Connexin50D47A Decreases Levels of Fiber Cell Connexins and Impairs Lens Fiber Cell Differentiation

Viviana M. Berthoud, Peter J. Minogue, Helena Yu, Richard Schroeder, Joseph I. Snabb, and Eric C. Beyer

Department of Pediatrics, University of Chicago, Chicago, Illinois

Correspondence: Viviana M. Berthoud, Department of Pediatrics, University of Chicago, 900 East 57th Street, KCB-5, Chicago, IL 60637; vberthou@peds.bsd.uchicago.edu.

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PURPOSE. Substitutions of aspartate-47 (D47) of Connexin50 (Cx50) have been linked to autosomal dominant congenital cataracts in several human pedigrees. To elucidate the lens abnormalities caused by a substitution at this position, we studied *No2* mice, which carry the Cx50D47A mutation and parallel the human pathology.

METHODS. Lenses from mice of different ages (neonatal to 4 months) were examined by dark-field and immunofluorescence microscopy. Protein levels were determined by immunoblotting using primary antibodies directed against connexins, other membrane proteins, crystallins, and proteins residing in different organelles.

RESULTS. Lenses of both heterozygous and homozygous Cx50D47A mice had cataracts and were smaller than those of wild-type littermates. Levels of Cx50 were severely reduced in mutant animals as compared with those in wild-type mice (<20% in heterozygotes and ≤3% in homozygotes). Levels of Cx46 and aquaporin0 were also decreased, but to a lesser extent. The immunostaining pattern of lens connexins was altered in mutant animals. The lenses of Cx50D47A mice showed persistence of nuclear remnants in deep regions of the lens and elevated levels of H3 histone and the mitochondrial protein, Tom20. γ -Crystallin levels were decreased in lenses of all mutant mice, and β -crystallins were reduced in homozygotes.

CONCLUSIONS. These data suggest that mice expressing Cx50D47A develop cataracts due to a severe decrease in the abundance of functional connexin channels. They also implicate Cx50 in fiber cell differentiation, since mutant lenses showed impaired degradation of organelles and decreased levels of some crystallins.

Keywords: connexin, congenital cataract, gap junction, organelle degradation, crystallins

Mechanisms of cataract formation may be elucidated by studying the abnormalities induced by genes whose mutations cause cataracts. Inherited cataracts account for a third of all childhood cataracts and are genetically and phenotypically heterogeneous. In most cases, nonsyndromic congenital cataracts are inherited as autosomal dominant traits. They have been mapped to mutations in several genes including those encoding α -, β -, and γ -crystallins, aquaporin0 (AQP0 or *MIP*) and the lens fiber connexins, Cx46 (*GJA3*), and Cx50 (*GJA8*).¹

Connexins belong to a family of homologous proteins that form plasma membrane specializations known as gap junctions. Gap junctions contain clusters of channels that allow direct intercellular passage of ions and molecules between adjacent cells. Because the lens is an avascular organ and fiber cells lose their organelles during differentiation, the lens relies on gap junction-mediated communication among its cells to maintain homeostasis and transparency.

Several cataract-associated mutations of human Cx46 and Cx50 have been studied in exogenous expression systems to determine their biochemical, cellular, and/or functional abnormalities.² The most commonly observed abnormality is loss (or severe reduction) of intercellular channel function associated with absence (or severe reduction) of gap junction formation. Unfortunately, lens tissue from humans carrying connexin mutations is not readily available. Therefore, sequential morphologic, histologic, and

biochemical evaluation of such tissues is not possible. Some of these obstacles can be overcome by studying animal models, which develop cataracts such as mice with targeted deletion of Cx46 or Cx50³⁻⁵ or rodent lines carrying lens connexin mutations.⁶⁻¹¹ However, most of these mouse and rat lines contain alterations in amino acid residues that do not correspond to those mutated in human pedigrees.

The great majority of the known cataract-associated human Cx50 mutants are missense substitutions. Among them, there are several mutants in the first extracellular loop, including Cx50D47N, which is associated with nuclear pulverulent cataracts.^{12,13} This mutant does not traffic properly to the plasma membrane and does not induce gap junctional currents.¹² A line of mice (originally called ENU-326 or *No2*) that develops bilateral nuclear and zonular opacities in both heterozygous and homozygous animals¹⁴ was subsequently shown to carry a missense substitution at the same amino acid, Cx50D47A.¹⁵ Like the human mutant, mouse Cx50D47A shows impaired trafficking and does not form functional gap junction channels.^{12,16} Therefore, the *No2*/Cx50D47A mouse represents an ideal model to study associated lens abnormalities.

