MECHANISM OF SMALL HEAT SHOCK PROTEIN FUNCTION IN VIVO: A KNOCKIN MOUSE MODEL DEMONSTRATES THAT THE R49C MUTATION IN αA-CRYSTALLIN ENHANCES PROTEIN INSOLUBILITY AND CELL DEATH

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αA-crystallin (cryaa/HSPB4) is a small heat shock protein and molecular chaperone that prevents non-specific aggregation of denaturing proteins. Several point mutations in αA-crystallin gene cause congenital human cataracts by unknown mechanisms. We took a novel approach to investigate the molecular mechanism of cataract formation in vivo by creating gene knockin mice expressing the arginine 49 to cysteine mutation (R49C) in αA-crystallin (αA-R49C). This mutation has been linked with autosomal dominant hereditary cataracts in a four generation Caucasian family. Homologous recombination in embryonic stem cells was performed using a plasmid containing the C to T transition in exon 1 of the cryaa gene. αA-R49C heterozygosity led to early cataracts characterized by nuclear opacities. Unexpectedly, αA-R49C homozygosity led to small eye phenotype and severe cataracts at birth. Wild type littermates did not show these abnormalities. Lens fiber cells of αA-R49C homozygous mice displayed an increase in cell death by apoptosis mediated by a five-fold decrease in phosphorylated Bad, an anti-apoptotic protein, but an increase in Bcl-2 expression. However, proliferation measured by in vivo BrdU labeling did not decline. The αA-R49C heterozygous and homozygous knockin lenses demonstrated an increase in insoluble αA-crystallin and αB-crystallin and a surprising increase in expression of cytoplasmic γ-crystallin, while no changes in β-crystallin were observed. Co-immunoprecipitation analysis showed increased interaction between αA-crystallin and lens substrate proteins in the heterozygous knockin lenses. To our knowledge this is the first knockin mouse model for a crystallin mutation causing hereditary human cataract and establishes that αA-R49C promotes protein insolubility and cell death in vivo.

INTRODUCTION

αA-crystallin is a member of the small heat shock protein family which comprises 10 proteins in humans characterized by a conserved α-crystallin domain of ~90 amino acid in their carboxy-terminal region (1). Point mutations in small heat shock protein genes are associated with pathological conditions.
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such as cataracts and desmin-related myopathy (2-8). Mechanisms leading to these pathologies are currently under intensive investigation. The R116C and the R49C mutations in αA-crystallin cause hereditary cataracts (2,3). However, in vivo models that recapitulate the heterozygosity of human patients have not yet been developed.

The eye lens is an ideal model system to study small heat shock protein function because it is a simple cellular system with only two cell types- an anterior layer of cuboidal lens epithelial cells covering layers of uniquely elongated differentiated lens fiber cells. The development of the eye lens is a highly coordinated process involving intricate control of cell cycle regulation and differentiation (9,10). During differentiation, lens fiber cells express a high abundance of crystallins, cytoplasmic proteins that are divided into two major families in vertebrate lenses, α-crystallins and βγ-crystallins. Essential for lens transparency, α-crystallin is a large multimeric complex with an average aggregate molecular mass of ~500 kDa, and is obtained as a complex of αA-crystallin and αB-crystallin when isolated from lens fiber cells (11,12). αA-crystallin constitutes nearly 20% of the soluble protein in newborn human lenses and acts as a molecular chaperone (13,14). Gene knockout mice have provided significant knowledge about αA-crystallin functions and demonstrated that besides its role in refraction, it is an active polypeptide which has anti-apoptotic properties important for maintaining the survival of lens epithelial cells in vivo (15-17).

