

Aged Nrf2-Null Mice Develop All Major Types of Age-Related Cataracts

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PURPOSE. Age-related cataracts affect the majority of older adults and are a leading cause of blindness worldwide. Treatments that delay cataract onset or severity have the potential to delay cataract surgery, but require relevant animal models that recapitulate the major types of cataracts for their development. Unfortunately, few such models are available. Here, we report the lens phenotypes of aged mice lacking the critical antioxidant transcription factor *Nfe2l2* (designated as Nrf2 $-/-$).

METHODS. Three independent cohorts of Nrf2 $-/-$ and wild-type C57BL/6J mice were evaluated for cataracts using combinations of slit lamp imaging, photography of freshly dissected lenses, and histology. Mice were fed high glycemic diets, low glycemic diets, regular chow ad libitum, or regular chow with 30% caloric restriction.

RESULTS. Nrf2 $-/-$ mice developed significant opacities between 11 and 15 months and developed advanced cortical, posterior subcapsular, anterior subcapsular, and nuclear cataracts. Cataracts occurred similarly in male mice fed high or low glycemic diets, and were also observed in 21-month male and female Nrf2 $-/-$ mice fed ad libitum or 30% caloric restriction. Histological observation of 18-month cataractous lenses revealed significant disruption to fiber cell architecture and the retention of nuclei throughout the cortical region of the lens. However, fiber cell denucleation and initiation of lens differentiation was normal at birth, with the first abnormalities observed at 3 months.

CONCLUSIONS. Nrf2 $-/-$ mice offer a tool to understand how defective antioxidant signaling causes multiple forms of cataract and may be useful for screening drugs to prevent or delay cataractogenesis in susceptible adults.

Keywords: age-related cataract, antioxidant, nutrition, glycemic index, caloric restriction

Cataracts account for 51% of global blindness and are especially prevalent in the aging population with 68.3% of Americans over the age of 80 years developing cataracts.¹⁻³ Cataracts are broadly defined as the opacification of the ocular lens and involve a myriad of pathogenic mechanisms. Leading risk factors for cataractogenesis include age, genetic predisposition, diabetes mellitus, and oxidative damage.⁴ Age-related cataracts are the

most prevalent form of cataract and are commonly present in three forms, as visualized by slit-lamp imaging: nuclear, posterior subcapsular, and cortical cataracts.⁵⁻⁷ These various forms of cataracts imply variable susceptibility to cataracts in different areas of the lens and can present separately or often as a mixed cataract.^{4,5} Given the growing global aging population, the personal burden of lost sight, burgeoning public health costs associated with loss of sight

and remediation, along with the dearth of sufficient ophthalmologic capacity to remediate cataracts, there is a dire need for appropriate models of age-related cataracts and their variable presentations to develop interventions to prevent cataract formation.

It is widely accepted that oxidative stress is a leading cause of protein aggregation and subsequent lens opacification causing vision loss.^{8–13} There is strong evidence demonstrating an age-related decline in antioxidant mechanisms, which when coupled with diminished proteolytic capacities to remove them, increases the risk for cataract development.^{5,6,14–17} Oxidative stress involves the production of excess reactive oxygen species (ROS), which can lead to numerous biochemical changes, including dysregulation of proteostasis and apoptosis.^{12,15,18–23} ROS production can occur from a variety of environmental triggers, including diminished antioxidant reserves, light exposure, mitochondrial activity, and hyperglycemia, as observed in diabetes mellitus.^{22,24} This association between oxidative stress and disease has led to a better understanding of the cellular mechanisms of protection against oxidative stress and means of enhancing these activities.

Nrf2 (nuclear factor erythroid 2-related factor 2 [Nfe2l2]) is a well-characterized transcription factor that acts as a primary cellular defense mechanism against oxidative stress. Under normal conditions, Nrf2 is located in the cytoplasm, bound to Kelch-like ECH-associated protein 1 (Keap1), ubiquitinated, and targeted for degradation by the proteasome.^{25,26} Upon oxidative stress, Nrf2 is released from Keap1, translocates to the nucleus, binds to the promoter for a set of antioxidant response element (ARE) genes, and initiates their transcription to facilitate a rapid response to oxidative stress.^{26,27} Genes activated by Nrf2 are largely cytoprotective, involving pathways like glutathione synthesis, detoxification, and elimination of ROS, and drug excretion.²⁶ Thus, it is not surprising that the inhibition of Nrf2 subsequently decreases the capacity for the cell to respond to oxidative stress, which is exacerbated with increasing age.^{26,28} The association between reduced downstream antioxidant effects due to decreased Nrf2 activity, evident with age and in age-related cataracts, has garnered attention for Nrf2 as a therapeutic target for cataract treatment and prevention.²⁷

Several studies have used human lens epithelial cells (HLECs), treated with suppressors of Nrf2 activity, to recapitulate molecular markers of protein aggregation as expected for cataractogenesis.^{18,19,29–33} In animal cataract models, treatment with antioxidant compounds, including those that induce Nrf2 transcription and activation protect against selenite-induced cataracts.^{31,34} Similarly, activators of Nrf2 can protect HLEC from features of cataract that are induced under conditions of oxidative stress with high glucose, hydrogen peroxide, or homocysteine treatments.^{20,32,33,35–37} Analysis of age-related cataractous lenses from human diabetic or non-diabetic samples showed decreased methylation of the Keap1 promoter, which in turn upregulates Keap1 expression, and inhibits Nrf2 nuclear translocation and antioxidant activity.^{28,38} Similarly, Nrf2 gene variants in humans have been associated with premature surgical treatment of cortical and posterior subcapsular cataracts.³⁹

At present, there are several mouse cataract models, but the majority of these exhibit early onset lens defects or congenital cataracts (i.e. those present at birth).^{40,41} These include genetic models for cataracts that contain well-characterized human cataract mutations, altered functionality of critical protein quality control pathways, like ubiqui-

tin proteolysis pathway, autophagy, mitochondrial dynamics, and unfolded protein response, and manipulation of developmental regulatory pathways.^{42–52} Other models for cataracts use chemical induction, the most common being sodium selenite that induces acute oxidative stress, triggering cataract formation.³¹ These models are suitable for studying severe cataracts but do not capture the gradual and varied onset that occurs with aging.