To gain insights into the mechanism of cataract formation due to a mutation of a lens fiber connexin that mimics a human mutation, we investigated histologic and biochemical characteristics of the lenses of *No2* mice. We have identified multiple

abnormalities, including ones that help to explain the dominant inheritance pattern of the cataracts.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Animals

No2 (ENU-326) mice were originally isolated by Favor using ethylnitrosourea mutagenesis and screening for the cataract phenotype.¹⁴ We obtained these mice from the International Mouse Strain Resource at Harwell (UK) and maintained them in the C3H mouse strain to stay as close as possible to the original background. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and followed the Institutional Animal Care and Use Committee guidelines from the University of Chicago.

Antibodies

Rabbit polyclonal anti-human Cx50 antibodies have been previously described.¹⁷ Rabbit polyclonal antibodies against a synthetic peptide sequence from within the intracellular loop of mouse Cx46 were generated and affinity purified by YenZym Antibodies, LLC (South San Francisco, CA). Rabbit polyclonal anti-human aquaporin 0 (AQP0) antibodies were obtained from Alpha Diagnostic International, Inc. (San Antonio, TX). Monoclonal rabbit anti-PDI and anti-H3 histone antibodies were obtained from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-Cx43 antibody was obtained from ThermoScientific (Pittsburgh, PA). Rabbit polyclonal anti- α A- and anti- α B-crystallin antibodies were obtained from Enzo Life Sciences (Farmingdale, NY). Rabbit polyclonal anti-Tom20 and mouse monoclonal anti- β -crystallin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti- γ -crystallin antibodies were a kind gift from Dr Samuel Zigler (Wilmer Eye Institute at Johns Hopkins University, Baltimore, MD). Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG antibodies were obtained from Life Technologies (Grand Island, NY). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Light Microscopy Analysis

Dark-field photomicrographs were obtained with a Zeiss Stemi-2000C dissecting scope (Carl Zeiss, München, Germany). Light microscopy analysis of the lenses was performed as previously described.¹⁸

Immunofluorescence

Lenses from mice aged 1 month or older, eyeballs from 10-day-old mice, and newborn mouse heads were fixed in 4% paraformaldehyde in PBS, pH 7.4. Then, they were transferred to 30% sucrose in PBS and left at 4°C until they sank. Cryostat sections (20 μ m) were obtained and then processed for immunofluorescence as previously described.¹⁹ Sections were subsequently incubated with 4',6-diamino-2-phenylindole dihydrochloride (DAPI; Invitrogen, Carlsbad, CA) to stain nuclei or with TRITC-conjugated phalloidin (Sigma Chemical Co.) or Alexa Fluor 488-conjugated phalloidin (Invitrogen) to stain filamentous actin followed by DAPI.

For immunofluorescence of epithelial cells, lenses were fixed in 4% paraformaldehyde in PBS for 12 minutes. Then, the fiber cell mass was removed and the epithelium placed on a polylysine-coated slide (Polysciences, Inc., Warrington, PA) and subjected to double labeling immunofluorescence.

Specimens were studied in an Axioplan 2 microscope (Carl Zeiss) equipped with a mercury lamp. Images were acquired with a Zeiss AxioCam digital camera using Zeiss AxioVision software.

Confocal images were obtained using a Leica SP2 AOBs laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) or an Olympus FluoView 1000 laser scanning confocal microscope (Olympus America, Inc., Center Valley, PA) using the factory settings for excitation and emission wavelengths of Alexa Fluor 488, Alexa Fluor 594, TRITC, and DAPI fluorescence. Images were collected by sequential scanning using single laser-line excitation to eliminate bleeding between channels.

Figures were assembled using Adobe Photoshop CS3 Extended (Adobe Systems, Inc., San Jose, CA).