Among each of the autosomal dominant mutations that have been linked with hereditary cataracts, only the R49C mutation in αA-crystallin has been found to lie outside the conserved C-terminal α-crystallin domain common to all small heat shock proteins (3). This mutation was linked with hereditary cataract in a four-generation Caucasian family. The C to T transition in codon 49 of exon 1 in the gene encoding αA-crystallin results in the non-conservative substitution of arginine 49 to cysteine (R49C). No phenotypic and molecular characteristics of the cataract are available. The positive charge on arginine 49 in αA-crystallin has been highly conserved during evolution, and is essential for the in vitro chaperone activity of αA-crystallin (18,19). Transfection studies of the αA-R49C protein in lens epithelial cell cultures have shown a 15-fold increase in basal cell death suggesting a gain of function phenotype of the mutant protein (3). Staurosporine-induced levels of cell death increased 8-fold in the αA-R49C transfectants compared to wild type αA-crystallin. While much can be learned from studying the mutant protein in vitro or in transgenic models, these studies have a number of limitations (3,8,20-23). Because how mutations affect protein interactions at low concentrations may have little relevance to how they associate in vivo at higher concentrations in the lens, an optimal design of a model must investigate the effect of the mutation in vivo. Point-mutation gene-knockin mice are a powerful tool for dissecting gene function. Point mutation gene knockin mice are those animals that have a point mutation in an endogenous gene that does not ablate the gene, but merely changes its function (24). Gene knockin mice have been used with great success to genetically dissect the role of specific genes, for example, the role played by specific lens connexin genes has been convincingly delineated with knockin mice (25,26), and have a number of advantages. First, it is possible to analyze the effect of the mutation in every cell. Second, it allows a comparison of the wild type with heterozygous and homozygous animals and study gene dosing effects. Finally, if successful, generation of such a mouse model would represent the first knockin mutation in vivo for small heat shock protein associated with inherited clinical pathology.
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In the current work, we created the first gene-knockin mice to investigate the mechanism by which the R49C mutation in αA-crystallin produces cataract in vivo. We demonstrate that αA-R49C expression enhances protein insolubility and lens cell death in vivo leading to a small eye, small lens and severe cataracts. Assessment of cell survival and signaling proteins suggests a link between in vivo cell death and dephosphorylation of the protein Bad, a central molecule involved in cell survival. Furthermore, the αA-R49C heterozygosity increased the interaction of key lens substrate proteins with the chaperone suggesting a higher level of unstable proteins in the mutant lenses. Together, these results extend our understanding of how αA-crystallin functions in vivo and they support the idea that αA-crystallin is an active polypeptide critical to the development of a transparent lens phenotype.

EXPERIMENTAL PROCEDURES

Generation of knockin mice- Knock-in mice were generated by removing the normal gene from one allele by homologous recombination in 129SvJ male embryonic stem (ES) cells (SCC-10) to modify the αA-crystallin (cryaa) gene such that exon 1 contained the R49C mutation, while the second copy of the gene was wild type. Mouse genomic DNA clone derived from a 129Sv strain containing αA-crystallin gene was generously provided by Dr. Eric Wawrousek. The 2.9 kb 5’ arm was cloned into a cloning plasmid with the neomycin cassette. Exon 1 was mutated from C to T by site-directed mutagenesis (QuickChange kit, Stratagene). Next, the 4.8 kb 3’ arm of the cryaa gene was cloned into the plasmid (Figure 1). The plasmid was electroporated into ES cells and ES cell selection, colony picking, freezing, expansions, and cryopreservation of homologous recombinant clones expressing the R49C mutant αA-crystallin gene were performed at the Washington University ES Cell Core facility. Clones positive for neomycin were selected with G418, and 150 ES cell colonies were screened for correct gene targeting by Southern blot analysis. Four clones were found to be correctly targeted. The results presented here are from clone 85 which was positive for the mutation. The C to T mutation was verified by sequencing genomic DNA of the positive ES clones (Figure 1B). Correct insertion of the knockin allele was tested by probing 5’ and 3’ ends of the cryaa gene in the plasmid construct, and with primers outside the cryaa gene. The neomycin-positive ES clones were analyzed by Southern blotting. ES cells positive for the mutation were karyotyped to check for normal chromosomes, and injected into C57BL6 blastocytes and implanted into pseudopregnant ICR females (27). Chimeric founders were mated with wild type C57BL6 mice and their progeny that genotyped positive for germ line transmission were bred. First generation offspring that inherited the targeted allele with neomycin were subsequently back crossed into C57BL/6J. The knockin mice with the neomycin cassette were identified by PCR based genotyping described in Figure 1A (PCR product of 224 bp with pcr3 and pcr4 primers). Next, the heterozygous knockin mice were bred with Cre EIIa transgenic mice in C57BL/6J genetic background to specifically delete the neomycin cassette (28,29). All mice were further genotyped by Southern blot analysis of XhoI digested DNA and hybridization with a 5’ end probe. Heterozygous offspring within each mating scheme were subsequently bred to yield homozygous mice. PCR genotypes of heterozygous and homozygous knockin mice after deletion of the neomycin cassette are shown in Figure 1D. Two independent lines of mice, R49CKI3 and R49CKI4, expressing the mutation in cryaa were bred. Because we started with a 129Sv mouse clone which is
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known to express a deletion mutation in CP49, a lens fiber cell specific protein, we backcrossed our αA-R49C knockin mice with C57BL6 mice. These mice were further genotyped to exclude the presence of a deletion mutation in the gene for lens phakinin (CP49), which is characteristic of the 129 strain from which ES cells were derived. This analysis was done to verify that the cataract was not the result of the absence of wild type CP49 (30). Only those mice that were wild type for CP49 expression were analyzed. No mutant CP49 gene was detected in these mice (Supplemental files, Figure S1).