Wild-type mouse lines of various strains develop age-related cataracts when aged out to 30 months; these animals demonstrate a variety of opacifications, including anterior opacities, ring cataracts, and cortical haziness.⁵³ However, it is difficult and expensive to keep a majority of mice out to such old ages. Therefore, there is a need for an accelerated timeline to visualize this same range. Several genetic models for age-related cataracts have been developed, although these models often develop one subtype of age-related cataracts. For example, β B2-crystallin (*Crybb2*) knockout mice are born with transparent lenses but develop cortical cataracts with age and show decreased oxidative stress response capacity.⁵⁴ In the LEGSKO model, in which de novo glutathione (GSH) production is abolished specifically in the lens, it was reported that mice develop severe nuclear cataracts by 9 months.⁵⁵ GSH is a critical lens antioxidant whose synthesis is transcriptionally controlled by Nrf2 in response to oxidative stress.²⁶ GSH is also responsible for the regulation of the major protein quality control machinery.⁵⁶ Similarly, a mouse knockout for glutaredoxin 2 (*Grx2*), a redox sensor that is activated under oxidative stress, also develops nuclear cataracts at an accelerated rate and severity by 13 to 16 months.⁵⁷ Although the majority of human cataracts present during the process of aging, there is no “gold standard” mouse model for age-related cataracts that presents the variety of subtypes seen in humans.

Given that many of these cataract models result in a decreased oxidative stress response capacity and that Nrf2 is an important transcription factor in activating cellular protection mechanisms against oxidative stress, we hypothesized that alterations to Nrf2 expression would lead to cataract development. A whole-body Nrf2 knockout (*Nrf2* $-/-$) streptozotocin-induced diabetic animal model showed accelerated cataract development compared to diabetic *Nrf2* $+/+$ control animals.⁵⁸ However, the possibility of cataracts in non-diabetic *Nrf2* $-/-$ mice was not reported. Animals lacking Nrf2 are viable but experience a shortened lifespan compared to wild-type mice, which can be extended by caloric restriction.⁵⁹ We recently described the protection of mouse retina from accelerated age-related macular degeneration-like features with the feeding of a low glycemic diet in an *Nrf2* $-/-$ context.⁶⁰ Here, we characterize the lens phenotypes of aged *Nrf2* $-/-$ mice and propose them to represent an accelerated model of age-related cataracts that captures a wide range of pathologies observed in humans.

MATERIALS AND METHODS

Animals

Study 1: Eleven to 18-month male *Nrf2* $-/-$: *Nrf2* $+/-$, and *Nrf2* $-/-$ mice (allele: *Nfe2l2*^{*tm1Ywk*}),⁶¹ maintained for at least 10 generations on a C57BL/6J genetic background, were obtained from Michael Freeman (Vanderbilt University) and The Jackson Laboratory (Bar Harbor, ME), respectively, and were interbred to generate *Nrf2* $-/-$ mice. The *Nrf2* $-/-$

gene targeting resulted in replacement of part of exon 4 and exon 5, containing the DNA binding domain and leucine zipper motif, with an in-frame LacZ-neomycin cassette.⁶¹

Animals were fed standard chow ad libitum (Teklad 7012; Harlan Laboratories, Madison, WI, USA) until 3 months of age, at which time they were placed on a study diet. Thirty-nine Nrf2 $-/-$ male mice were randomized into groups of 20 high glycemic (HG)-fed mice and 19 low glycemic (LG)-fed mice. At the time that slit lamp imaging was performed, 17 HG-fed mice and 18 LG-fed mice were still alive. A full description of dietary treatment is published elsewhere.⁶⁰ Diets contained identical macronutrient compositions with the exception that the HG starch was composed of 100% amylopectin (Amioca starch; Ingredion Inc., Bridgewater, NJ, USA), whereas the LG starch was composed of 70% amylose/30% amylopectin (Hylon VII starch; Ingredion Inc.). For comparisons with wild-type C57BL/6J mice, a separate cohort of 3 male mice fed standard chow ad libitum was studied at 18 months. All animal work was performed at the Tufts University Human Nutrition Research Center of Aging (HNRCA) and approved by the Tufts University Institutional Animal Care & Use Committee (IACUC) in adherence with the ARVO statement for the use of animals in ophthalmic and vision research.

Study 2: Neonatal and 3-month old wild-type and Nrf2 $-/-$ mice: wild-type (Nrf2 $+/+$) and Nrf2 $-/-$ mice (allele: *Nfe2l2*^{tm1Mym})⁶² on a C57BL/6J genetic background were housed at a temperature range of 20 to 23°C under 12:12-hour light-dark cycles with free access to water and food, as described previously.⁶³ The Nrf2 $-/-$ mice were generated by replacing a portion of exon 5 of Nrf2 with lacZ cDNA, resulting in the deletion of the conserved carboxyl 280 amino acid residues DNA binding domain and producing a nonfunctional Nrf2-LacZ fusion protein.⁶² Mice were used in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine, and mouse studies adhered to the ARVO statement for the use of animals in ophthalmic and vision research.

Study 3: Twenty-one month male and female Nrf2 $-/-$ mice: Nrf2 $-/-$ mice (allele: *Nfe2l2*^{tm1Yuk}), maintained for at least 10 generations on a C57BL/6J genetic background, were obtained from The Jackson Laboratory (Bar Harbor, ME) and were backcrossed to C57BL/6J mice. Mice were bred at the National Institute on Aging (NIA) and fed chow diets (NIH-31 diet; #T.9717.15, Envigo) ad libitum or were given a 30% caloric restriction, beginning at 9 months of age until 21 months of age, as described previously.⁵⁹ Mice were used in accordance with protocols approved by the Institutional Animal Care and Use Committee of the NIA, and mouse studies adhered to the ARVO statement for the use of animals in ophthalmic and vision research.

Statistical Analyses

Analysis for age-diet and age-genotype interactions was performed in SPSS as a univariate general linear model using a type III sum-of-squares method. Pairwise comparisons of cortical nuclear depth were performed in SPSS using Student's *t*-test. Cataract frequency was compared using Fisher's exact test.

Cataract Imaging and Scoring

Study 1: Morphological changes in the lenses of the mice were monitored by a slit-lamp (Zeiss Slit Lamp Biomicroscopy, Santa Rosa, CA, USA). After dilation with eye drops containing 1% tropicamide and 0.5% phenylephrine hydrochloride, the animals were handheld unanesthetized and were presented to the slit lamp observer (author S.J.) randomly. The slit lamp was first frontal view, followed by 30 degree angle viewing. The degree of lens opacity in each eye was graded and classified according to the LOCS II system.⁶⁴ Total cataract scores of 0 to 4 were based on The Framingham Eye Study, where scores of 4 and 5 were combined into a single score due to limitations of resolution due to the smaller eye size.⁶⁵ Scores are reported per animal, with individual eyes combined. The examiner was blinded to the age and diet groups of the mice.