Immunoblotting

Lenses were dissected in PBS, and homogenized in PBS, 4 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, and cComplete EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) in a glass-glass homogenizer, and then sonicated. Homogenates were stored at -80°C until use. Protein concentrations were determined using the BioRad Protein Assay (BioRad, Hercules, CA) based on the Bradford dye-binding procedure.²⁰ Immunoblots were performed as described in Minogue et al.²¹ using anti-Cx50, anti-Cx46, anti-AQP0, anti-H3 histone, anti-PDI, anti-Tom20, or anti-crystallin antibodies. Except as noted, immunoblots were performed in triplicate, quantified by densitometry, and are reported in the text as percentages of the values determined from wild-type samples.

Assessment of DNase II Activity

DNase II activity was assessed essentially as described by De Maria and Bassnett.²² Homogenates prepared from 1.6-month-old animals were incubated at pH 5.9 with linearized plasmid DNA (Cx43 subcloned in pSFFV-neo) for 2 hours. Then, DNA degradation was assessed by electrophoresis through a 1% agarose gel.

RESULTS

Expression of Cx50D47A Caused Cataracts and Reduced Lens Size in Heterozygous and Homozygous Mice

To examine the appearance of the lenses of *No2* mice, we observed the lenses under dark-field illumination. Lenses from Cx50D47A heterozygous and homozygous animals both had cataracts. They were much more severe in the homozygous than in the heterozygous mice (Fig. 1). The homozygous mice had nuclear opacities that were dense in the center, but appeared punctate in more peripheral regions; lenses of heterozygotes showed punctate cataracts (Fig. 1). Opacities were present in all mice carrying the Cx50D47A mutation.

Expression of Cx50D47A also affected lens size (Fig. 1). We consistently observed smaller lenses in heterozygous and homozygous animals. An estimation of the differences in lens volume can be calculated from the dark-field images by assuming that the lenses are spheres. Using this computation, the estimated volumes of 1-month-old heterozygous and homozygous lenses were 3.8 and 2.5 mm³, respectively,

FIGURE 1. Photographs of lenses from 1-month-old wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) mice. Both heterozygous and homozygous lenses show nuclear cataracts and are smaller than the lenses from wild-type mice. These defects are much more pronounced in homozygous than in heterozygous animals. Scale bar: 590 μ m.

compared with a volume of 5.6 mm³ for wild-type lenses. These values represent a decrease in lens volume of 33% in heterozygous animals and 56% in homozygous animals. These decreases in lens size are more pronounced than those reported for Cx50 null mice, which show no change in lens mass in heterozygotes and a 40% to 46% decrease in homozygotes.^{4,5,23}

No2 Mice Had Decreased Levels of Cx50

To test the effects of Cx50D47A expression on the abundance of Cx50, we performed immunoblotting on lens homogenates. Levels of Cx50 were significantly decreased in both heterozygous and homozygous animals (Fig. 2). In lenses from heterozygous mice, Cx50 levels averaged only 10% of the levels in wild-type animals, and in homozygous lenses, Cx50 levels were reduced to only 1% of the levels detected in wild-type mice.

The severe decrease in Cx50 levels led us to investigate the distribution of Cx50. Immunoreactive Cx50 was detected in gap junction plaques in superficial cortical fiber cells in lens

sections from all genotypes (Fig. 3). However, the intensity of the Cx50 immunostaining in the cortical region (extending inward from the periphery of the lens) decreased much more abruptly in lenses from heterozygous and homozygous animals than in lenses from wild-type mice (Fig. 3). Indeed, high intensity immunoreactive Cx50 was observed in many fiber cell layers in wild-type lenses compared with only a few cell layers in heterozygous lenses and even fewer cell layers in homozygous lenses (Fig. 3).

To test whether the decrease in Cx50 in Cx50D47A-expressing lenses could be detected early in postnatal life, we performed immunofluorescence in sections from neonates and 10-day-old lenses. These experiments revealed that Cx50 immunoreactivity was decreased in Cx50D47A heterozygous and homozygous neonates (Fig. 4). However, the decrease in the number of cell layers showing immunoreactive Cx50 was more pronounced in lenses of 10-day-old mice than in neonates (Fig. 4).

Cx50D47A Mice Showed Alterations of Connexins in the Epithelium

Lens epithelial cells express Cx50 and Cx43.^{5,24} We examined the distribution of these connexins in flat mounts of the epithelium after removal of the fiber cell mass. In epithelial cells from wild-type mice, Cx43 and Cx50 both localized in a punctate distribution along appositional membranes (Figs. 5A–C). The relative intensities of the Cx43 and Cx50 signals varied in different areas of the epithelium (Fig. 5, compare panels A, B, and C). Some (but not all) of the puncta showed colocalization of Cx43 and Cx50 in co-expressing cells. In more peripheral regions of the epithelium, where differentiating cells are located, cells showed increased Cx50 staining at the plasma membrane with a more linear appearance and some intracellular staining.