Mice were maintained at Washington University by the Division of Comparative Medicine, by trained veterinary staff. All protocols and animal procedures were approved by the Washington University Animal Studies Committee.

Genotyping- Genomic DNA was prepared from tail biopsy using the DNeasy spin column kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions and was quantified by absorbance at 260 nm. Mice were genotyped by PCR amplification (50 µl) of tail DNA (1 µg). To identify the knockin construct containing neomycin cassette, the following primers were used: pcr3 (Cryaa Genotype 30mer forward primer): TCCTGTCTTCCACCATCAGCCCCTACTAC; pcr4 (Cryaa Genotype 30mer reverse primer): ACATTTCCCCGA AAAGTGCCACCTAAGCTT. This primer set generates a 224 bp spanning the 5' end of exon 1 of cryaa into the neomycin cassette. To detect mice homozygous for the knockin construct the primers were: pcr1 (5’ forward primer) TGGACGTCACCATTCAGCATCCTTGGT and pcr2 (3’ reverse primer) AAGCAGCTTGCCTCGCCATGTAATC. The expected size of the PCR product was 550 bp (no neomycin cassette) and 2.5 kb (with neomycin cassette). Primers for detecting Cre-recombinase were: 5’ forward primer GCATTACCCTGATGCAACCGAG TGATGAG; 3’ reverse primer GAGTGAAACGAACCTGCGAAAAATCGAT GCG with an expected 408 bp PCR product. The lox P site was 34 bp long and two 8 bp multi cloning sites were present on either side, giving a 50 bp longer PCR product for the knockin mice than the wild type mice after breeding with Cre recombinase expressing transgenic mice. The wild type allele in heterozygous mice without the neomycin cassette was identified using genomic DNA and primer set pcr1 and pcr2 as a PCR product of 500 bp. DNA isolated from homozygous mice with the neomycin cassette deleted produced a 550 bp PCR product (Figure 1D). Lack of the wild type cryaa allele in the homozygous mice was confirmed by absence of the 500 bp PCR product (Figure 1D). PCR products (5 µl) were separated on 2 % agarose gels (Bio-Rad) containing 0.05% ethidium bromide and visualized (302 nm) with a gel documentation system (AlphaImager 3400; Alpha Innotech).

Preparation of peptides from wild type and αA-R49C mutant lens epithelial and cortical fractions for mass spectrometric analysis. Lens epithelial and cortical fiber cells were freshly dissected from wild type and αA-R49C knockin mutant lenses, and protein samples in 20 µl of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 7.4) were precipitated using the 2D clean-up kit (GE HealthSciences, Piscataway, NJ). The pellets were dissolved in 10 µl of 0.1 M Tris-HCl, pH 8.5 containing 8 M urea. The proteins were reduced in 5 mM TCEP by the addition of 2 µl of a 50 mM solution in water. After 30 min, iodoacetamide (1 µl) was added to a final concentration of 10 mM. After alkylation (30 min, in dark), the sample was digested with endoprotease LysC for 18 h at 37°C. The sample was then diluted 8-fold and digestion was continued with GluC (0.1 µg) overnight at 37°C, followed by trypsin digestion for the same duration. The combined
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digest was diluted with an equal volume of water and the peptides were extracted with a
NuTip porous graphite carbon tip (Glygen Part No. NP2CAR.96). The tips were
activated with 60% acetonitrile/0.1% formic acid by drawing up 5 µl and expelling to
waste five times. The tips were equilibrated with 0.1% formic acid by ten sequential
pipetting cycles (drawing and expelling) to waste. The digest was slowly drawn into the
conditioned tip and expelled for 50 cycles. The tip was then placed into a microfuge tube
containing 2 times the digest volume and washed for four cycles. This step was repeated
with another clean microfuge tube of wash solution (0.1% formic acid). The peptides
were then eluted with two sequential 10 µl aliquots of 60% acetonitrile/0.1% formic acid.
The samples were evaporated to near dryness in a Speed-Vac centrifuge. The samples were
then transferred to low-bind autosampler vials and the tubes were rinsed with 10 µl of 1%
formic acid/1% acetonitrile for nanoLC-

