First Mutation in the βA2-crystallin Encoding Gene is Associated with Small Lenses and Age-Related Cataracts

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Purpose. A new mouse mutant with small lenses was identified within a mutagenesis screen. The aim of the study was to determine its molecular and morphologic characterization.

Methods. The offspring of paternally *N*-ethyl-*N*-nitrosourea (ENU)-treated C57BL/6J mice were analyzed for eye-size parameters by noninvasive in vivo laser interference biometry.

Results. A new mutant characterized by a clear, but significantly smaller lens without any changes for cornea thickness, anterior chamber depth, or aqueous humor size, was identified. The smaller size of the lens was more pronounced in the homozygous mutants, which were fully fertile and viable. The mutation was mapped to chromosome 1 between the markers *D1Mit251* and *D1Mit253*. Using a positional candidate approach, the βA2-crystallin encoding gene *Cryba2* was sequenced; a T→C exchange at cDNA position 139 led to a p.S47P amino-acid alteration. The eyes of newborn homozygous mutants showed no gross changes. At the age of three weeks, some clefts appeared at the cornea, but the lens and retina appeared without major changes. At the age of 25 weeks, the lenses of the heterozygous mutants develop a subcapsular cortical cataract, but the lenses of homozygous mutants were completely opaque.

Conclusions. These findings demonstrate the first mutation in the *Cryba2* gene. In contrast to the closely linked *Cryg* gene cluster, no congenital cataract mutation could be attributed to the *Cryba2* gene. Therefore, the human *CRYBA2* gene should be considered as a strong candidate gene for age-related cataracts, and the slightly smaller size of the lens might be recognized as an early biomarker for age-related cataracts. (*Invest Ophthalmol Vis Sci.* 2011;52:2571–2576) DOI:10.1167/iovs.10-6443

General estimates for blindness total ~50 million people worldwide. Recent data from the World Health Organization (WHO) indicate that cataracts represent the most frequent reason for blindness worldwide (39%), followed by uncorrected refractive error (18%; excluding presbyopia), glaucoma (10%), and age-related macular dystrophy (AMD; 7%). The proportion of blindness due to cataracts among all eye diseases ranges from 5% in Western Europe, North America, and the Western Pacific region up to 65% in poorer regions.[1] In addition to the implications for health care delivery and health care costs, cataract has been shown to be associated with falls and increased mortality, possibly because of associated systemic conditions. Major risk factors for age-related cataracts are diabetes and UV light (for a review see Ref. 2). Genetic components have been frequently discussed for age-related cataracts[3,4]; however, only a few facts could be presented up to now.

In the mouse, two models for age-related cataracts are well established: the senescence-accelerated mouse (SAM) and the Emory mouse. The SAM was identified at Kyoto University in 1970 on an AKR/J background strain. There are eight senescence-prone (SAM-P) strains; cataracts have been found in the SAM-P/1 and SAM-P/9 strains. At the late stages of life, the lens cortex became liquefied and developed into a mature cataract.[5] The Emory mouse has been first described in 1981/1982[6] as a spontaneous dominant cataract, which appears between 5 and 8 months and increases in severity with age, resulting finally in complete lens opacification. Emory mouse cataracts are associated with changes in numerous biochemical parameters and gene expression levels in the lens (see Refs. 7, 8 and references therein). However, in both cases, the underlying primary genetic defect still remains to be elaborated.

The first genetically characterized mouse model for age-related cataracts is the *Crybb2* knockout mouse, which forms anterior and posterior cortical cataracts after several months of life with increasing severity at higher age.[9] A rather complex model is the *Gja3*-knockout mouse, which needs a particular calpain 3 isoform to develop age-related cataracts.[10]

In humans, several SNPs have been associated with the formation of age-related cataracts, including the genes coding for αA-crystallin,[11,12] ephrin receptor A2,[13] solute carrier of the monocarboxylate transporter,[14] connexin50,[15] or galactokinase 1.[16] In most of these cases, the risk for cataract formation increases with higher age and the presence of a particular allele; however, there are also mutations reported that co-segregate in families (e.g., the F71L mutation in *CRYAA*,[12]; mutations in the 5′-UTR of *SLC16A12* leading to a significant upregulation of the corresponding gene expression[14]).