Front view slit lamp photography was carried out using a Zeiss slit lamp microscope. Narrow focal slit lamp photography was carried out using the Micron III system with an attached slit lamp light source (Phoenix Research Laboratories). The Micron III system was used for fundus photography. Freshly dissected lenses were photographed on a Leica dissection microscope.

Study 3: Dissected lenses were imaged on a dissecting microscope, either mounted against a 300-mesh electron microscopy grid (Electron Microscopy Sciences, Hatfield, PA, USA, Catalog No. 6300H-Cu), a wire grid, or placed on a dark background. Lenses were evaluated from two large cohorts of mice. The first cohort of mouse lenses, representing mostly male mice, was evaluated and scored at the University of Delaware, whereas the second cohort of mice, consisting of mostly female mice, was evaluated and scored at the National Eye Institute (NEI). Opacities were determined ex vivo and scored in a binary fashion. Lenses with visible opacification and distorted appearance in grid imaging were considered as defective.

Histology and Lens Immunofluorescence

Study 1: Eyes ($n = 5$, Nrf2 $-/-$ mice [3 HG and 2 LG]; $n = 3$ wild-type mice [regular chow]) were fixed by immersion in fixative (2.5% glutaraldehyde, 2% paraformaldehyde, with 0.025% [w/v] CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.4) and lenses were dissected. Fixed lenses were processed by freeze-substitution followed by embedment in paraffin, as described in ref. 66. In brief, eyes were plunged into dry iced-cooled liquid propane for 1 minute, very rapidly transferred to 20 mL 97% methanol, 3% acetic acid, and cooled on dry ice. Freeze-substitution was allowed to proceed for 48 hours at -80°C . Samples were gradually warmed up, and then processed through graded ethanols to three changes of 100% ethanol. Samples were then processed conventionally into paraffin. The 5 μm sections were harvested, deparaffinized, and hydrated. To visualize membranes, sections were blocked in 10% normal goat serum in PBS, then exposed to Wheat Germ Agglutinin, Alexa Fluor 488, diluted 1:20, for 1 hour (Thermo Fisher).

Study 2: Heads of P1 pups ($n = 3$, Nrf2 $-/-$ mice and $n = 3$ wild-type mice) were fixed by immersion in 4% paraformaldehyde for 2 hours at 4°C and transferred to PBS. Tissue was transferred to 30% sucrose for cryopreservation and then embedded in OCT. Cryosections were obtained at a thickness of 12 μm , dried overnight, and stored at -80°C . For immunofluorescence, tissue sections were rehydrated in PBS

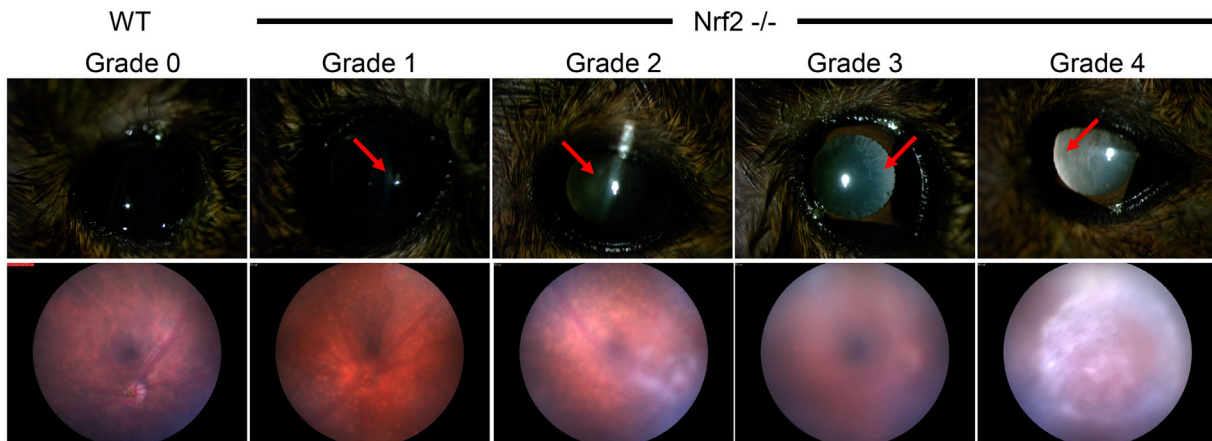


FIGURE 1. Mature 11 to 15 month Nrf2 $-/-$ mice develop cataracts of various severities. Top panels are slit lamp images of cataracts representative of different LOCSII grades. Arrows indicate regions of opacification. Bottom panels show effects of different grade cataracts on fundus images, taken from different mice.

containing 0.1% (v/v) Triton X-100 (PBT). Next, slides were blocked using normal donkey serum (Jackson Immuno-research), incubated with primary antibodies (goat anti- γ -crystallins; Santa Cruz Biotechnology) for 2 hours, washed with PBT, and incubated with secondary antibody conjugated to either Cy3 or Alexa Fluor 488 (Jackson Immuno-research). Slides were mounted in Prolong Gold Antifade with DAPI (Molecular Probes) and photographed on a Zeiss Axiovert fluorescence microscope and digital camera.

Study 3: Intact ocular globes ($n = 37$, Nrf2 $-/-$ mice and $n = 13$, wild-type mice) were fixed by immersion in formalin and embedded in methacrylate for conventional hematoxylin and eosin stain (H&E) histology.

RESULTS

Cataract phenotypes have not previously been reported in aged Nrf2 $-/-$ mice. Using slit lamp or fundus imaging, we observed a full range of LOCSII cataract scores in these 11 to 15 month old Nrf2 $-/-$ mice. The grades ranged from 1 (mild) to 4 (severe; Fig. 1).⁶⁴ Cataracts scoring a grade of 3 or 4 sufficiently obscured the light to the back of the eye such that fundus images did not reveal the retina or its vasculature. None of the Nrf2 $-/-$ mice had completely transparent lenses at 11 to 15 months, which were observed in wild-type C57BL/6J mice of the same age (see Fig. 1).

Because epidemiologic data suggested a correlation between HG diets and risk for cataracts,^{67,68} we evaluated cataracts in our Nrf2 $-/-$ cohort that had consumed HG or LG diets.⁶⁰ We have previously shown that dietary treatment with an LG diet ameliorated retinal phenotypes.⁶⁰ Cataract grades were lowest in the youngest mice we scored (11 months of age) and increased with aging such that almost all of the mice had cataract grades of 4 at 15 months (Fig. 2). The trend to increased cataract score with age was highly significant ($P = 2.3 \times 10^{-4}$). In contrast, the effect of diet on cataract score was not significant ($P = 0.97$). Cataracts were present bilaterally, with no animals scoring more than one grade apart between eyes. Cataract prevalence was also determined based on a cutoff grade of two (Table 1). Again, cataract frequency increased with age but was not associated with diet (see Table 1).