Similar to wild-type lens epithelia, heterozygous and homozygous Cx50D47A lens epithelia showed immunopositive staining for Cx43 and Cx50 with variations of intensities in different areas (Figs. 5D, 5E). However, the extent of colocalization between Cx43 and Cx50 immunostaining was decreased, especially in homozygous lenses. Heterozygous and homozygous lens epithelia showed increased intracellular Cx50 immunostaining, a phenomenon that was most pronounced in epithelia of homozygous Cx50 mice (Figs. 5D, 5E). Furthermore, the linear staining at appositional membranes in peripheral regions was more diffuse in homozygous Cx50D47A lens epithelia (Fig. 5E).

FIGURE 2. Levels of Cx50 in lenses from 3.9-month-old mice. (A) Aliquots of lens homogenates were subjected to immunoblotting with anti-Cx50 antibodies. (B) Graph represents the densitometric values of the Cx50 bands obtained in three independent experiments expressed as a percentage of the values determined from wild-type littermates. Levels of Cx50 are severely reduced in heterozygous and homozygous mice expressing Cx50D47A.

FIGURE 3. Immunolocalization of Cx50 in lenses from wild-type and Cx50D47A-expressing mice. **(A)** Photomicrographs show the distribution of immunoreactive Cx50 (green) and nuclei (DAPI, blue), in longitudinal sections of lenses from 1-month-old wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) mice. **(B)** Confocal images show the distribution of immunoreactive Cx50 (green), nuclei (DAPI, blue), and filamentous actin (TRITC-phalloidin, red) in cross sections of lenses from 1.5-month-old wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) mice. The appositional membrane staining of Cx50 shows a dramatic decrease from the periphery toward the center in lenses of mice expressing Cx50D47A. These lenses also show an increase in diffuse cytoplasmic staining in the epithelium and the most peripheral fiber cells. Scale bar: 127 μ m for all panels in (A), and 27 μ m for wild-type and heterozygous lens sections and 22 μ m for the homozygous lens sections in (B).

Expression of Cx50D47A Also Affected Cx46 and AQP0

We also considered the possibility that expression of Cx50D47A affected the second lens fiber connexin, Cx46. Immunoblots showed that levels of Cx46 in lenses were decreased on average to 61% (heterozygotes) and 37% (homozygotes) of the levels in wild-type lenses (Fig. 6). This reduction was also observed by immunofluorescent staining of lens sections. The number of cell layers of the lens cortex containing punctate Cx46 staining was reduced, and the intensity of the immunostaining decreased from the periphery towards the center of the lens (Fig. 6).

We assessed whether another major lens membrane protein, AQP0, was also affected in the *No2* lenses. Immunoblotting of lens homogenates showed that levels of AQP0 were reduced to 77% of wild-type levels in heterozygotes and to 14% of wild-type levels in homozygotes (Fig. 7A). In lens areas where it was detectable, the plasma membrane distribution of AQP0 on all sides of the fiber cells appeared unaffected by expression of Cx50D47A as revealed by immunofluorescent

staining of cross-sections of the lenses (Figs. 7B–D). However, the number of cortical cell layers extending inward from the periphery of the lens that showed AQP0 immunostaining decreased drastically in homozygous as compared with wild-type lenses (compare the top panels in Figs. 7B, 7D). A slight decrease in the intensity of AQP0 immunostaining was observed between Cx50D47A heterozygous and wild-type lenses (compare the top panels in Figs. 7B, 7C).