Here we demonstrate the first mutation in the *Cryba2* gene of the mouse. We identified the mutation in a breeding colony of mice characterized by smaller lenses. We show that the mutation causes cortical cataracts and leads finally in homozygous mutants to a total cataract at the age of 25 weeks. Therefore, we suggest that the new mutant line can be used as a well-characterized model for age-related cataracts.

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Material and Methods

Mice

Mice were kept under specific pathogen-free conditions at the Helmholtz Center, Munich, Germany. The use of animals was in accordance with the German Law of Animal Protection, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the tenets of the Declaration of Helsinki. Male C57BL/6J mice were treated with ENU (80 mg/kg body weight applied by intraperitoneal injection in three weekly intervals) at the age of 10 to 12 weeks as previously described[17] and mated to untreated female C57BL/6J mice.[18] The offspring of the ENU-treated mice were screened at the age of 11 weeks for abnormalities of the eye size. Briefly, the sizes of ocular parameters were examined using optical low coherence interferometry (ACMaster; Carl Zeiss Meditec, Jena, Germany). Briefly, mice were anesthetized with an intraperitoneal injection of 137 mg ketamine and 6.6 mg xylazine per kilogram body weight. The anesthetized mouse was placed on a platform and oriented in an appropriate position using light signals from six infrared LEDs arranged in a circle that must be placed in the center of the pupil. Central measurements of lens thickness, axial length, corneal thickness, and anterior chamber depth, as well as data evaluation, were performed essentially as described previously.[19,20] Mice with phenotypic deviations were tested for a dominant mode of inheritance.

Linkage Analysis

Heterozygous carriers (first generation) were mated to wild-type C3HeB/FeJ mice, and the offspring (second generation) were backcrossed to wild-type C3HeB/FeJ mice. DNA was prepared from tail tips of affected offspring of the third generation (G3). For linkage analysis, genotyping of a genome-wide mapping panel consisting of 153 single nucleotide polymorphisms (SNP) was performed using a mass spectrometry high-throughput genotyping system (MALDI-TOF [matrix-assisted laser/desorption ionization, time of flight analyzer] MassExtend; Sequenom, San Diego, CA).[21] Fine mapping was performed with the microsatellite markers *D1Mit7*, *D1Mit46*, *D1Mit251*, and *D1Mit253*.

Genotyping and Sequencing

Genomic DNA was isolated from tail tips of C57BL/6J, C3HeB/FeJ, JF1, and CFW wild-type mice or homozygous/heterozygous mutants according to standard procedures. For sequencing of the cDNA of the positional candidate gene, *Cryba2*, the primer pair *Cryba2-L3* (5′-AGCGAAC-ACCAGGGTCGTGC-3′) and *Cryba2-R2* (5′-GAGCTTTTAT-TGAGAATCTTACTGGTGATGAC-3′) were used. PCR was performed with a thermocycler (PTC-225; MJ Research, Waltham, MA,). Products were analyzed by electrophoresis on 1.5% agarose gel. Sequencing was performed commercially (GATC Biotech, Konstanz, Germany) after direct purification of the PCR products (Nucleospin Extract II; Macherey-Nagel, Düren, Germany).

To confirm the mutation in the genomic DNA, a 337-bp fragment was amplified from genomic DNA using the primer pair *Cryba2-Ex2-L1* (5′-CAGGCTTAGGCTAAGTAGAGTGTTCC-3′) and *Cryba2-Ex2-R1* (5′-GCACTGGAGTGAAGGTTGAAGTTCC-3′) and digested by the restriction enzyme *Bse*NI. For initial description of protein alterations we used the proteomics tools at the Expasy server (www.expasy.ch).

Histologic Analysis

Eyes of embryos and adult mice were analyzed histologically for retinal irregularities and lens pathologies. Embryo heads or prepared eyes were fixed for seven days in Davidson solution and embedded in plastic medium (JB-4; Polyscience Inc., Eppelheim, Germany) according to the manufacturer's protocol. Sectioning was performed with an ultramicrotome (OMU3; Reichert-Jung, Walldorf, Germany). Serial transverse 3-μm sections were cut with a glass knife and stained with methylene blue and basic fuchsin. The sections were evaluated with a light microscope (Axioplan; Carl Zeiss Meditec). Images were acquired by means of a scanning camera (AxioCam; Jenoptik, Jena, Germany) and imported into an image-processing program (Photoshop 10.0; Adobe, Unterschleissheim, Germany).