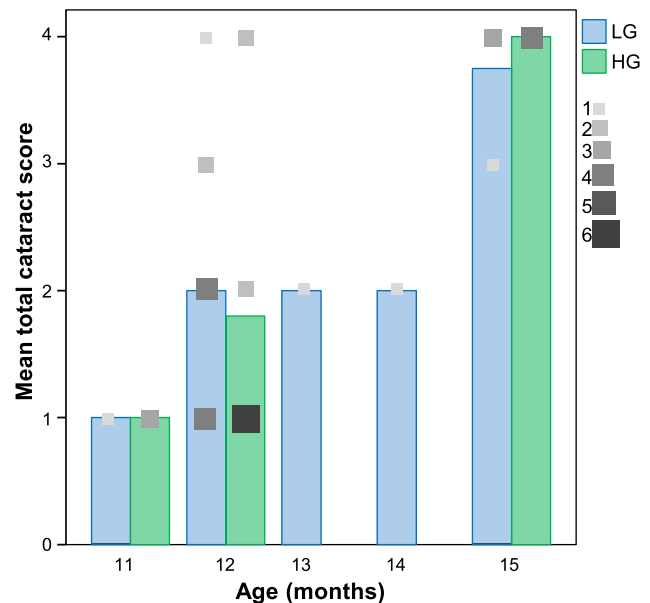


FIGURE 2. Nrf2 $-/-$ cataract scores get progressively worse with age irrespective of diet. Bar graphs for the diet groups indicate mean total cataract score. Lenses with a respective cataract score are indicated by grayscale squares that are scaled to the number of lenses with that LOCSII grade (total sample size: $N = 18$, low glycemic diet [LG]; $N = 17$, high glycemic diet [HG]).

TABLE 1. Summary Data for Cataract Frequency in Male Nrf2 $-/-$ Mice Scored Between 11 and 15 Months

	11 mo.	12 mo.	13 mo.	14 mo.	15 mo.	P_{Age}
HG	0/3	4/10			4/4	0.029
LG	0/1	7/11	1/1	1/1	4/4	0.11
Total	0/4	11/21	1/1	1/1	8/8	0.0022
P_{Diet}	1	0.39	N/A	N/A	1	

Cataract was defined as a LOCSII grade of ≥ 2 . Ages were compared between 11 and 12 months versus 13 to 15 months.

HG, high glycemic diet; LG, low glycemic diet.

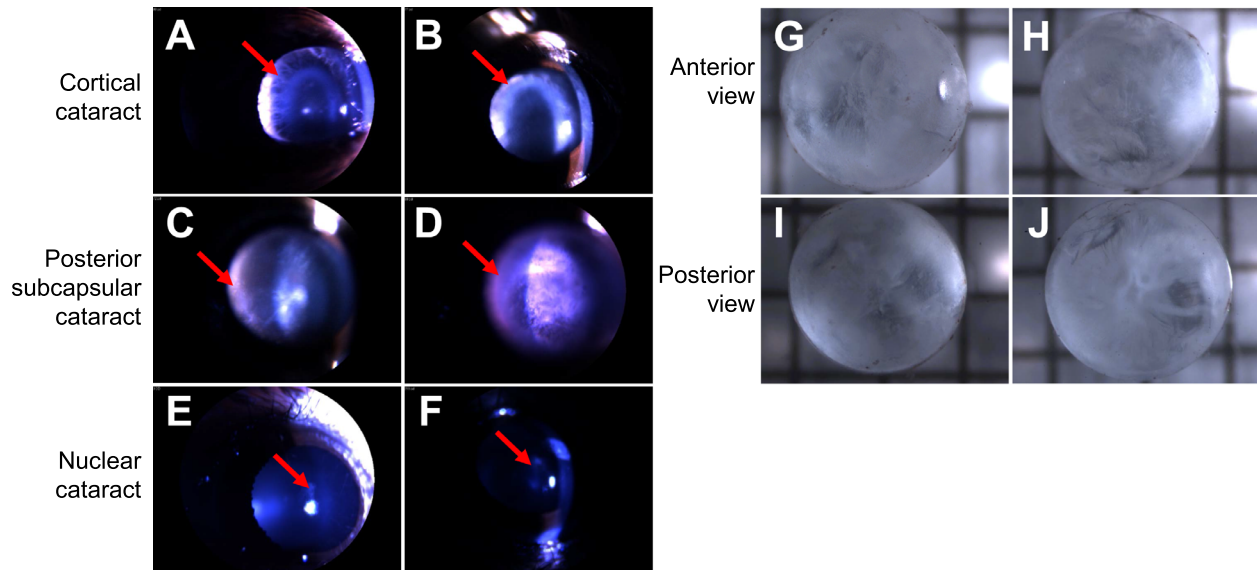


FIGURE 3. *Nrf2*^{-/-} mice develop multiple types of cataracts. Slit lamp images from 15 month old mice reveal cortical cataracts (A, B), posterior subcapsular cataracts (C, D), and nuclear cataracts (E, F). Extracted fresh lenses reveal heterogeneous anterior and posterior subcapsular cataracts (G–J). Arrows indicate the diagnostic opacities associated with each cataract type.

Further analysis of 15 month *Nrf2*^{-/-} mouse lenses using slit lamp photography revealed that cataracts resembled the 3 major human age-related cataracts: cortical cataracts (Figs. 3A, 3B), posterior subcapsular cataract (see Figs. 3C, 3D), and nuclear cataract (see Figs. 3E, 3F). Cataracts were further scored for cataract subtype and total cataract score using the modified LOCSII scoring system. The majority of cataracts presented with significant (defined as grade 2 or higher) cortical cataracts and/or posterior subcapsular cataracts (19/35). Nuclear cataracts were observed in 5 of the 35 mice. Most of the cataracts and all of the nuclear cataracts consisted of at least two kinds of cataracts simultaneously. Ex vivo analysis of cataractous lenses at 18 months revealed significant and heterogeneous opacification of the anterior capsule (see Figs. 3G, 3H) and the posterior capsule (see Figs. 3I, 3J).

