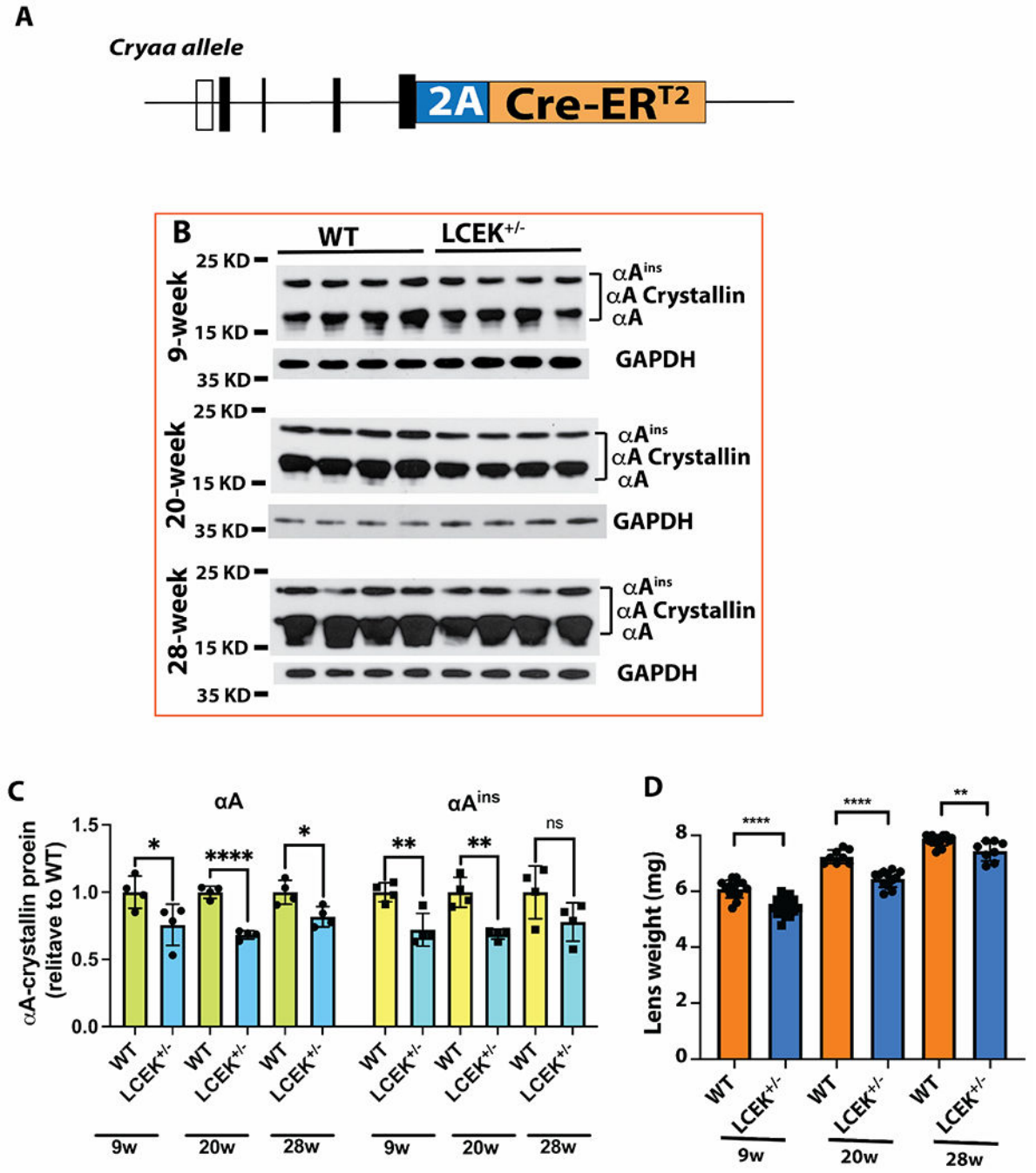

Figure 1.

LCEK knock-in mouse design and αA crystallin expression. (A) *Cyaa-2A-CreER^{T2}* knock-in allele design. (B) Lens αA crystallin protein expression at 9, 20, and 28 weeks of age. The lower band close to the 15KD marker is native αA crystallin, and the upper band close to 25KD is the αA crystallin insertion isoform. GAPDH serves as a loading control. (C) Semi-quantitative results of αA crystallin protein expression based on densitometry (n=4). (D) Lens wet weight (n≥8). Results are expressed as mean ± SD. αA crystallin expression was analyzed using one-way ANOVA with Sidak's multiple comparison test, and lens wet

weight was analyzed using unpaired two-tailed Student's t-test. Only $p<0.05$ is considered significant. *<0.05, **<0.01, ***<0.001, ****<0.0001, n.s, not significant.