In Situ Hybridization

In situ hybridization of sections from neonatal mice 1 (P1) and 7 (P7) or 21 (P21) days old mice were performed essentially as described previously.[22] Briefly, the eyes were fixed in paraformaldehyde and embedded (Jung Histowax; Cambridge Instruments, Nussloch, Germany). Sections (7–10 μm) were cut with a microtome (RM-2065; Leica, Nussloch, Germany) and mounted onto slides. After hybridization and detection of the heteroduplices using anti-Digoxigenin antibodies, the sections were evaluated with a light microscope (Axioplan; Carl Zeiss Meditec). Images were acquired by means of a scanning camera (Progress 3008; Jenoptik) and imported into an image-processing program (Photoshop 6.0, Adobe Illustrator 9.0; Adobe).

Vision Test

Vision tests were performed between 9 am and 4 pm using a virtual optomotor system (Cerebral Mechanics, Lethbridge, Canada) as described previously.[23] Briefly, a rotating cylinder covered with a vertical sine wave grating was calculated and drawn in virtual three-dimensional space on four computer monitors facing to form a square. Visually unimpaired mice track the grating with reflexive head and neck movements (head-tracking). Vision threshold of the tested mice was quantified by a simple staircase test. Rotation speed and contrast were set to 12.0 d/s and 100%, respectively. Since no significant threshold differences were observed between males and females ($P > 0.05$; calculated by Mann-Whitney U test), data of both sexes were combined. Thresholds of wild-type C57BL/6J and homozygous *Aca30* mice were compared using the Mann-Whitney U test.

General

Chemicals and enzymes were purchased from Fermentas (Sankt Leon-Rot, Germany), Merck (Darmstadt, Germany), or Sigma Chemicals (Deisenhofen, Germany). Oligonucleotides were synthesized by Sigma Genosys (Steinheim, Germany).

Results

We screened offspring from ENU-treated male mice by laser interference biometry to detect eye size anomalies. One of the confirmed mutants was characterized by a clear, but significantly smaller lens without any changes for cornea thickness, anterior chamber depth, or aqueous humor size; it was assigned the laboratory code *Aca30*. At the age of 11 weeks, the mean lens axis length of wild-type mice is 2.1 mm (±0.01 mm), and 1.9 mm (±0.03 mm) for heterozygous *Aca30* mutants. The smaller size of the clear lens was more pronounced in homozygous *Aca30* mutants (1.7 mm ± 0.03 mm). It is shown for male and female mice in Figure 1.

The mutants are fully fertile and viable. In a genome-wide linkage analysis using SNP markers, the mutation was mapped to chromosome 1 close to the marker rs3678148 (76.2 Mb, Build 37.1). Fine mapping using microsatellite markers revealed a critical interval of 7.5 kb between the markers *D1Mit251* (70.4 Mb) and *D1Mit253* (77.9 Mb; Fig. 2a) making the βA2-crystallin encoding gene *Cryba2* a very interesting candidate gene (Fig. 2b). Sequencing of the *Cryba2* cDNA identified a T→C exchange at position 139 leading to a p.S47P amino acid exchange (Fig. 3a). The mutation was not detected in wild-type mice of several strains (C3HeB/FeJ, C57BL6, CFW, DBA, JF1), but was present in all tested mutant mice (Fig. 3b).

Computer-assisted analysis of the mutated protein predicted an increase of the random-coil structure (from aa 31–42 in the