NanoLC-FTMS Analysis –Mass spectrometry was performed using a linear quadrupole ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ-
FTMS, Thermoelectron, San Jose, CA) as described previously (31). The nanoliquid chromatograph (Eksigent nanoLC, Eksigent, Livermore, CA) was interfaced to the LTQ-
FTMS with a Pico-View nanocapillary source from New Objective (Woburn, MA). Sample
injection was performed with an autosampler (Endurance, Spark, Plainsboro, NJ). The
column was a C-18 PicoFrit (75 µm X 10 cm) (New Objective, Woburn, MA). The mobile
phases were HPLC grade water (Fisher Scientific, Pittsburgh, PA) containing 1%
formic acid (Sigma-Aldrich, St. Louis, MO) (Solvent A) and acetonitrile (Honeywell,
Burdict & Jackson, Muskegon, MI) containing 1% formic acid (Solvent B). The sample (5 µL)
was loaded at 600 nL/min at 1% B for 10 min. The flow was then
decreased to 200 nL/min with isocratic elutions for 15 min that was followed by a
linear increase in Solvent B (0.3%/min) for 30 min. The LTQ FT (7 Tesla) mass
spectrometer was operated in the data dependent mode. The survey scans (m/z =
350 – 2000) were acquired using FTICR with a resolution of ~100,000 at m/z = 421.75 with
a target value of ~500,000. The ten most intense ions from survey scans were isolated
in the ion trap and analyzed after reaching a target value of ~10,000. The MS/MS isolation
width was 2.5 Da and the normalized collision energy was 35%. Electrospray ionization was
accomplished with a spray voltage of 2.2 kV without sheath gas. The ion transfer tube
temperature was 200°C.

MS Data Analysis– The MS and MS/MS data from the nanoLC-FTMSMS
were collected in the profile mode. The “raw” files were processed using MASCOT Distiller
(Matrix Science, Oxford, U.K.) with the following settings: 1) MS processing: 200
data points per Da; no aggregation method; maximum charge state = +8; minimum
number of peaks = 1. 2) MS/MS processing: 200 data points per Da; time domain
aggregation method enabled; minimum number of peaks = 10; precursor charge and
m/z, try to re-determine from the survey scan (tolerance = 2.5 Da); charge defaults = +2/+3;
maximum charge state = +2. 3) Time domain parameters: minimum precursor mass = 700;
maximum precursor mass =16,000; precursor m/z tolerance for grouping =0.1; maximum
number of intermediate scans = 5; minimum number of scans in a group = 1. 4) Peak
Picking: maximum iterations = 500; correlation threshold = 0.90; minimum signal-
to-noise = 3; minimum peak m/z = 50; maximum peak m/z = 100,000; minimum peak
width = 0.001; maximum peak width = 2; and expected peak width = 0.01. The resulting
Mascot generic files (.mgf) were exported to MASCOT, version 2.1.6. The tandem MS data
were searched against the NCBI protein

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database with the following constraints: MS tolerance = 10 ppm, MS/MS tolerance = 0.8 Da with fixed modifications of cysteins (carbamidomethylation) and methionine (oxidation) residues.

Slit lamp examination and recording-
Slit lamp biomicroscopy was used on non-anesthetized mice in a masked fashion. Pupils were dilated with a mixture of 10% phenylephrine hydrochloride and 1% tropicamide (Alcon, Fort Worth, Texas). After 3 minutes, the animal was placed directly facing the slit lamp, by holding the mouse gently by the scruff of the neck. The left eye of the animals was examined. The knockin mice were examined at postnatal ages between 3 weeks (eye opening) and 36 weeks. To confirm lens opacity in newborn mice, newborn mouse pups were sacrificed and examined by slit lamp biomicroscopy.

Assessment of lens opacity- Cataract formation was scored by slit lamp biomicroscopy according to a modified LOCS III method. Stage 0: Clear lens; Stage 1: Loss of normal appearance of anterior and posterior lens and prominence of y-suture line. Changes appear by 3 weeks in heterozygous lenses. Stage 2: Discrete posterior changes accompanied by light nuclear opacity. Changes evident at 4 months in heterozygous lenses. Stage 3: Nearly mature cataract, involving approximately three-fourths of the lens with vacuoles and opacity. Changes evident by 8 months in heterozygous and 3 weeks in homozygous lenses. Stage 4: Completely mature cataract involving the cortex with vacuoles, evident in heterozygous lenses not before 1 year and in homozygous lenses at 2 months.