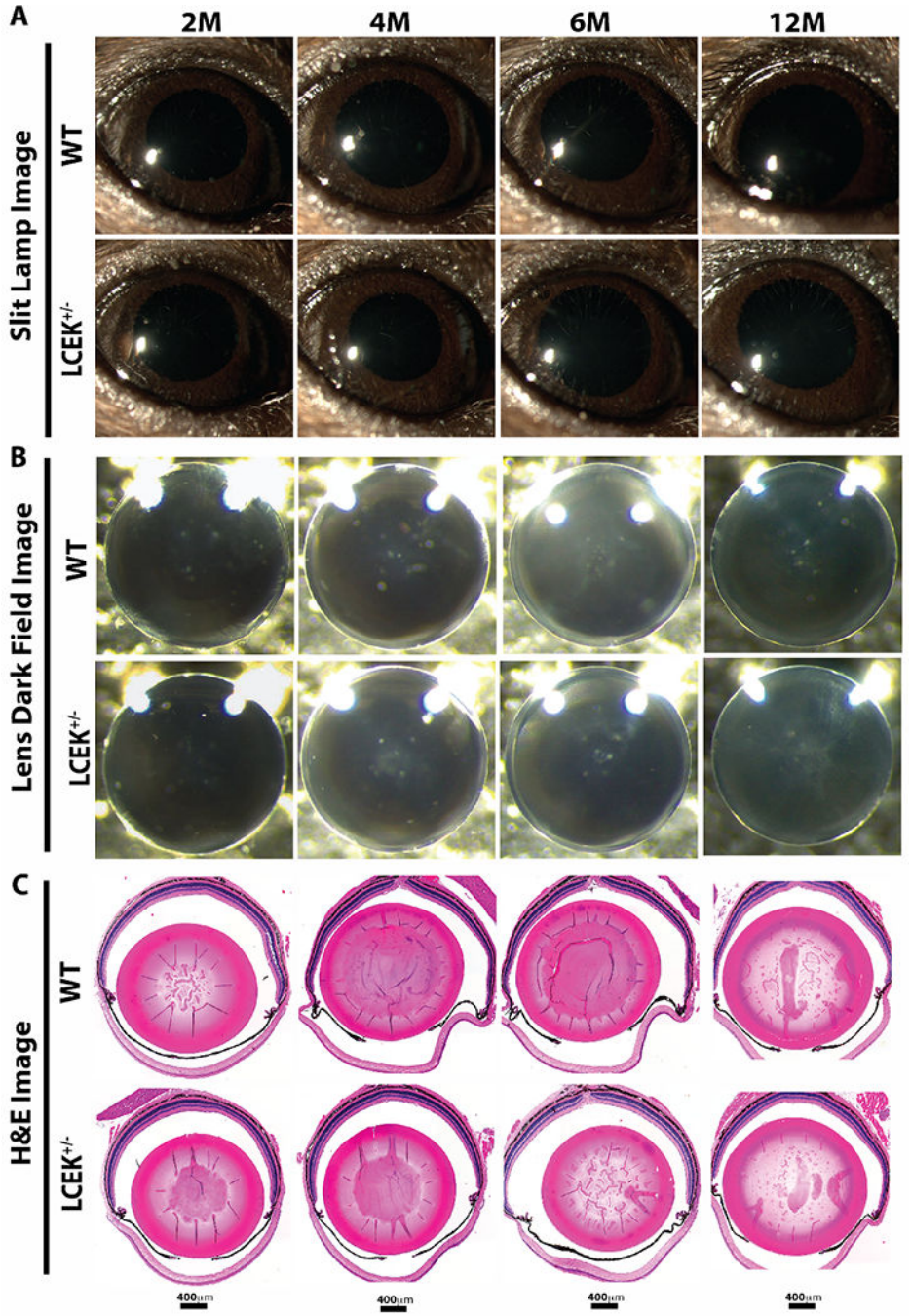

Figure 2.