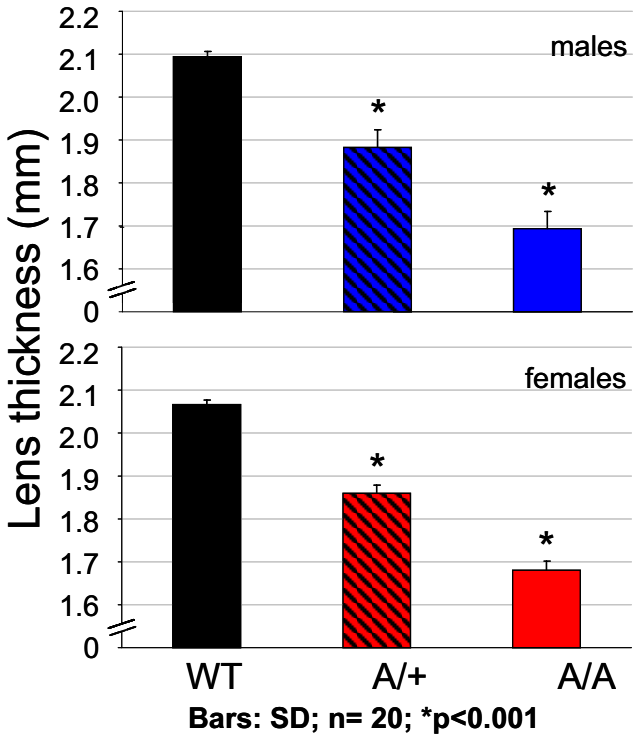

FIGURE 1. Lens thickness in *Aca30* mutants. Lens thickness is given for all three genotypes (WT [wild type], A/+ [heterozygous], and A/A [homozygous mutants]), both for males and females. Female lenses are slightly smaller than male lenses, but it is obvious that the lens size of heterozygotes is intermediate between wild-type and homozygous mutant lenses indicating a semi-dominant mode of inheritance.

wild-type protein to position 47 in the mutant βA2-crystallin). Correspondingly, the score for formation of the first Greek key motif (from aa 12–49) decreases from 7.7 to 6.5 (for comparison: the scores for the other Greek key motifs range from 9.1 to 14.0).

Histologically, the eyes of newborn homozygous mutants showed no obvious pathologic changes (Fig. 4a). At the age of three weeks, some clefts appeared in the cornea. The lens and the retina are without major changes (Fig. 4b). In situ hybridization (Fig. 5) demonstrated that *Cryba2* is expressed in the mouse during the first three weeks mainly in the outer cortical regions of the lens and also in the lens epithelial cells. During the first week, an expression gradient can be observed increasing from the center of the lens to the outer layers of the secondary fibers. At the age of three weeks, *Cryba2* mRNA is restricted to the equatorial and posterior regions of the lens, but it remains present in the entire lens epithelium.

The vision test using the virtual optokinetic drum did not reveal any difference at the age of four weeks, indicating the functional integrity of the eye and the entire visual system (data not shown). However, at the age of 25 weeks, the lenses of the heterozygous mutants developed a subcapsular cortical cataract, and the lenses of homozygous mutants were completely opaque (Fig. 6).

DISCUSSION

Our findings described here demonstrate the first mutation in the *Cryba2* gene in any organism so far. The *Cryba2* gene is very closely linked to the γ-crystallin gene cluster and belongs to the same superfamily of β/γ-crystallins. Mutations in the corresponding genes have been shown in many cases to cause congenital dominant cataracts in mouse and man (for a recent review see Ref. 24). Surprisingly, no cataract mutation could be attributed to the *Cryba2* gene up to now. Here we show that a point mutation in the *Cryba2* gene leads to smaller lens in juvenile mice and later to cortical cataracts in heterozygotes and to a total cataract in homozygous mutants.

The *Aca30* mutation characterized by its small lenses was linked to mouse chromosome 1, but the region of the *Cryg* gene cluster at 65 to 66 Mb was excluded. In the critical 7.5 Mb-interval between 70.4 and 77.9 Mb the *Cryba2* gene was by far the most likely candidate gene. The p.S47P mutation in *Cryba2* is not present in other mouse strains, but cosegregates with the pathologic phenotype in the *Aca30* line. Therefore, the p.S47P mutation is most likely causative for the small-lens phenotype of the *Aca30* mutant line. Based on the regular histology of the mutant lenses and the expression pattern of *Cryba2* in the lenses of the very young animals (mainly in the epithelial cells and the early secondary fiber cells), it might be speculated whether βA2-crystallin contributes to the thickness (volume) of the secondary lens fiber cells or to the speed of the terminal differentiation process in the transition zone from epithelial cells to the secondary fiber cells.