Assessment of proliferation- In vivo labeling with 5-bromo-2’-deoxyuridine (BrdU) was performed as described previously (17). Mice were injected with BrdU intraperitoneally (0.1 ml of a 10 mM solution of BrdU in sterile PBS) at 10 AM and were sacrificed 1 hour later. Eyes were dissected and fixed, and mid-sagittal sections were labeled with a primary antibody to BrdU and a horseradish peroxidase-conjugated secondary antibody. Labeled nuclei were detected with an Olympus microscope. The total number of nuclei were measured by haematoxylin staining of the sections. The labeling index was determined by the ratio of the BrdU positive nuclei and the total number of nuclei per section. For each sample, 3 sections per lens were analyzed, and labeling index was determined for 6 lenses per genotype.

Assessment of MIP (AQP0) immunofluorescence- Lenses were embedded in paraffin and 4 µ sections were stained with a polyclonal antibody to MIP (Alpha Diagnostics International, and an Alexa-568-conjugated secondary antibody, and visualized by confocal microscopy in a Zeiss 510 confocal microscope.

Assessment of lens protein expression- Whole lenses were homogenized in PBS containing protease inhibitor cocktail (Sigma) and centrifuged at 15,000 g to separate soluble and insoluble fractions (20,32). The protein concentration was measured by the Pierce BCA assay, and equal protein (15 µg) was loaded on the gel, unless otherwise noted. SDS-PAGE and immunoblot analysis was performed using previously described antibodies to αA-crystallin, αB-crystallin, total β-crystallin and total γ-crystallin (16,20,33). MIP expression was detected using a polyclonal antibody to MIP (17) (Alpha Diagnostics International). Densitometric analysis was performed with a Storm 860 system (Molecular Dynamics).

Assessment of disulfide crosslinking. Whole lenses were homogenized in PBS without 10 mM dithiothreitol (DTT) and lens proteins were separated into soluble and insoluble fractions by centrifugation as described above. SDS-PAGE and immunoblot analysis with antibodies to αA-crystallin and γ-crystallin was performed.
Assessment of αA-crystallin interaction with lens proteins by immunoprecipitation – A co-immunoprecipitation assay was used to investigate proteins interacting with αA-crystallin in wild type lenses and to investigate the effect of the αA-R49C mutation on the interaction of αA-crystallin with lens proteins. Lenses were homogenized in PME buffer (80 mM PIPES, 1 mM MgCl₂ and 1 mM EGTA, pH 6.8) and centrifuged for 30 min at 10,000 g. Supernatants were lysed for 30 min on ice with immune precipitation buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and protease inhibitor mixture (Sigma) and centrifuged for 10 min at 10,000 × g. Supernatants were treated with a primary antibody to αA-crystallin and immunoprecipitated with Protein A/G-agarose beads (Santa Cruz Biotechnologies). A monoclonal antibody to αA-crystallin (1:20) was used. Immunoprecipitates were washed three times with a lysis buffer containing 20 mM Tris-HCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, and protease inhibitor mixture (Sigma), resuspended in SDS-PAGE sample buffer, and analyzed on 15% acrylamide gels as described previously (21). Immunoblot analysis with antibodies to αB-crystallin, β-crystallin, γ-crystallin, and β-tubulin was performed.

Assessment of cell survival and signaling protein expression – Freshly dissected lens epithelial and cortical fiber cells were analyzed by immunoblot analysis. Samples were extracted in 44.4 mM n-octyl β-D-glucopyranoside, 1% Triton X-100, 100 mM NaCl, 1 mM MgCl₂, 5 mM EDTA, and 10 mM imidazole containing 1 mM sodium vanadate, 0.2 mM H₂O₂ and protease inhibitor cocktail (Sigma). Antibodies against Bel-2 (Santa Cruz Biotechnology, Inc.), phospho-Bad Ser (112) and phospho-Bad Ser (136) (Biosource, Camarillo, CA 93012), Akt and p-Akt (Cell signaling Technology, Inc. Danvers, MA), p38 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and phospho-38 ERK1/2 and phosphorylated ERK1/2 (Promega, Madison, WI) were used for immunoblot analysis. Equal amounts of total cellular protein (15 µg) were separated on Tris-glycine gels (Novex, San Diego, CA), electrophoretically transferred to a membrane (Immobilon-P; Millipore Corp., Bedford, MA) and immunoblotted as described previously (34). All gels were run under reducing conditions. Densitometric analysis was performed using Kodak 1D software (Eastman Kodak Company, Rochester, NY).