Lenses from 18 month old mice were histologically evaluated using wheat germ agglutinin to visualize the shape of lens cells (Fig. 4, green) and DAPI to visualize the nuclei (see Fig. 4, red). In these older animals, cells in the anterior lens (see Figs. 4A–C), cortex (see Figs. 4D–F), and posterior (see Figs. 4G–I) revealed widespread disruptions to the lens fiber architecture, in association with opacification. The most severe abnormalities were observed in the cortical region, consistent with the degree of cortical cataract (see Figs. 4D, 4E). The lens nucleus architecture was largely intact, also consistent with nuclear cataract being a less penetrant phenotype. Alongside changes in lens cell architecture, we also observed aggregates throughout the lens. Strikingly, we observed nuclei abnormally present in *Nrf2*^{-/-} mouse lenses well beyond the outer cortical region. Ectopic nuclei were observed in the posterior part of *Nrf2*^{-/-} mouse lenses (see Figs. 4G, 4H) but not wild-type mouse lenses (see Fig. 4I). Many of these nuclei appeared pyknotic or rounded-up (see Figs. 4D–F, insets). We did not observe ectopic nuclei in the deeper parts of the lens nucleus in either *Nrf2*^{-/-} or wild-type mouse lenses, suggesting that nuclear degradation occurred normally in primary fiber cells.

The process of lens fiber cell denucleation (LFCD), whereby the nucleus of the fiber cell is systematically disassembled and degraded, begins during mid-gestation in the center of the lens, and continues throughout the life of the organism in a stereotypical pattern beginning at the lens outer cortex and proceeding to the inner cortex.^{69,70} Usually, retention of lens nuclei is noted in congenital cataracts and is most obvious in the center of the lens.^{46,47,71} In order to determine whether embryonic LFCD occurred normally in *Nrf2*^{-/-} mice, we evaluated lenses at P1, when LFCD has already occurred in wild-type mice (Supplementary Fig. S1A). *Nrf2*^{-/-} mouse lenses at P1 showed appropriately timed LFCD (see Supplementary Fig. S1B). Lens differentiation also occurred appropriately, with normal timing and levels of gamma crystallins expression in *Nrf2*^{-/-} mouse lenses (see Supplementary Figs. S1C, S1D). We did observe some fracturing of the *Nrf2*^{-/-} mouse lens upon sectioning, which may indicate some defective packing of primary fiber cells (see Supplementary Fig. S1D).

In order to gain further insight into the early steps of cataractogenesis in *Nrf2*^{-/-} mice, we also evaluated young adult lenses in 3 month wild-type and *Nrf2*^{-/-} mice. *Nrf2*^{-/-} mouse lenses were transparent with no obvious gross phenotypes. Histological evaluation of the lenses showed that lenses were properly formed and lens fiber cell differentiation occurred normally at the transition zone (Supplementary Figs. S2D–F). However, the precise geometry of the bow region appeared subtly different in *Nrf2*^{-/-} mouse lenses (see Supplementary Fig. S2F). We also observed some abnormalities in the anterior part of the lens, where delaminated nucleated cells were observed in conjunction with disorganization of the lens fiber cells in the region, a possible initiating event in the formation of an anterior subcapsular cataract (see Supplementary Figs. S2H, S2I).

The abnormalities in 18 month *Nrf2*^{-/-} mouse lenses in the bow region prompted us to evaluate potential connections between retained cortical nuclei, age, and genotype. Figure 5 shows measurements of the distance from

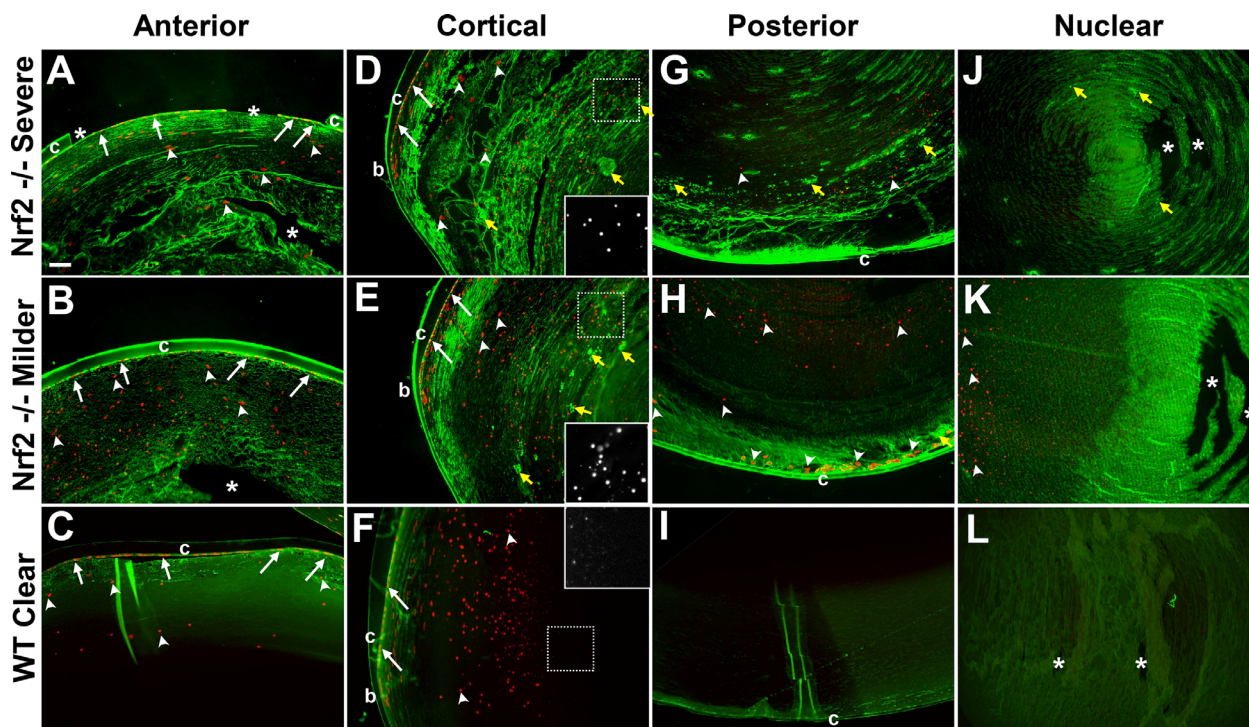


FIGURE 4. Histological analysis of cataracts in 18-month *Nrf2* ^{-/-} or wild-type lenses. Samples from the anterior region (A–C), cortical region (D–F), posterior region (G–I), or the nuclear region (J–L) are stained with wheat germ agglutinin (green) and DAPI (red) from representatives of severe cataracts (A, D, G, J), milder cataracts (B, E, H, K), or age-matched wild-type clear lenses (C, F, I, L). Abnormalities are observed in all parts of the *Nrf2* ^{-/-} lens, especially the cortex, which shows severe fiber cell disorganization. Aggregates are also observed (yellow arrows) as are ectopic nuclei, which mostly appear pyknotic (white arrowheads – see insets in D–F), in contrast to the normal nuclei of the lens epithelium and bow region (white arrows). Asterisks indicate tears in the lens that are likely histological artifacts. Abbreviations: b, bow region; c, lens capsule. Scale bar in (A) is 100 μm.