LCEK knock-in mouse and WT mice lens phenotypic exam. (A) Slit lamp image of WT and heterozygous LCEK mice at 2, 4, 6, and 12 months of age. (B) Darkfield image of WT and heterozygous LCEK mice lenses at 2, 4, 6, and 12 months of age. (C) H&E staining of WT and heterozygous LCEK mice lenses at 2, 4, 6, and 12 months of age. N=5 in each group.

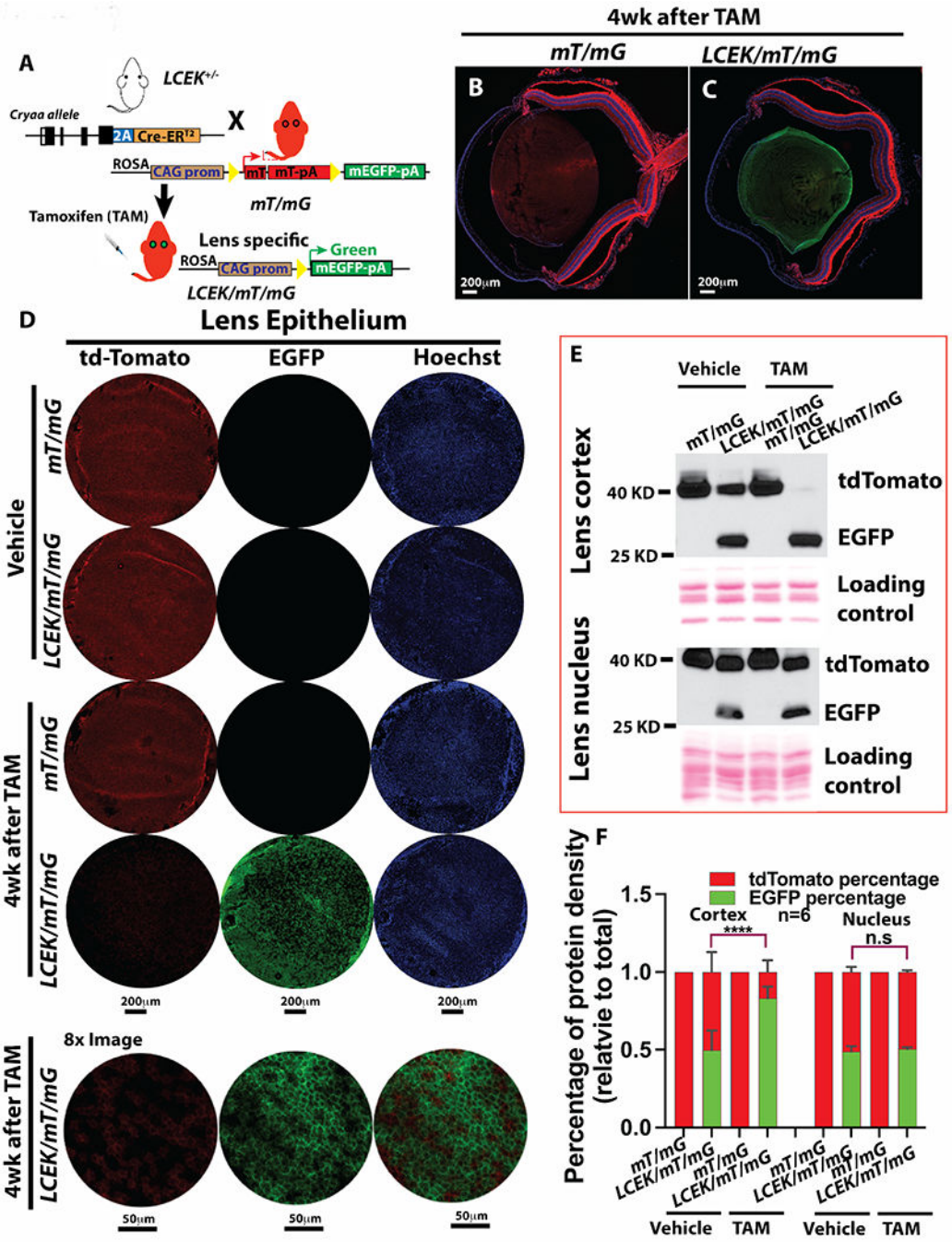

Figure 3.