Denucleation was Impaired in *No2* Lenses

During normal lens differentiation, cells that will become fiber cells lose their nuclei and other organelles. This process leads to a typical distribution of nuclei in epithelial cells and in differentiating fibers in the bow region (characterized by nuclei and nuclear fragments localized anteriorly to the bow region in the most superficial cortical cell layers) and the absence of nuclei in the center of the lens. Such a pattern was observed after staining sections of wild-type lenses with DAPI (Fig. 8). In the cortical region of heterozygous Cx50D47A lenses, the pattern of DAPI-stained nuclei resembled that of the

FIGURE 4. Immunolocalization of Cx50 in lenses from neonates and 10-day-old mice. Photomicrographs in the *top panels* show the distribution of Cx50 (*green*) in sections of lenses from neonatal wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) mice. Confocal images in the *bottom panels* show the distribution of Cx50 (*green*), filamentous actin (*red*) and nuclei (DAPI, *blue*) in lens sections from 10-day-old animals of all three genotypes. The decrease in Cx50 immunostaining in mutant mouse lenses between the periphery and the center of the lens is already apparent at these early ages. *Scale bar*: 333 μ m for the *top panels* and 79 μ m for the *bottom panels*.

wild-type lenses; however, nuclear remnants were also observed in a central region corresponding to that containing denucleated mature fiber cells in wild-type lenses (Fig. 8). The most striking pattern of DAPI staining was observed in homozygous Cx50D47A lenses. In addition to the normal distribution of nuclei in the epithelium and differentiating fiber cells, these lenses contained abundant nuclear remnants deep within the lens. In many sections, these nuclear remnants had a unique distribution, forming several concentric arcs (Fig. 8).

To examine further the impairment of denucleation in Cx50D47A lenses, we used whole lens homogenates to assess the levels of H3 histone, one of the core proteins of chromatin. Levels of H3 histone were markedly increased in lenses from heterozygous and homozygous Cx50D47A mice as compared with the levels detected in wild-type lenses (Fig. 9A).

During lens fiber cell maturation, denucleation is dependent on DNA degradation by the acid nuclease, DNase II β .²⁵ To test whether the impaired denucleation in the *No2* lenses was due to the absence of acid DNase activity, we determined the activity of this enzyme in lens homogenates using a linearized

plasmid as substrate. Incubation of this DNA with lens homogenates resulted in a decreased intensity of the plasmid DNA band and the appearance of a comet-like smear of degraded DNA underneath it (Fig. 9B, lanes 3–5). The intensity of the remaining intact plasmid DNA was similar between the wild-type and heterozygous lens homogenates, but slightly reduced in the homozygous lens homogenate (Fig. 9B, compare lane 3 with 5).

No2 Lenses Showed Impaired Degradation of Other Organelles

The impaired denucleation observed in Cx50D47A-expressing lenses suggested that degradation of other organelles might also be reduced. Therefore, we performed immunoblotting on lens homogenates using antibodies against resident proteins of the endoplasmic reticulum (ER) (protein disulfide isomerase, PDI), and mitochondria (a major receptor of the preprotein translocation system, Tom20).

In lens homogenates from 1-month-old mice, levels of PDI were minimally different between mutant and wild-type mice (increased by 13% in the heterozygous and 21% in the homozygous lenses in the blots shown in Fig. 9C). However, levels of Tom20 were increased on average by 108% in heterozygous lenses and by 279% in homozygous lenses. These findings suggest that there was a modest retention of ER components, but a significantly impaired degradation of mitochondria in the mutant lenses, especially in the homozygous animals.

Mutant Lenses Showed Alterations in Crystallin Levels

Since the expression of crystallins changes with differentiation of lens cells, we used immunoblotting to determine whether expression of Cx50D47A changed the abundance of the different families of crystallins. The levels of γ -crystallins were decreased in 1-month-old heterozygous and homozygous animals compared with the levels in wild-type animals; they averaged 86% in heterozygotes and 55% in homozygotes (Fig. 10). Homozygotes also showed a decrease in the levels of β -crystallins (average: 42%; $n = 3$) and an increase in the levels of α B-crystallins (average: 236%; $n = 3$) as compared with wild-type animals (Fig. 10). The levels of α A-, α B-, or β -crystallins were not consistently altered in heterozygotes, nor were α A-crystallins affected in homozygotes (Fig. 10).