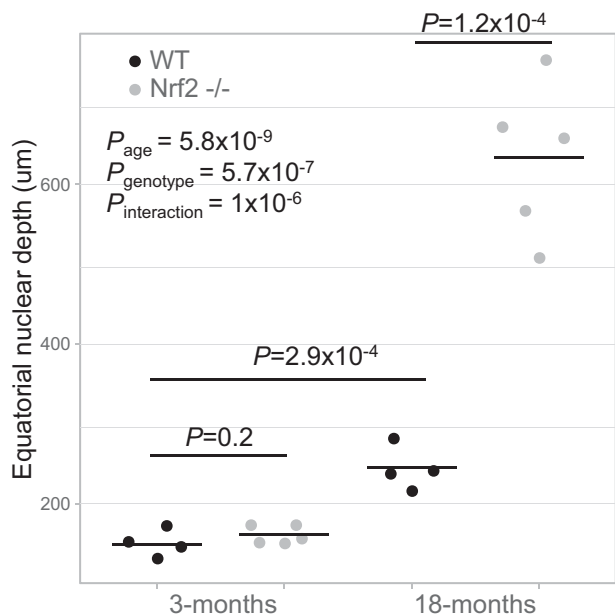


FIGURE 5. Age and genotype affect equatorial nuclear depth. Pair-wise comparisons show statistically significant differences between 3 month old and 18 month old lenses and between wild-type and *Nrf2* ^{-/-} 18 month old mouse lenses. A generalized linear model was used to show statistically significant age and genotype effects on equatorial nuclear depth, as well as an age-genotype interaction.

TABLE 2. Summary Data for Cataract Incidence in Female 21-Month-Old Mice

	AL	CR	Total	P _{diet}
Wild-type	3/11	1/13	4/24	0.30
<i>Nrf2</i> ^{-/-}	17/19	14/20	31/39	0.24
P _{genotype}	9.7 × 10 ⁻⁴	8.8 × 10 ⁻⁴	1.2 × 10 ⁻⁶	

Wild-type and *Nrf2* ^{-/-} mouse lenses, as scored via photography of dissected lenses mounted on grids.

the lens equator to the furthest intact nucleus. We observed both age-related increases in nuclear depth and genotype-related increase, but only in older *Nrf2* ^{-/-} mice. These findings indicate a statistically significant age-genotype interaction in equatorial nuclear depth that may underlie some of the opacities observed in older *Nrf2* ^{-/-} mouse lenses.

Given the increasing severity in cataracts between 11 and 15 months (see Fig. 2) and the severity of cataract phenotype at 18 months in male *Nrf2* ^{-/-} mice (see Figs. 3G–J, Fig. 4), we tested whether further aging to 21 months would lead to increased severity of cataracts in an independent cohort of male and female *Nrf2* ^{-/-} mice. This cohort also allowed us to test whether delaying aging via a 30% caloric restriction compared to ad libitum feeding would impact cataractogenesis in *Nrf2* ^{-/-} mice,⁵⁹ a finding we observed using Emory mice.^{72,73} Cataracts were observed at high frequency in both male and female mice (Supplementary Fig. S3, Tables 2–3, Figs. 6A–D). Furthermore, observed cataracts were hetero-