Cre recombinase expression patterns and induction efficiency in ROSA$^{mT/mG}$/LCEK$^{+/-}$ mice. (A) Breeding strategy for ROSA$^{mT/mG}$/LCEK$^{+/-}$ production. (B, C) Four weeks after tamoxifen induction, Cre-loxP recombination manifested with EGFP expression only occurs in the lens. (B) mT/mG mice, (C) LCEK$^{+/-}$/mT/mG mice. (D) Lens epithelium Cre-loxP recombination with or without tamoxifen induction. Upper panel: mT/mG and LCEK$^{+/-}$/mT/mG mice lens epithelia without tamoxifen induction. Lower panel: mT/mG and LCEK$^{+/-}$/mT/mG mice lens epithelia with tamoxifen induction (4 weeks). EGFP was

only seen in tamoxifen-induced epithelia. Hoechst 33342 was used for nuclei staining. (E) EGFP and tdTomato protein expression in lens cortex and nucleus with and without tamoxifen induction. Ponceau S staining served as a loading control. (F) The ratio between EGFP and tdTomato proteins in the lens cortex and nucleus. The protein level was determined by semi-quantitative measurement of protein immuno-blot densitometry from (E) images. N=6 in each group. TAM: tamoxifen. Results are expressed as mean ± SD. Two-Way ANOVA with Sidak's multiple comparison test was used to compare each group in (F). Only $p<0.05$ is considered significant. *<0.05, **<0.01, ***<0.001, ****<0.0001, n.s, not significant.