The lenses of the *Aca30* mutants are clear at the time when the lens size was measured (at 11 weeks); however, a cortical cataract is visible at 15 weeks progressing in homozygous mutants to total cataracts at 25 weeks of age. We expect progression to a similar clinical feature also in the heterozygous mutants at higher age. This brings the *Cryba2*

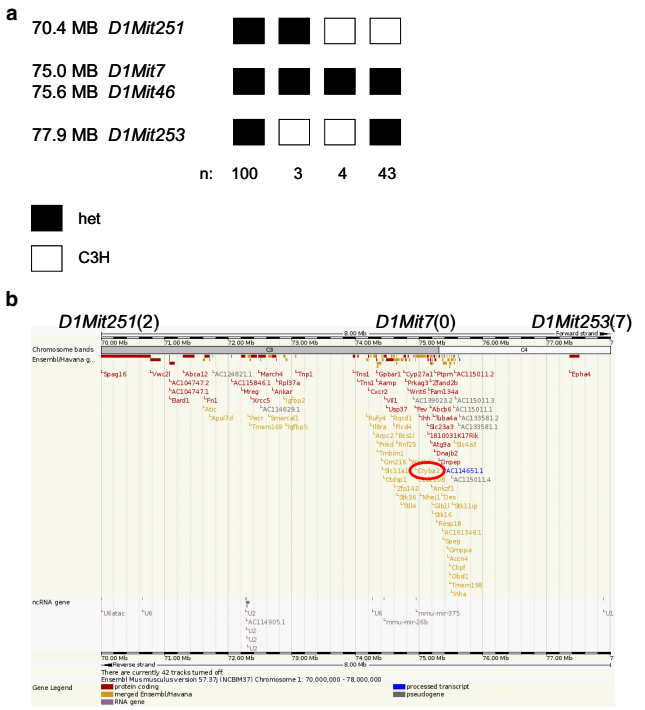

FIGURE 2. Fine mapping of *Aca30*. (**a**) Haplotype analysis revealed a critical interval of 7.5 Mb between the markers *D1Mit251* and *D1Mit253*. The markers *D1Mit7* and *D1Mit46* did not show any recombination with *Aca30*, indicating the close vicinity to the underlying gene. het, marker is heterozygous; C3H, marker is homozygous for C3H. (**b**) The overview of the entire critical region demonstrates *Cryba2* as an excellent candidate gene for the *Aca30* mutation. The graphics of the genomic area is from the ENSEMBL website based on the m37 assembly (http://www.ensembl.org/Mus_musculus/Info/Index).