FIGURE 5. Immunofluorescence of Cx43 and Cx50 in the epithelium. Confocal images show the distribution of Cx50 (*green*) and Cx43 (*red*) in lens sections from 1.9-month-old wild-type (+/+) (A–C), heterozygous (+/D47A) (D), and homozygous (D47A/D47A) (E) animals. (A–C) Images from the wild-type lens epithelium illustrate the variations in relative proportions of Cx43 and Cx50 and variations in their colocalization in different cells. In some areas, cells had an increased proportion of Cx43 ([A], *bottom right corner*); other cells showed an increased proportion of Cx50 punctate and intracellular staining ([A], *upper left corner*). In other areas, cells showed a high degree of colocalization between the two connexins (B) or a more uniform punctate staining with some colocalization between Cx43 and Cx50 (C). (D) Image from a heterozygous Cx50D47A lens epithelium shows decreased colocalization of punctate Cx43 with Cx50 and increased intracellular Cx50 immunostaining. (E) Image from a homozygous Cx50D47A lens epithelium shows an increase in intracellular Cx50 immunostaining and a more diffuse linear plasma membrane staining; overall, punctate Cx43 staining and its colocalization with Cx50 were decreased. *Scale bar*: 25 μ m (A–D) and 23 μ m (E).

FIGURE 6. Levels and immunolocalization of Cx46 in lenses from wild-type and Cx50D47A-expressing mice. (A) Aliquots of lens homogenates from 3.9-month-old mice were subjected to immunoblotting with anti-Cx46 antibodies. (B) Graph represents the densitometric values of the Cx46 bands obtained in three independent experiments expressed as a percentage of the values determined from wild-type littermates. Levels of Cx46 are more severely reduced in homozygous than in heterozygous mice expressing Cx50D47A. (C–E) Photomicrographs show the distribution of immunoreactive Cx46 (green) and nuclei (DAPI, blue) in longitudinal sections of lenses from 1-month-old wild-type (+/+) (C), heterozygous (+/D47A) (D), and homozygous (D47A/D47A) (E) mice. Cx46 has a punctate distribution at appositional membranes in both wild-type and mutant mouse lenses, although its abundance (especially in deeper layers of the lens) is decreased in mutant compared with wild-type mouse lenses. Scale bar: 68 μ m.

DISCUSSION

In this study, we have characterized the consequences of expression of a mutant mouse Cx50, Cx50D47A, which has similar characteristics to a cataract-associated mutant Cx50 in humans, Cx50D47N. The mutations occur at the same position, the mutant proteins do not traffic properly through the protein synthetic/export pathway, and they are nonfunctional. Moreover, the presence of nuclear cataracts in heterozygous mice implies a similar inheritance pattern to that observed in humans (autosomal dominant).

In addition to the presence of cataracts, mice expressing Cx50D47A had smaller lenses than wild-type mice, and lenses of homozygotes were even smaller than those of heterozygotes. Mouse lines carrying three other Cx50 mutants also have

FIGURE 7. Levels and distribution of AQP0 in lenses from wild-type and Cx50D47A-expressing mice. (A) Aliquots of lens homogenates from 3.9-month-old wild-type (+/+) , heterozygous (+/D47A), and homozygous (D47A/D47A) mice were subjected to immunoblotting with anti-AQP0 antibodies. AQP0 levels are dramatically decreased in lenses from homozygous Cx50D47A mice. (B–D) Top panels show photomicrographs illustrating the localization of AQP0 (green) and nuclei (blue) in longitudinal sections from lenses of 1-month-old wild-type (+/+) , heterozygous (+/D47A), and homozygous (D47A/D47A) mice. Bottom panels show confocal images of cross sections from lenses of 1.2-month-old wild-type (+/+) , heterozygous (+/D47A), and homozygous (D47A/D47A) mice. AQP0 localizes in a similar linear distribution in fiber cells in both wild-type and mutant lenses, but the abundance of staining is reduced in lenses from homozygous Cx50D47A mice. In cross sections, AQP0 distributes along both the short and long sides of fiber cells in all three genotypes. Scale bar: 36 μ m for the top panels (B–D), 20 μ m for the bottom panel (B), and 13 μ m for the bottom panels (C, D).

FIGURE 8. Distribution of nuclei and nuclear fragments in lenses from wild-type and Cx50D47A-expressing mice. Photomicrographs show DAPI staining in longitudinal sections of 1.5-month-old lenses from wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) mice. In addition to the normal staining of nuclei in the epithelium and equatorial region, heterozygous mice show some staining in the center of the lens, and homozygous mice show abundant staining from the periphery to the center of the lens. These observations imply that nuclear degradation is impaired. *Scale bar:* 318 μ m.

small lenses. In mice expressing Cx50R205G, heterozygous lenses are slightly smaller than those of wild-type animals, whereas homozygotes have much smaller lenses.¹¹ Heterozygous and homozygous mice expressing Cx50S50P have very small lenses that rupture posteriorly.¹⁰ Lenses from Cx50V64A mice are smaller than those of wild-type animals, but show no major differences in size between heterozygotes and homozygotes.⁶ This pattern of lens size contrasts with that of Cx50-null mice, since only lenses with homozygous (but not heterozygous) deletion of Cx50 are small.^{4,5} These observations imply that heterozygosity for a Cx50 mutant protein is worse than haploinsufficiency.