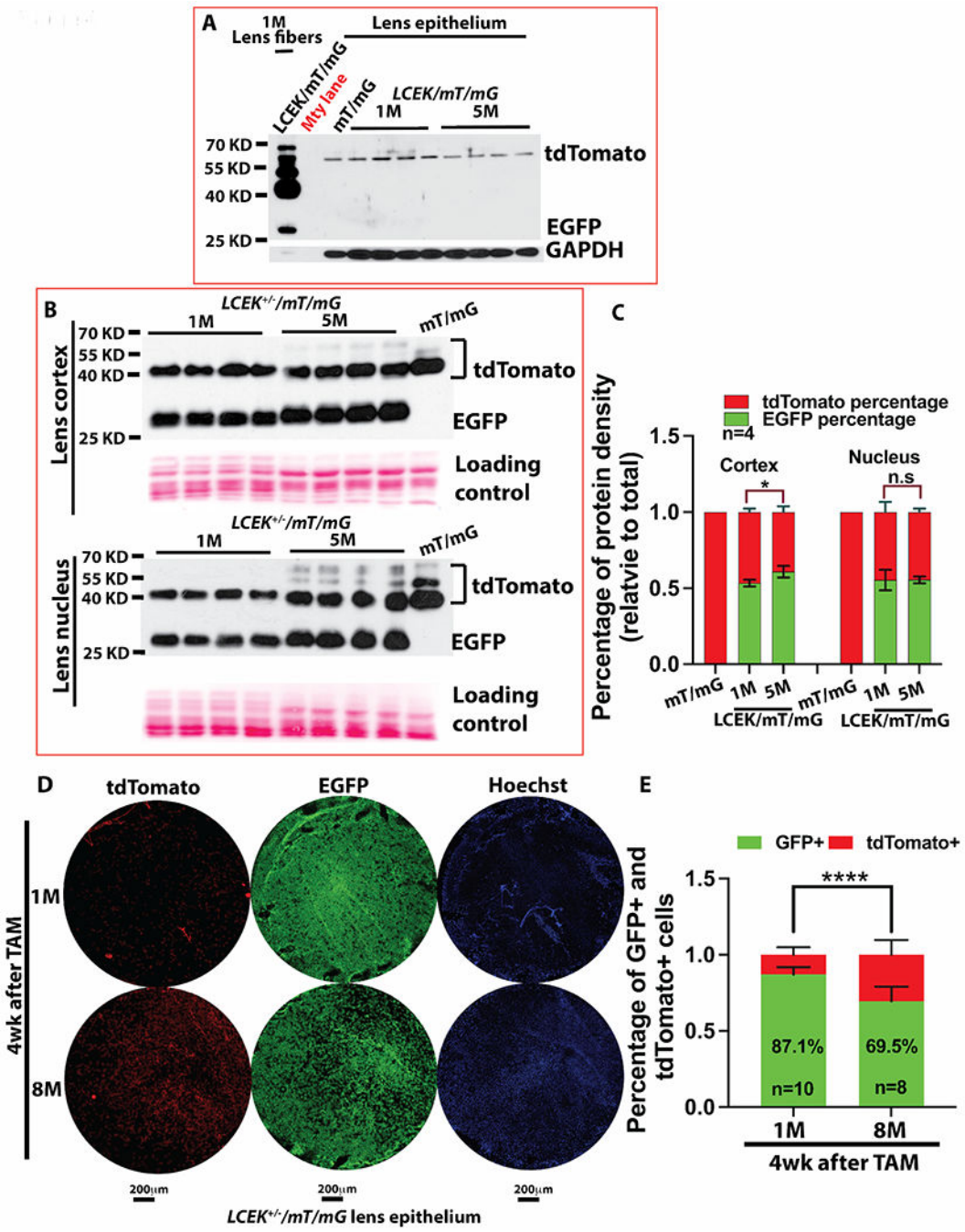

Figure 4.

Age-related changes of Cre recombinase expression and induction efficiency in ROSA$^{mT/mG}$/LCEK$^{+/-}$ mice. (A) Lens epithelium has no Cre recombinase leakage in 1- and 5-months old mice without tamoxifen induction. tdTomato was measured by immuno-blot assay, and GAPDH served as a loading control. Lane 1: mT/mG/Cre lens fiber homogenate without tamoxifen induction. We found several tdTomato bands from fiber homogenate (also see in Fig 3F); Lane 2: Mty lane: empty lane (avoid spillover from lane 1); Lane 3: mT/mG lens epithelium; lanes 4-7 are 1-month-old mT/mG/CreERT2 lens epithelium,

and lanes 8-12 are 5-month-old mT/mG/CreERT2 lens epithelium. (B) EGFP and tdTomato protein expression in the lens cortex and nucleus region of 1- and 5-month-old mice without tamoxifen induction. Ponceau S staining served as a loading control. (C) The ratio between EGFP and tdTomato proteins in the lens cortex and nucleus without tamoxifen induction. The protein level was determined by semi-quantitative measurement of protein immuno-blot densitometry from (B) images, n=4/group. (D) The tamoxifen-induced EGFP expression in the lens epithelium of 1- and 8-month-old mice. The EGFP expression was recorded 4 weeks after tamoxifen administration. Hoechst 33342 was used for nuclei staining. (E) The ratio of green (EGFP) and red (tdTomato) expression was estimated by the number of green and red fluorescent cell counts. 500 cells were counted in each lens epithelium from 5 sites. Results are expressed as mean ± SD. Two-Way ANOVA with Sidak's multiple comparison test was used to compare each group in (E). Only $p<0.05$ is considered significant. *<0.05, **<0.01, ***<0.001, ****<0.0001, n.s, not significant.