Real time quantitative reverse-transcriptase PCR – Total RNA from wild type and αA-R49C knockin mouse lens cortical fractions was isolated according to manufacturer’s protocol (Qiagen) and treated with DNase I. cDNA was prepared using a kit from Invitrogen. Q-RT-PCR assays of RNA isolated from lenses were performed in 50 µl reactions containing 1X SYBR Green Supermix (BioRad) and 200 nM gene-specific primers for mouse γB, γC and γD-crystallin genes shown in supplemental Table S1. Assays were performed in triplicate using an I-Cycler (BioRad) and three independent experiments were performed. Primers were designed and synthesized by Integrated DNA Technologies. To optimize the primers, reverse-transcriptase polymerase chain reaction (RT-PCR) was performed and products were run on 1.5% agarose gels to ascertain that a single band of the correct size was obtained. For comparison between wild type and knockin samples, a standard curve of cycle thresholds for several serial dilutions of RNA sample was established and then used to calculate the relative abundance levels of messenger RNA (mRNA). The expression level of each γ-crystallin mRNA was determined relative to GAPDH of the same sample (35,36).

TUNEL staining – TUNEL labeling was used to examine cell death in lens epithelial
wholemounts from wild-type and αA-R49C mouse lenses. Paraformaldehyde sections were fixed in 4% para-formaldehyde, pH 7.4 for 30 minutes, permeabilized for 1 min in 0.1% Triton X-100/PBS at room temperature, and apoptotic nuclei were detected using a horseradish peroxidase-conjugated TUNEL labeling mix according to the manufacturer’s instructions (Roche Biochemicals). TUNEL staining was visualized in an Olympus microscope. The number of TUNEL-positive cells was counted in lens epithelial and fiber cells for each of 4 sections per lens. Statistical analysis was performed using the t-test. Lens epithelial wholemounts were stained with fluorescein-conjugated TUNEL reagents as described previously, and TUNEL stained nuclei visualized in a confocal microscope (16,17).

RESULTS

Generation of αA-R49C knockin mice - A cloning vector containing a point mutation in αA-crystallin exon 1 was made (Figure 1A) and electroporated into 129Sv ES cells, and neomycin-resistant clones were isolated. The targeting vector contained an XhoI restriction site. The mutation in exon 1 of the cryaa gene changed the codon for arginine 49 (CGC) to cysteine (TGC). The DNA from ES clones was sequenced and the C to T mutation in exon 1 of mouse cryaa gene was verified (Figure 1B). Four positive ES clones were analyzed (Figure 1C). They showed a distinct 14.5 kb band by Southern blot analysis, representing the correctly targeted ES clone. The wild type lane shows a wild type ES clone with no insertion. Additional Southern blots for the 5’ and 3’ ends were performed to verify the correct insertion, and restriction digest analysis was used with an internal control to verify a single insertion of the plasmid (data not shown). We next injected ES clonal cells positive for the mutation into C57BL6 blastocysts and implanted them into pseudopregnant ICR female mice. Pups born 17 days after blastocyst implantation were screened by coat color (agouti -129, and black - C57BL6). The percent agouti color indicated how much of the ES cells contributed to the developing embryo. Next we mated the progeny with C57BL6 mice and selected pups with brown coat color indicating germline transmission. We tested all progeny for the mutation to show that they carried the mutant allele. PCR screening was used to genotype the mice. The neomycin cassette was deleted by breeding αA-R49C homozygous knockin mice with Cre-EIIa expressing transgenic mice. PCR genotyping was used to identify wild type, homozygous and heterozygous mice (Figure 1D).