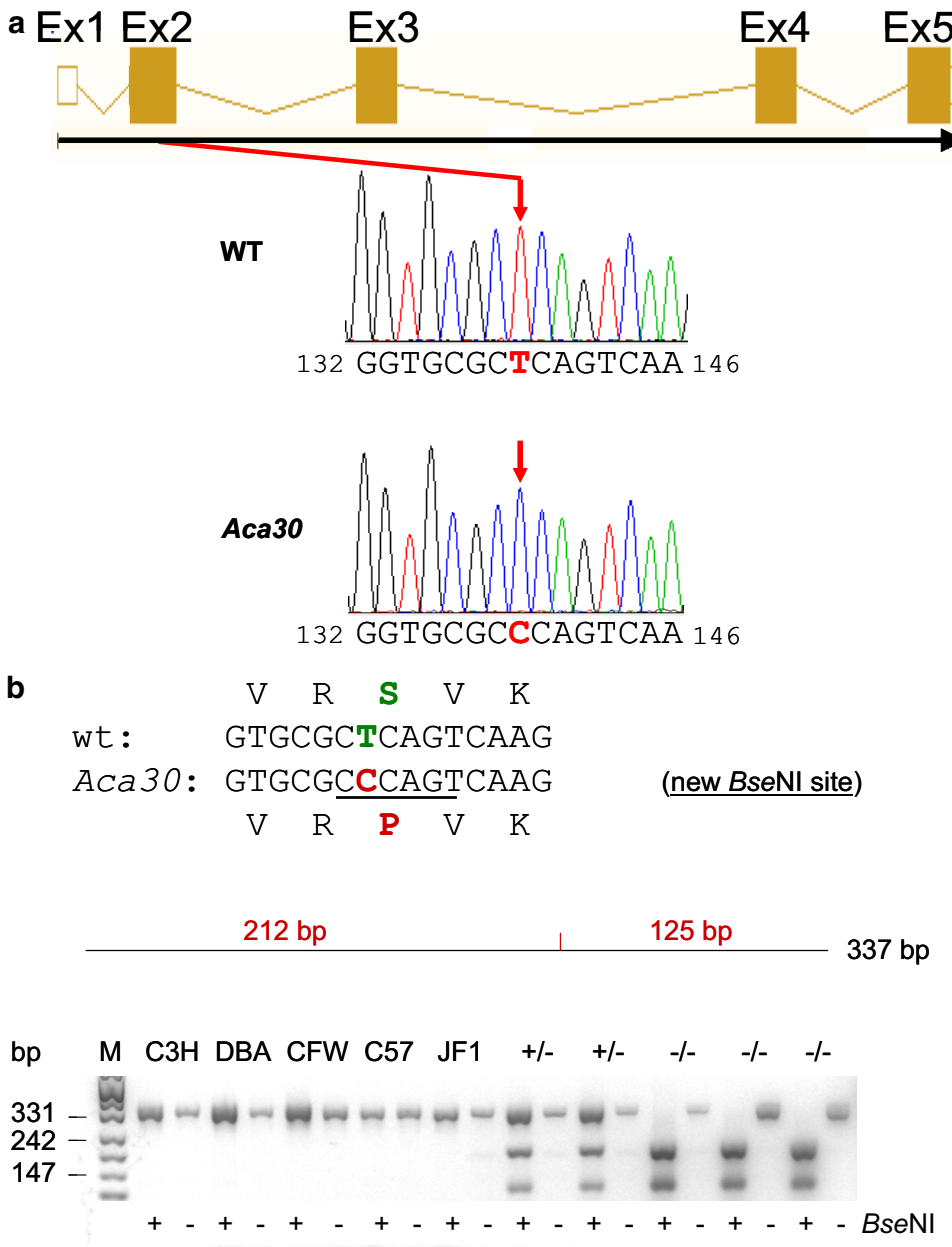

Figure 3. *Aca30* mutation affects the *Cryba2* gene. (**a**) Sequence analysis demonstrates a T>C exchange at cDNA position 139 leading to an amino acid exchange from Ser to Pro in codon 47. Moreover, a new *Bse*NI restriction site is created in the mutant. (**b**) The *Bse*NI restriction enzyme cuts only in the mutants a genomic fragment of 337 bp containing parts of *Cryba2* exon 2 and its flanking regions resulting into two fragments of 212 and 125 bp. DNA restriction analysis in different wild-type strains of mice showed the absence of the T139C mutation; however, it is present in five different *Aca30* mutant mice randomly collected from the actual running breeding (in two heterozygotes and three homozygotes). C3H, C3HeB/FeJ; DBA, DBA/2J; C57, C57BL/6J; "+" or "–" *Bse*NI indicates digested or undigested DNA.

gene in close relation to other *Cryb* genes leading also to progressive cataracts in mice. The most prominent progressive cataract mutation is the Philly mouse forming the first sign of cataract in heterozygotes at 15 days after birth and having mature cataract at the age of 45 days[25]; similar features have been observed in the other two *Crybb2* alleles, *Aey2*[26] and *O377*.[27] In contrast to these dominant findings in point-mutations of the *Crybb2* gene, the phenotype of the null mutant leads to recessive cataracts in the mouse being formed in the posterior and anterior cortex several months after birth; the severity of these cataracts increased with age.[9] Another progressive cataract (*Po*) was characterized by a mutation in the *Cryba1* gene and leads to a cortical opacity when the mice open their eyes (i.e., 12 days after birth). The cataract progresses to a perinuclear zonular opacification and finally to a total cataract at the age of eight weeks. In homozygous mutants, the total cataract is already developed at eye opening.[28] No mutants of the remaining 3 *Cryb* genes (*Cryba4*, *Crybb1* and *Crybb3*) are reported; there exist only ES cell lines with targeted mutations (http://www.informatics.jax.org; Aug 12, 2010).

In humans, the situation is a bit different: the only *CRYB* gene with no mutation reported so far is the *CRYBA2* gene. Mutations in all other *CRYB* genes are associated with domi-