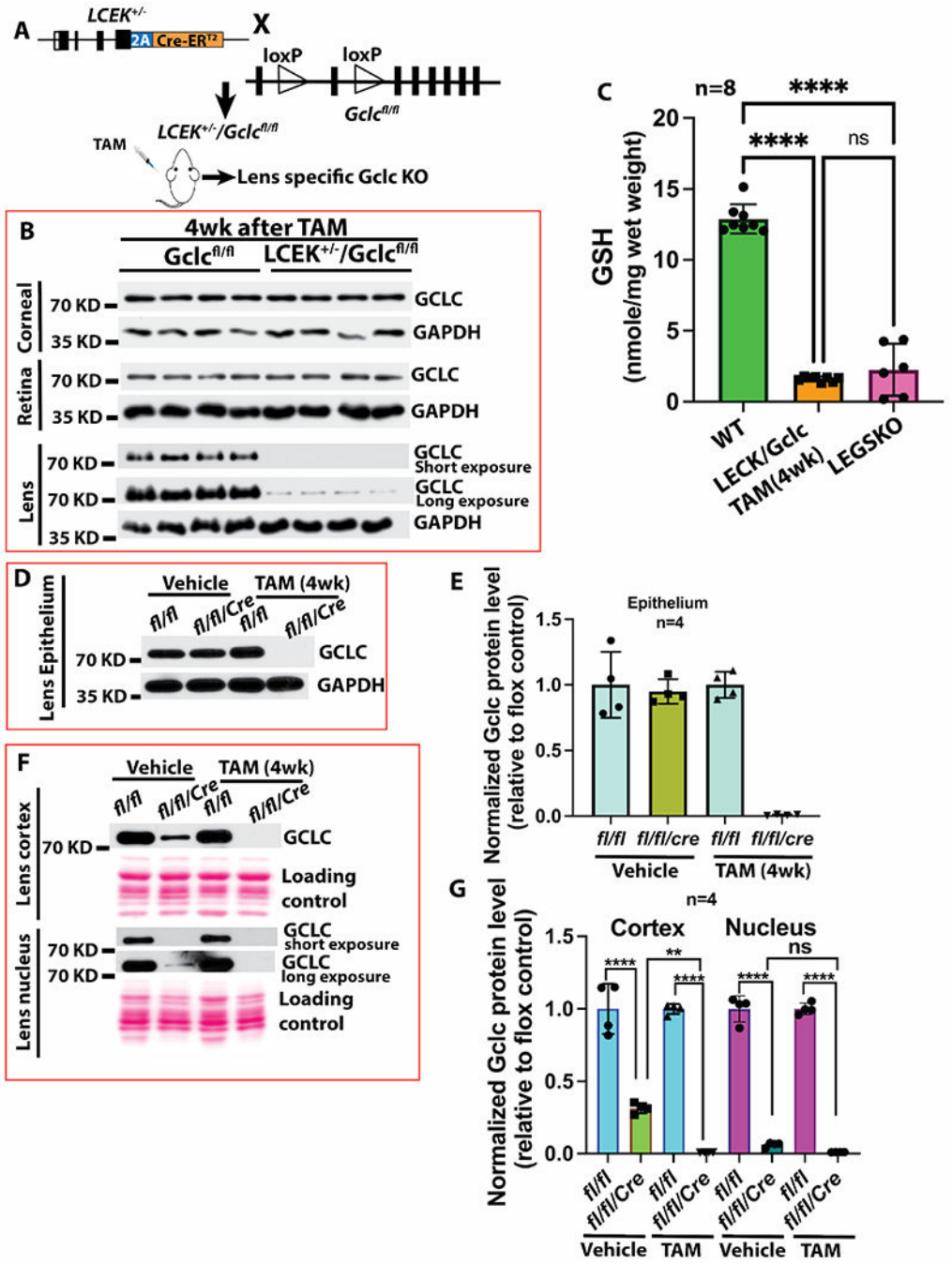

Figure 5.

LCEK mediated Gclc gene targeting in the lens. (A) Breeding strategy for LCEK$^{+/-}$/Gclc$^{fl/fl}$ production. (B) Level of GCLC protein expression in corneal, retina, and lens after tamoxifen induction. Only lens GCLC was targeted. GAPDH was used as a loading control. (C) Lens glutathione (GSH) levels in WT, LEGSKO, and tamoxifen-induced LCEK$^{+/-}$/Gclc$^{fl/fl}$ mice lenses. (D) Almost complete GCLC deletion was seen in the lens epithelium after tamoxifen induction. GCLC was determined by an immuno-blot assay. (E) Semi-quantitative results of GCLC protein expression based on densitometry (n=4).

(F) About 70% and 80% reduced GCLC protein levels were seen in the lens cortex and nucleus without tamoxifen induction, respectively. After tamoxifen induction, almost complete GCLC deletion was seen in the lens cortex, but no significant changes were seen in the lens nucleus. (G) Semi-quantitative results of GCLC protein expression based on densitometry (n=4). Results are expressed as mean ± SD. GCLC expression was analyzed using one-way ANOVA with Sidak's multiple comparison test. Only $p<0.05$ is considered significant. *<0.05, **<0.01, ***<0.001, ****<0.0001, n.s, not significant.