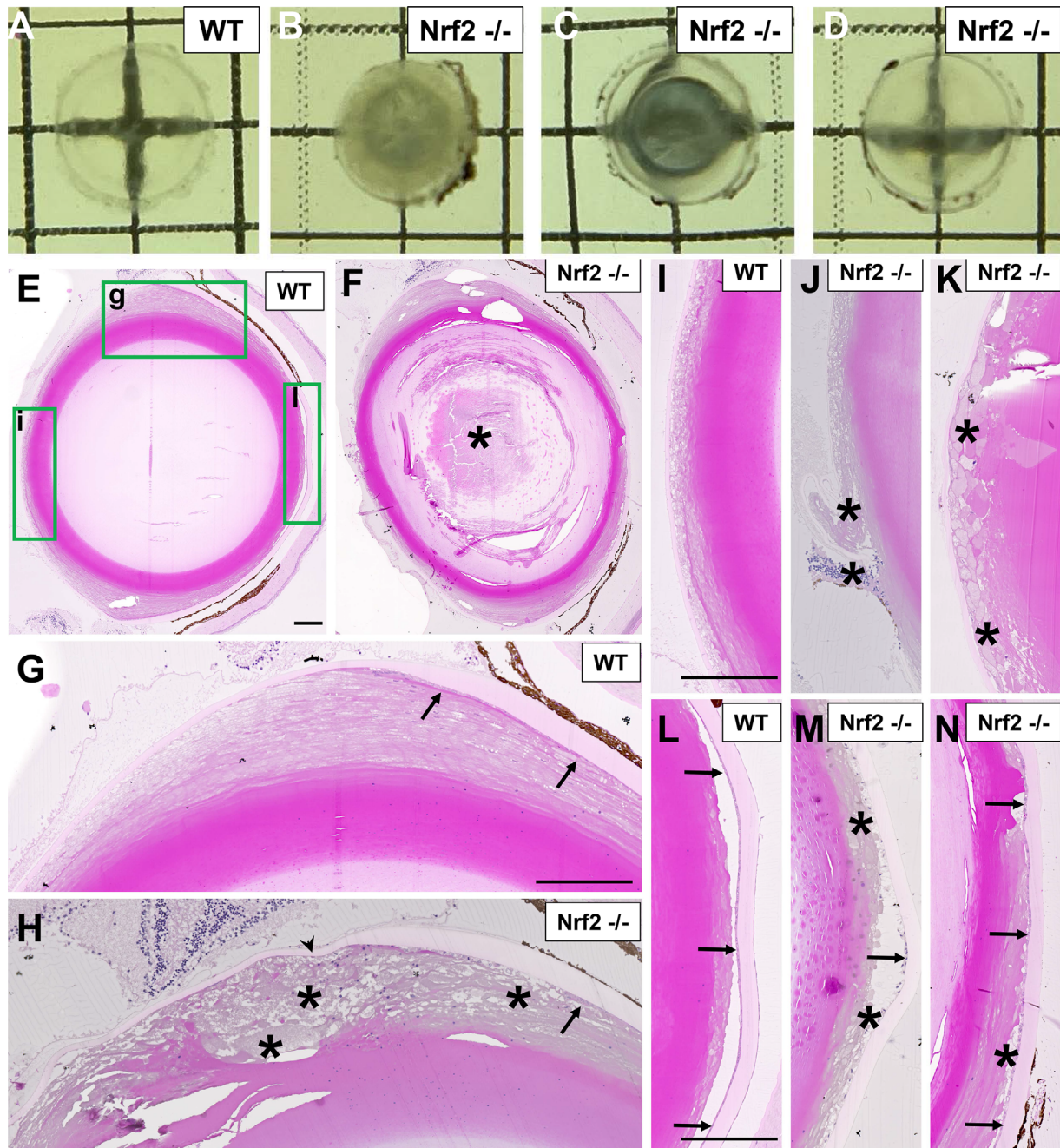


FIGURE 6. Appearance and histology of cataracts from female 21-month old mice. (A–D) Freshly dissected lenses from wild-type mice (A) or Nrf2 $-/-$ mice (B–D) reveal a diverse array of cataracts with different degrees of severity in Nrf2 $-/-$ mouse lenses. Cataracts shown are from mice fed their diets ad libitum. (E–N) Histological images are shown from wild-type mouse lenses (E, G, I, L) or Nrf2 $-/-$ mouse lenses (F, H, J, K, M, N). High-magnification images from (E) are shown in (G, I, L), as indicated by green boxed regions, covering the lens equatorial region (G, H), posterior capsular region (I–K), or anterior capsular regions (L–N). Asterisks indicate histological abnormalities (e.g. cellular disorganization, vacuolation) corresponding to lens opacities. Arrows point to lens epithelial cells and arrowheads point to the lens capsule. Scale bar in (E, G, I, L) is 100 μ m.

geneous, sometimes leading to complete opacification of the lens (see Supplementary Fig. S3B, Fig. 6B) or varying degrees of regional opacities (see Supplementary Figs. S3C, S3D; Figs. 6C, 6D). Both male and female mouse Nrf2 $-/-$ mice showed a significantly higher frequency of cataracts than wild-type mice, and CR did not significantly reduce the frequency of cataracts relative to ad libitum feeding (see Tables 2, 3).

Histological analysis was performed on contralateral eyes from 21 month female wild-type and Nrf2 $-/-$ mice. Whereas wild-type mouse lenses were clear and showed no histologic abnormalities (see Figs. 6E, 6G, 6I, 6L), nuclear cataracts were observed both in lens sections from Nrf2 $-/-$ mice (see Fig. 6F), consistent with the images from whole lenses (see Figs. 6B, 6C). Cortical lens defects were also observed histologically (see Fig. 6H). These cortical

TABLE 3. Summary Data for Cataract Incidence in Male 21-Month-Old Mice

	AL	CR	Total	P _{diet}
Wild-type	1/5	3/12	4/17	1
Nrf2 $-/-$	11/11	12/15	23/26	0.24
P _{genotype}	2.8×10^{-3}	7.1×10^{-3}	4.4×10^{-5}	

Wild-type and Nrf2 $-/-$ mouse lenses, as scored via photography of dissected lenses mounted on grids.

defects showed aberrant fiber cell differentiation, similar to that observed in 18 month mouse lenses (see Fig. 4D). Most Nrf2 $-/-$ mouse lenses exhibited posterior and anterior subcapsular defects (see Figs. 6J, 6K, 6M, 6N). Posterior subcapsular defects were associated with cellular (see Fig. 6J) or acellular plaques (see Fig. 6K). Anterior subcapsular defects were associated with degeneration of the lens epithelium (see Fig. 6M), normally a monolayer of flat cells (see Fig. 6L), or vacuolation of the subcapsular region (see Fig. 6N).

DISCUSSION

There are few highly reproducible models of age-related human cataracts. Because cataracts in humans often occur in combinations of different types and they appear later in life, the property of Nrf2 $-/-$ mice to similarly exhibit different types of cataracts in an age-dependent manner (see Tables 1–3), present them as a good model of human cataracts, while also providing a useful and suitable model for age-related cataracts of various specific types.

Cataracts in these Nrf2 $-/-$ mice can be compared with the few prior models of age-related cataracts. The Emory mouse develops progressive cortical and nuclear cataracts but penetrance is low and severity is limited, making it a costly and difficult model.^{72,74} The Grx2 knockout first develops a nuclear opacity, which progresses to affect cortical and subcapsular regions, but this does not model the typical human cataract pattern.⁵⁷ Few rodent models recapitulate highly prevalent posterior subcapsular cataracts, which are best modeled in chemically treated rabbits or ionizing radiation-treated mice.^{75,76} The LEGSKO mouse, lacking the glutathione synthesis enzyme, GCLC, develop age-related nuclear cataracts.⁴⁰ Similarly, mice lacking Gpx1, glutathione peroxidase 1, develop age-related nuclear cataracts, as did mice lacking Grx2, glutaredoxin 2.^{57,77} Nrf2 directly activates the rate-limiting enzymes responsible for glutathione synthesis, GCLC, and GCLM, and regulates glutathione regeneration.⁷⁸ Thus, it is somewhat surprising that Nrf2 $-/-$ mouse cataracts do not develop nuclear cataracts at a higher prevalence. Genetic redundancy with Nrf1, also present at high levels in the lens, may be an additional factor.^{79,80} Whether phenotypic disparities relate to different genetic backgrounds, genetic redundancies, or baseline levels of glutathione should be sorted out as we determine which are the best models of human age-related cataracts.

Nrf2 $-/-$ mice offer an opportunity to understand the sequence of events behind cataract development, especially considering that Nrf2 functions as a nuclear transcription factor, and thus likely performs its critical functions in lens epithelial cells and/or newly developing cortical fiber cells prior to LFC, when the lens cell nucleus is disassembled and degraded.⁶⁹ Our observation that young Nrf2 $-/-$ mouse lenses, although transparent, progress to cataracts

suggests that they may have underlying defects in fiber cell formation, findings hinted at in histological analysis of 3 month old Nrf2 $-/-$ mouse lenses. Careful analysis of Nrf2 $-/-$ mouse lens epithelial cells may reveal aspects of accelerated aging, altered stress response pathways, or abnormal metabolism and homeostasis. Future studies also warrant the study of Nrf2 binding partners like MafG and MafK, whose deficiency is also associated with cataracts.⁸⁰ The notion of subtle alterations in fiber cells escalating into progressive cataracts is supported by the *Crybb2* knockout mouse, where the loss of a β -crystallin manifests as age-related cortical cataracts,⁵⁴ and in *Bfsp2* knockout mice, where loss of the intermediate protein filensin results in more rapid age-related cataracts.⁸¹