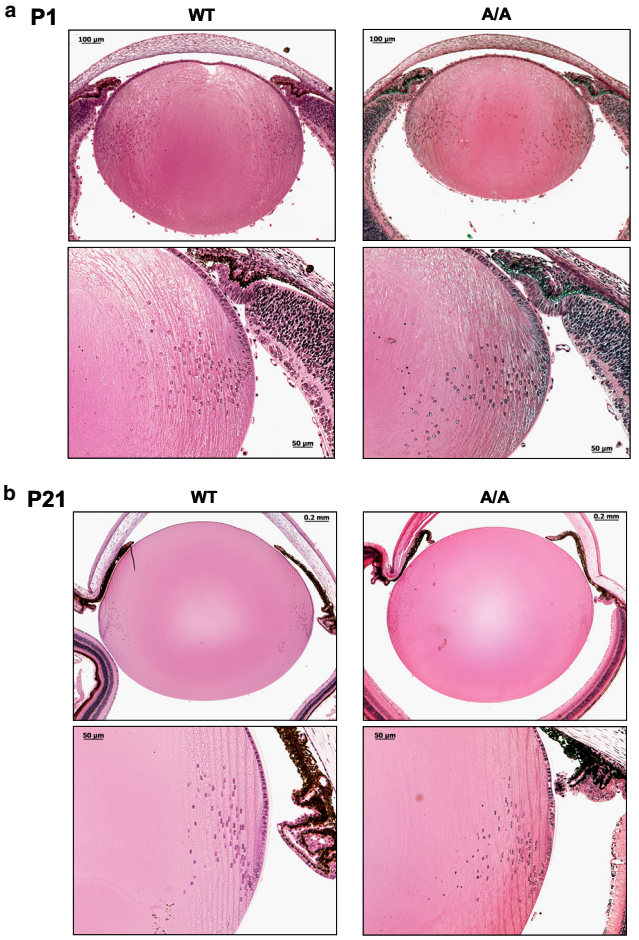

FIGURE 4. Histologic analysis of *Aca30* eyes. Wild-type and homozygous mutant eyes are compared at postnatal day (P)1 and P21. (**a**) Eyes of newborn homozygous mutants do not show major alterations. (**b**) At the age of three weeks, some clefts in the cornea appear. The lens and the retina do not show major alterations.

nant (or in a few cases also recessive), but congenital cataracts of different types (for a recent review see Ref. 24 and references therein). Therefore, it will be interesting to see whether a human mutation in *CRYBA2* is associated with a rather age-related cataract (like in the *Aca30* mouse) or with congenital cataract (like mutations in the other human *CRYB* genes). Moreover, it might be important to test the hypothesis, if a slightly smaller lens might be understood as an early biomarker for age-related cataracts.

Since the overall lifespan of a mouse is approximately two years, a six-month-old mouse (~25% of the total lifespan) might correspond to a 20-year-old human. However, this calculation does not account for the differences in the timing of developmental and aging processes among these two species. For example, mice are born with closed eyes and open their eye lids approximately two weeks after birth. In humans, this happens at the end of the first trimester of pregnancy. Moreover, the reproductive lifespan of (female) mice ranges from eight weeks to 20–35 weeks,[29] suggesting the age of a six-month-old mouse might correspond to a 50-year-old (female) human.

Finally, data from the Allen Brain Atlas (http://mouse.brain-map.org) demonstrate that *Cryba2* is also expressed in various brain regions of the mouse; the midbrain shows the highest signal density. We have identified *Cryba2* transcripts in cDNA preparations of the entire brain and in particular of the cerebellum (data not shown). This is in line with findings in other *Cryb* genes, particularly, with the βB2-crystallin–encoding gene.[27,30,31] However, according to the Allen Brain Atlas, the highest expression of mouse *Cryb* genes shows the *Crybb1* gene, particularly in the cerebellum, the cerebral cortex, and in the olfactory bulb. In contrast, almost no expression in the brain was found for the *Cryba1* and *Cryba4* genes as well as for most of the γ-crystallin–encoding genes (except for *Crygb*, which is expressed also in some brain region with its highest expression level in the dorsal region of the striatum). Even if the function of β-crystallins in the brain remains mainly speculative, ophthalmologists might be aware of additional neurologic deficits in cataract patients suffering from mutations in β-crystallin encoding genes, and additional research in this field should be initiated.