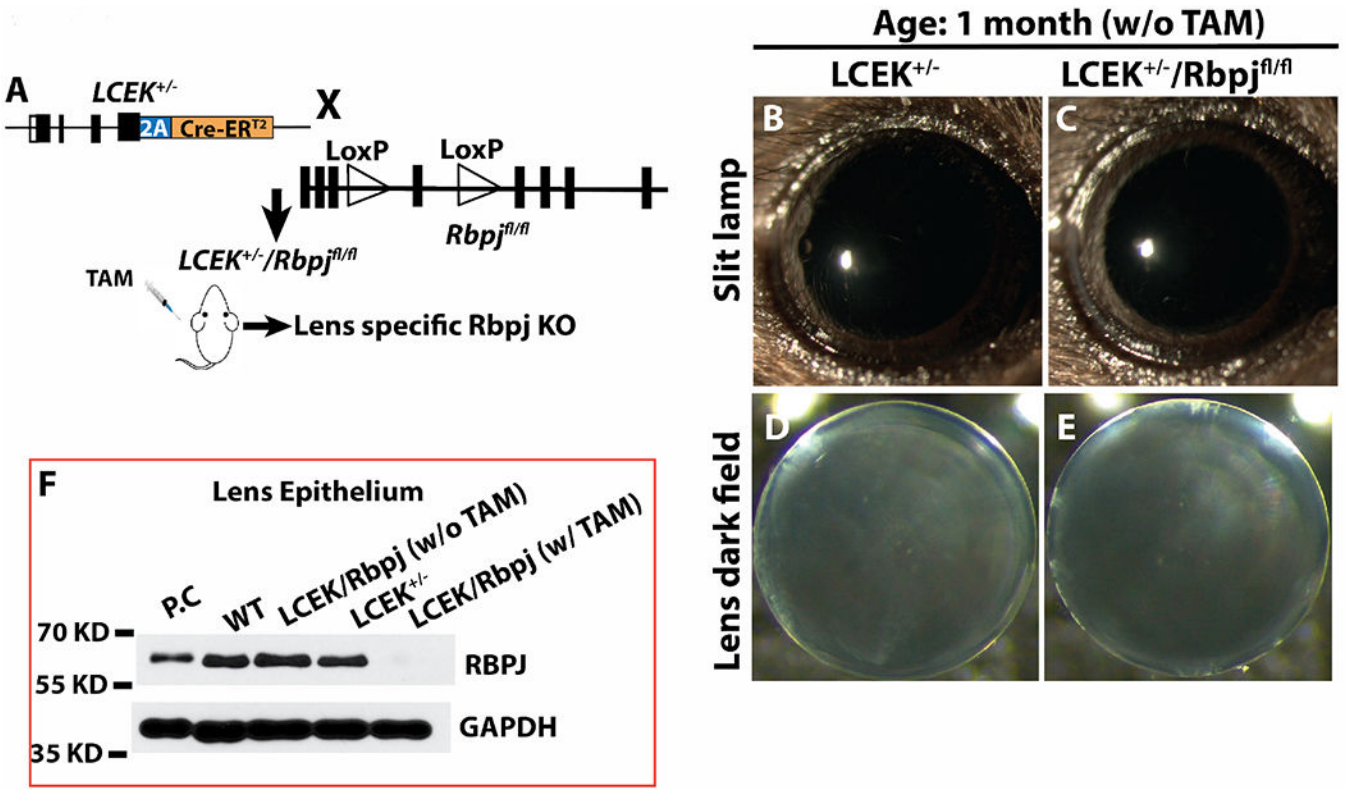

Figure 6.

LCEK mediated Rbpj gene targeting in the lens. (A) Breeding strategy for LCEK$^{+/-}$/Rbpj$^{fl/fl}$ production. (B, C) Slit lamp image of 1-month-old LCEK$^{+/-}$ and LCEK$^{+/-}$/Rbpj$^{fl/fl}$ mice eyes. Both mice showed normal lens morphology (n=5). (D, E) Lens darkfield image of 1-month-old LCEK$^{+/-}$ and LCEK$^{+/-}$/Rbpj$^{fl/fl}$ mice lenses. Both mice showed normal lens morphology (n=5). (F) Near complete RBPJ protein deletion was seen in LCEK$^{+/-}$/Rbpj$^{fl/fl}$ mice lens epithelium after tamoxifen induction (n=4). GAPDH served as a loading control.

Table 1.

Antibody list

Antibody Name	Catalog Number	Vendor	Dilution
Alpha A Crystallin, mouse monoclonal	200-301-E84	Rockland Immunochemicals Inc.	1:50,000
GFP, mouse monoclonal	600-301-215S	Rockland Immunochemicals Inc.	1:20,000
RFP, rabbit polyclonal	600-401-379S	Rockland Immunochemicals Inc.	1:20,000
GCLC, rabbit monoclonal	ab190685	Abcam	1:20,000
GAPDH, rabbit polyclonal	PA1-987	Thermo Fisher	1:5000
RBPJ, rat monoclonal	SIM-2ZRBP2	Cosmo Bio	1:300