Next it was of interest to establish that the mutant lenses produced the R49C mutant αA-crystallin by LC-MS/MS analysis. Figure 2 shows the MS and fragmentation spectra of the predicted peptide from a combined endoprotease digest (LysC + GluC + trypsin) of the wild type αA-crystallin. The sequence of the doubly-charged precursor ion (inset) at m/z = 828.9537 is confirmed by the series of y and b fragment ions (Panel A). The expected peptide from the αA-R49C knockin lens was observed as a doubly-charged ion at m/z = 1146.591. The sequence was deduced from the the series of y and b fragment ions (Panel B). The signal from the mutant peptide (m/z = 1146.591) was only observed in the mutant lenses (± 2 ppm). The expected endoprotease digested peptide with m/z of 828.9537 was detected in both wild type and heterozygous knockin lenses. However, the peptide with mass 1146.591 was only detected in the mutant lenses. The mutant protein loses a trypsin cleavage site as a result of the arginine 49 to cysteine mutation in αA-crystallin (Panel C). Instead of the wild type peptide sequence LLPFLSSTISPYYR, the sequence LLPFLSSTISPYYCQSLFR is created. We identified both mutant and wild type peptides by their accurate masses as well as unique sequence (Figure 2), thus proving that the αA-
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R49C mutant protein was indeed being expressed in the knockin mutant lenses in vivo.

*Phenotypic and molecular changes in αA-R49C knockin mice-* The αA-R49C knockin mice were viable and fertile. Mice were backcrossed into C57BL6 background by breeding. At all ages, cataract severity was the least in wild type. Homozygotes showed a rapid onset that stabilized between 8-12 weeks (Figure 3). Lens opacities were less severe in heterozygous lenses, and a clear dose-response was observed. Stage 3 and 4 lens opacities were observed in 5 and 16 week old αA-R49C homozygous mice, respectively (Table 1). Interestingly, in the lenses with stage 3 opacities, the lens nucleus was opaque, but the outer cortical fibers were relatively clear. An unexpected phenotype in mouse eyes homozygous for the αA-R49C mutation was severe micro-ophthalmia. αA-R49C homozygous eyes weighed 60-70% less than wild type and heterozygous littermates, which was mainly due to the decrease in lens weight (Figure 4).

*Mechanism of αA-R49C effects-* Measurement of BrdU labeling index in vivo showed a comparable level of cell proliferation in wild type, αA-R49C heterozygous and homozygous lenses (Figure 5). However, cell death measured by TUNEL staining in the homozygous lens epithelium (Figure 5 and 6) and lens fiber cells (Figure 5E and F) increased suggesting that the cell death is due to apoptosis in the αA-R49C homozygous lenses. TUNEL-positive cells increased 6-fold in the fiber cells (Wild type vs. αA-R49C homozygous lenses, n= 6 p< 0.01). These data suggest that the smaller αA-R49C homozygous lens is due to a decrease in the number of lens epithelial cells and hence in the number of lens fiber cells. In order to investigate the mechanism by which cell death was induced in the lens epithelial and fiber cell populations of the homozygous αA-R49C mice we examined whether the presence of the αA-R49C induced changes in expression or activation of molecules involved in cell death and cell survival (Figure 7). Overall, the signaling changes in the fiber zone were much more pronounced than in lens epithelial cells. In the lens epithelial cells there was no significant change in the activation of ERK or the expression of Bcl-2, both signals associated with cell survival. Increased activation of Akt and phosphorylation of its target on Bad (Ser 136) also occurred, consistent with activation of cell survival signals. The main cell death-associated signal detected in the epithelium of αA-R49C lenses was the inhibition of phosphorylation of Bad at serine 112. Substantial changes in activation or expression of survival and death inducing signaling molecules in the lens fiber cells of the R49C mice were observed (Figure 7). The phosphorylation of Bad was suppressed at both serine 112 and 136, a potent signal for apoptosis. Quantitative analysis showed that phosphorylation of Bad at serine 112 decreased 3-fold in the lens epithelium of αA-R49C homozygous lenses. Similarly, the phosphorylation of Bad at serine 112 decreased 3.7 and 5.3-fold in the lens fiber cells of αA-R49C heterozygous and homozygous lenses, respectively, and phosphorylation of Bad at serine 136 decreased 2.4-fold in the lens fiber cells of αA-R49C homozygous lenses. In addition, there was increased expression and phosphorylation of the stress signaling molecule p38. Interestingly, concurrent with activation of pathways associated with cell death, signaling pathways associated with cell survival including activation of ERK and Akt and induction of Bcl-2 also were induced in the lens fiber cells. However, activation of these pathways appeared ineffective in preventing induction of apoptosis in the lens fiber zone of the αA-R49C mutants. These results suggest that the αA-R49C mutation in the protein chaperone αA-crystallin alters the expression of key cell survival and apoptosis...
related proteins, suppressing some, while activating others, ultimately resulting in cell death in the homozygous lenses.