The alterations of connexin levels that we observed are likely to account for the presence of cataracts in heterozygous Cx50D47A mice (and people with corresponding mutations). Expression of either one or two Cx50D47A alleles led to very severe reductions in the levels of Cx50 (on average, by 90% in heterozygotes and by 99% in homozygotes). Thus, even the heterozygous animals were nearly Cx50-null and would have very reduced gap junction function due to this connexin. Moreover, we observed that levels of the co-expressed lens fiber cell connexin, Cx46, were also substantially reduced (on average, levels decreased 39% in heterozygotes and 63% in homozygotes). Thus, *No2* mice should also have reduced gap junction function due to this connexin. These results contrast with the findings in Cx50-null mice, since heterozygous mice with targeted deletion of Cx50 have only a 50% decrease in Cx50 levels, and both heterozygous and homozygous Cx50 knockout animals have normal (wild-type) levels of Cx46.^{4,5}

In lens fiber cells, the hexameric hemichannels that form gap junction channels contain Cx50 or Cx46 or both connexins. Thus, it is possible that Cx50D47A forms mixed oligomers with wild-type Cx50 and/or Cx46 in lenses of the *No2* mice. We have previously shown that Cx50D47A (as well as the human mutant Cx50D47N) does not traffic properly and localizes in compartments of the secretory pathway when expressed in mammalian cell lines.¹² It is likely that Cx50D47A (or Cx50D47N) is similarly retained within the secretory pathway in the lens, and most of it undergoes degradation during differentiation of epithelial cells into mature fiber cells. Co-oligomerized wild-type connexins would be degraded along with Cx50D47A. This may explain the observed decreases in

total levels of Cx50 and Cx46 and in immunoreactive connexins at appositional membranes in the *No2* mice.

The changes in the distribution of Cx50 in the epithelia from Cx50D47A-expressing lenses (i.e., increased localization of Cx50 immunoreactivity in intracellular compartments and more diffuse linear staining at appositional membranes, especially in homozygous Cx50D47A epithelia) probably reflect a trafficking problem of the mutant protein in epithelial lens cells, similar to that observed after its expression in transfected cells in culture.¹² These results suggest that cytoplasmically-retained Cx50D47A is targeted for degradation in epithelial cells.

The deleterious effects of expression of Cx50D47A were not limited to connexins, but also were manifested in other membrane proteins. Expression of Cx50D47A significantly reduced levels of lens AQP0 in homozygous animals. This contrasts with other models (Cx50 knockout mice and mice with Cx46 knocked into the Cx50 locus), which show no alterations in AQP0 levels.^{26,27} In homozygous *No2* mice, levels of AQP0 were significantly lower than those of heterozygous AQP0-null mice, which develop opacities by 24 weeks of age.²⁸ Thus, the reduction of AQP0 may contribute to cataractogenesis in Cx50D47A mice. In the lens, there is a spatial relationship between AQP0 and connexin channels with the tetragonally arranged AQP0 tetramers surrounded by densely packed nonordered connexin channels, and it has been suggested that this arrangement stabilizes AQP0 at the membrane.²⁹ Destabilization of AQP0 by loss of fiber cell connexins might explain the reductions of AQP0 (at least in homozygous *No2* mice). However, the decrease in AQP0 levels was much less pronounced in heterozygous Cx50D47A-expressing lenses even though Cx50 levels were significantly decreased; it is possible that the remaining Cx50 and Cx46 gap junction channels were sufficient to stabilize AQP0 in these lenses.