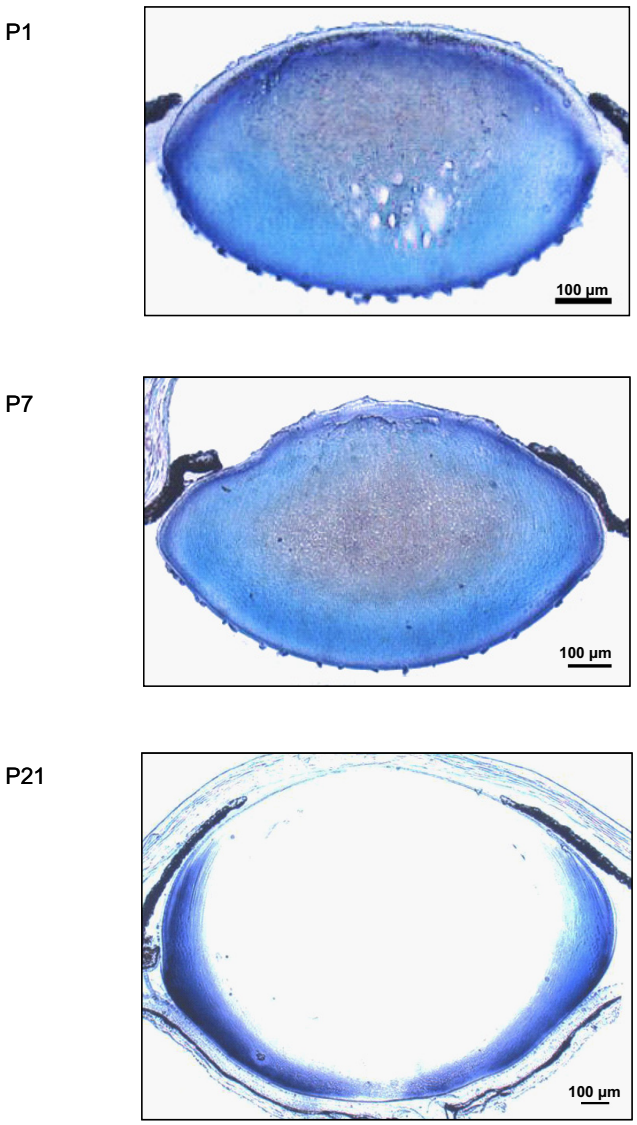

FIGURE 5. Expression analysis of *Cryba2* in mouse lenses. In situ hybridization of lenses from wild-type mice (C3H) at the age of 1, 7, or 21 days revealed a constant expression in the lens epithelial cells and a variable expression in the fiber cells. At P1 and P7, there is a gradient increasing from the lens center to the cortex; however, at P21 the center of the lens is free from *Cryba2* mRNA, which remains concentrated at the equatorial region of the lens. It is slowly decreasing toward the posterior pole. The anterior secondary lens fiber cells are free of *Cryba2* mRNA (anterior is always at the *top* of the figure).

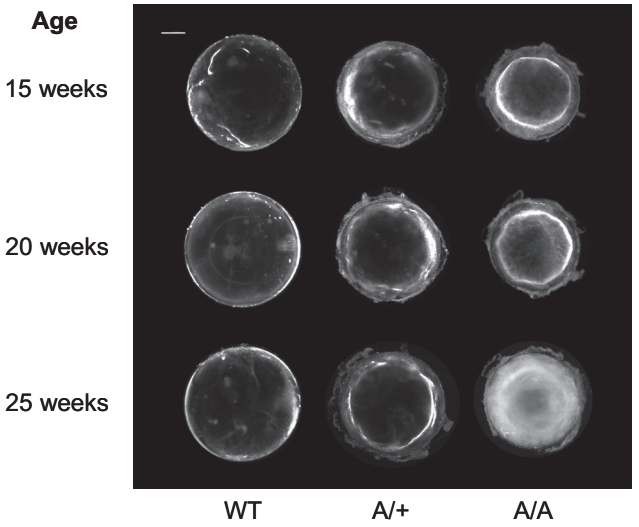

FIGURE 6. Progressive lens opacities in *Aca30* mutants. Isolated lenses of all three genotypes are shown for 15, 20, and 25 weeks of age. Lenses of wild-type mice remain transparent, but it is obvious that homozygous lenses develop total cataracts at the age of 25 weeks. In lenses of younger homozygous mutants, a cortical opacity is observed. Heterozygous mutants also develop cortical cataracts; however, a total cataract does not yet appear. Bar, 0.5 mm.

In conclusion, we report here the first mutation in the *Cryba2* gene leading to a smaller lens in juvenile mice and to cataracts later in life. Therefore, the *Aca30* mouse mutant might be used as a model for age-related cataracts in humans. Because of the expression of *Cryba2* in the brain, neurologic effects of the mutation cannot be excluded.

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