Our observation of large numbers of abnormal nuclei through the inner and outer cortex of Nrf2 $-/-$ mouse lenses indicates abrogation or truncation of secondary LFC. Some cataract models have shown delayed secondary LFC, with nuclei observed anteriorly, such as mutations in β B2-crystallin or loss of Connexin 50, but the fiber cells eventually do undergo denucleation.^{82,83} More commonly, impaired LFC occurs in primary fiber cells, thus preferentially affecting the central part of the lens.^{47,84} Impaired primary LFC is observed in many congenital cataract models, as well as certain age-related cataracts like the Grx2 knockout mouse.⁸⁵ The finding of normal primary LFC may explain why we do not observe more significant nuclear cataracts. However, retention of fiber cell nuclei in itself does not explain the degree of cortical opacification, as milder cataracts also retained fiber cell nuclei (see Figs. 4B, 4E, 4H, 4K). Our finding that LFC occurred normally at birth (see Supplementary Figs. S1A, S1B) and was not different between wild-type and Nrf2 $-/-$ mouse lenses at 3 months (see Fig. 2) raises the possibility that there may be alternative regulatory mechanisms for LFC during adulthood. Absent or incomplete secondary LFC may be a more general feature of age-related cataracts than previously described. Our observation of increased nuclear depth with age, even in wild-type mice (see Fig. 5), confirms results found during normal aging in many organisms.⁸⁶ Our data suggest that the rate-limiting steps in nuclear removal are impacted by normal aging and further exacerbated by a deficiency in Nrf2. The recent transcriptomic characterization of the aging mouse lens may offer potential mechanisms linked to cortical LFC.⁸⁷

The presence of anterior and posterior subcapsular cataracts in Nrf2 $-/-$ mice offers the potential to determine the etiology of these cataracts as well. Some subcapsular cataracts were associated with the presence of nucleated cells, as is typical during congenital TGF β -induced cataracts, and associated with pathological epithelial-to-mesenchymal transition (EMT).⁸⁸ Evidence of potential EMT was observed in 18 month cataracts (see Fig. 4H), as well as in young 3 month lenses (see Supplementary Figs. S2H, S2I). Nevertheless, other posterior and anterior opacities did not show such cellular plaques and had severely disrupted fiber cell architecture (see Figs. 4A, 4B, 4G; Figs. 6K, 6M, 6N). Our data support a model where subcapsular plaques consist of populations of fiber cells and myofibroblastic cells that may eventually die or degenerate.^{89,90} Plaque formation may be further linked to specific tissue damage, including breakage of the posterior lens capsule (see Fig. 6J), which may activate a subsequent fibrotic immune response.⁹¹

Our finding that Nrf2 $-/-$ mice developed severe age-related cataracts emphasizes the importance of the Nrf2

pathway in lens morphogenesis and cataractogenesis. In human cataractogenesis, disruption of the Nrf2 pathway appears to occur via altered epigenetic regulation of KEAP1, leading to a diminished Nrf2 response.³⁸ During the normal process of lens aging, downstream functions of Nrf2, including glutathione synthesis, thioredoxin reductase, glutathione reductase, thioltransferases, and levels of antioxidant enzymatic function, all decrease in association with cataractogenesis.^{5,6,8,14,15,92} Whether those decreases are caused directly or indirectly by loss of Nrf2 function remains to be determined.

This research also informs about potential ways to alleviate or remediate cataracts using Nrf2-related biology. The Nrf2 $-/-$ mouse cataract model presented here should be useful to screen anti-cataractogenic treatments, preferably in combination with other Nrf2 $+/+$ models. Existing approaches, including small molecule Nrf2 activators or plant-derived Nrf2 activators, like sulforaphane, should be studied further in Nrf2 $+/+$ and Nrf2 $-/-$ cataract models. In vitro data in lens epithelial cells or aging human lenses suggests that sulforaphane treatment can confer improved antioxidant levels and response and therefore may protect against cataracts.^{93,94} It will be beneficial to identify compounds that can protect the lens in an Nrf2-independent fashion, because human cataracts are associated with epigenetically diminished Nrf2 responses over the course of aging. Because multiple types of cataracts are observed, this Nrf2 $-/-$ mouse model may reveal treatments that preferentially improve different parts of the lens.

Prior laboratory and epidemiology studies indicated that LG diets protect against age-related ocular conditions, including age-related macular degeneration and some cataracts, and that caloric restriction delayed cataracts in Emory mice and some pigmented lines of mice and rats.^{60,68,72,73,95-97} We therefore explored the possibility of a dietary cataract intervention by feeding Nrf2 $-/-$ mice LG or caloric restriction diets. However, we did not find a significant difference in cataract onset, prevalence, or type of cataract in Nrf2 $-/-$ mice fed LG or caloric restriction diets. There was some hint from cataract frequency data that caloric restriction may reduce cataract frequency in 21 month male and female Nrf2 $-/-$ mice (approximate 20–25% reduction; see Tables 2, 3), but we were likely underpowered for this difference to reach statistical significance. This reduction may indicate separate roles for Nrf2 in regulating aging events related to cataracts, which are reduced by caloric restriction, separately from cell autonomous requirements for Nrf2 in the lens.

Our study design has strengths and limitations. Our comparison of multiple Nrf2 $-/-$ alleles, dietary treatments, ages, and cataract assessments may appear as a limitation. However, one strength of utilizing multiple cohorts of mice, reared and analyzed at different centers, is that we can be more confident of the reproducibility of cataracts in Nrf2 $-/-$ mice in both sexes of mice fed multiple dietary treatments, and whose cataracts are observable in slit-lamp imaging, gross appearance, and histology. We evaluated mice with two different knockout alleles of Nrf2, although we did not evaluate mice with the *Nfe2l2*^{tm1Mym} allele at older ages. It is conceivable that different alleles of Nrf2 bred to different genetic backgrounds may develop cataracts at different frequencies than what we report here. Future studies will evaluate proteomic and transcriptomic changes in Nrf2 $-/-$ lenses and direct measurements of antioxidant status in mild and severe cataracts.

In summary, aged Nrf2 $-/-$ mice develop all major types of human age-related cataracts at a high prevalence. Nrf2 $-/-$ lenses retain cortical fiber cell nuclei, suggesting a role for Nrf2 in terminal differentiation of late-born lens fiber cells. Understanding the roles of Nrf2 in normal lens homeostasis will generate new insights into the processes of cataractogenesis and will generate new leads into the development of anti-cataract treatments. This new model of highly prevalent age-related cataracts should facilitate drug discovery and testing.

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