One of the most striking defects observed in Cx50D47A-expressing lenses was the presence of many nuclear remnants in the organelle-free zone. While some abnormalities of denucleation have previously been observed in other Cx50 mutant mice, the derangements were much more severe in the Cx50D47A mice. Homozygous Cx50-null mice show delayed denucleation in interior lens fibers, but the cell nuclei are eliminated by 3 weeks of age.⁵ Homozygous Cx50G22R or

FIGURE 9. Levels of H3 histone, DNase activity, and ER and mitochondrial markers in wild-type and mutant mouse lenses. (A) Immunoblot showing levels of H3 histone in lens homogenates from 1-month-old wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) mice. Levels of H3 histone are dramatically increased in Cx50D47A-expressing lenses; they represent 549% in heterozygotes and 1052% in homozygotes compared with the values in wild-type littermates. (B) Photograph of a 1% agarose gel showing the degradation of linearized plasmid DNA after incubation with lens homogenates from wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) 1.6-month-old mice. The second lane (Time 0) contains unincubated, linearized plasmid DNA; its intensity appeared identical to the DNA that was incubated in the absence of lens homogenate (not shown). A 1 kb DNA ladder was electrophoresed in the first lane, and the sizes of some of its bands are indicated on the left. Because the amount of degraded DNA appeared similar in all three experimental samples, we infer that the impaired denucleation observed in these lenses is not due to lack of DNase II activity. (C) Immunoblots show levels of PDI and Tom20 in lens homogenates from 1-month-old wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) mice. Levels of PDI were slightly increased, whereas levels of Tom20 were significantly increased in lenses expressing the Cx50D47A mutant compared with lenses from wild-type animals.

Cx50S50P mice retain nuclei in some deeper layers than normal.³⁰ In contrast, in our study, nuclear remnants were present in all fiber cell layers of homozygous *No2* animals and in mature fiber cells of heterozygous *No2* animals. Moreover, they were not eliminated with age.

The denucleation deficiency in fiber cells from the homozygous *No2* lenses appears comparable with that seen in mice with targeted deletion of DNase II β , the major acid DNase in the mouse lens that is responsible for the degradation

FIGURE 10. Levels of crystallins in wild-type and mutant lenses. Representative immunoblots illustrate levels of α A-, α B-, β - and γ -crystallins in lens homogenates from 1-month-old wild-type (+/+), heterozygous (+/D47A), and homozygous (D47A/D47A) mice. While levels of α A-crystallins did not vary consistently, levels of α B-crystallins were increased in homozygotes. Levels of β -crystallins were decreased in homozygotes, and levels of γ -crystallins were decreased in both heterozygous and homozygous animals.

of nuclear DNA during lens cell differentiation.²⁵ However, we did not find evidence for a decrease in acid DNase activity in homogenates of Cx50D47A-expressing lenses. Therefore, our results imply that the impaired denucleation observed in *No2* lenses is not due to absence of DNase II β activity.

Our data indicate that the fiber cell differentiation program does not go to completion in *No2* lenses. Degradation of other organelles including mitochondria was clearly impaired, while that of ER was minimally affected. Impaired differentiation is also suggested by the reduction in levels of β - and γ -crystallins, which are normally much more abundant in fiber cells.³¹ These results imply that connexin functions are critical for proper lens cell differentiation, likely by allowing intercellular diffusion of signaling molecules among communicating cells. Indeed, since fibroblast growth factor receptor signaling is required for multiple aspects of fiber cell differentiation including expression of γ -crystallins,³² it is possible that expression of Cx50D47A impaired intercellular diffusion of second messengers generated through this pathway. Incomplete fiber cell differentiation might also decrease synthesis of other proteins that are predominantly present in lens fiber cells, including Cx46 and AQP0. The elevated levels of α B-crystallins in homozygous mutant lenses may be explained differently. Since increases in this crystallin subfamily can result from various stresses³³ in lens epithelial and other cell types,^{34–36} the increase in α B-crystallins may be part of a cellular response to expression of the Cx50D47A mutant.

In summary, we have identified several defects in animals expressing Cx50D47A that likely contribute to cataractogenesis. (1) The levels of both lens fiber connexins are reduced so drastically that they would severely decrease gap junctional intercellular communication and cause cataract formation even in heterozygous animals, (2) the decrease in AQP0 would also be sufficient to cause cataracts especially in homozygous animals, and (3) the retained mitochondria and nuclear remnants would act as light scattering particles and may contribute to the pulverulent appearance of the cataract.

Taken together, these results demonstrate that expression of one allele of a mutant Cx50 associated with autosomal dominant cataract such as Cx50D47A (or Cx50D47N) is more deleterious for the lens than deletion of a single lens connexin allele.

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