Next it was of interest to determine whether the αA-R49C mutant lenses still maintained the molecular markers associated with the lens phenotype. First we investigated the expression of lens cytoplasmic proteins α-, β- and γ-crystallins. Increased amounts of αA-crystallin and αB-crystallin were detected in the lens insoluble fractions, with a greater increase in homozygous than heterozygous lenses (Figure 8). β-crystallin expression remained unchanged in the αA-R49C knockin lenses. In contrast, γ-crystallin was upregulated in αA-R49C heterozygous and homozygous lenses. Real time quantitative RT-PCR with gene-specific primers for γB, γC and γD-crystallin showed that the upregulation of γ-crystallin occurred at the transcriptional level in heterozygous lenses (Table 2). Furthermore, MIP, a membrane protein important for lens function that is normally found exclusively in lens fiber cell membranes was detected at the same level in the lens insoluble fractions (Figure 8). Immunofluorescence analysis with an MIP antibody showed a severe disturbance of fiber cell morphology in αA-R49C homozygous lenses (Figure 9), suggesting that αA-R49C expression affects lens fiber cell membrane organization. Interestingly, in the equatorial region it appears that the fiber cells elongate, but later the morphology of the fiber cells becomes altered, and the fiber cell structure is disrupted.

Wild type αA-crystallin has only one cysteine residue in its amino acid sequence, whereas the αA-R49C has two. The second cysteine residue in the N-terminal region could contribute to protein insolubility and cataract by its potential to crosslink with itself and other proteins. Disulfide crosslinking was assessed by extracting lens proteins under non-reducing conditions. Immunoblot analysis with antibodies to αA-crystallin and γ-crystallin detected 60-80 kDa crosslinked immunoreactive bands in the αA-R49C homozygous lenses under non-reducing conditions, but not in the wild type or heterozygous lenses (supplemental Figure S2).

Since mutations in αA-crystallin are known to affect its chaperone activity and interaction with substrate proteins, co-immunoprecipitation of αA-crystallin and other crystallins was performed with an αA-crystallin monoclonal antibody and immunoblotting with antibodies to αB-crystallin, β-crystallin and γ-crystallin. The association of αB-crystallin, β-crystallin, γ-crystallin and β-tubulin increased two to three-fold in the αA-R49C heterozygous lenses (for example, a 2.3-fold increase for β-crystallin, wild type vs. αA-R49C heterozygous, n= 3, p= 0.02), suggesting that the concentration of unstable proteins increases with the mutation (Figure 10). In contrast, the interaction of these substrate proteins in αA-R49C homozygous lenses decreased or remained unaffected. Taken together, these results indicate that αA-R49C causes multiple effects in vivo by changes in interaction with other crystallins, activating lens fiber cell Bcl-2 expression, dephosphorylation of key cell survival protein phospho-Bad, and suppressing or activating specific MAP kinases important in cell survival and apoptosis, ultimately resulting in protein insolubilization, cell death and a severe loss of lens transparency.

DISCUSSION

We report severe cataracts and small eye phenotype in αA-R49C homozygous lenses and protein insolubility in αA-R49C heterozygous lenses establishing the first knockin mouse model for hereditary human cataracts caused by a crystallin mutation in vivo. The knockin mice contained the exon 1 mutation of arginine 49 to cysteine which is
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linked with hereditary cataracts in a 4-generation Caucasian family (3). αA-crystallin, a small heat shock protein and molecular chaperone, is a major component of lenses constituting 20% of newborn human lens protein. The αA-R49C homozygous mice developed cataracts at birth. The arginine residue at amino acid 49 in αA-crystallin amino acid sequence has been conserved in 56 different species during evolution (19). Investigators have demonstrated that αA-crystallin’s in vitro chaperone activity is abolished by replacement of arginine 49 with alanine (18). The αA-R49C knockin lenses in our study demonstrated a decrease of αA-crystallin and αB-crystallin in the lens water-soluble fraction with a concomitant increase of these proteins in the water-insoluble fraction. Mutation of other conserved arginine residues in αA-crystallin has also been shown to reduce protein solubility in vitro, and structural changes in αA-crystallin have been reported with the R116C mutation (37-39). Replacement of the positive charge on arginine 120 in αB-crystallin, (R120G-αB) causes partial protein unfolding, reducing its stability and promoting its aggregation (40).

Unlike previous studies however, the current work is the first to show the effect of the mutation of an arginine residue in the N-terminal region of αA-crystallin on the protein’s solubility, and further shows that as αA-crystallin becomes insolubilized, so does its aggregation partner in the lens, αB-crystallin. Several mechanisms may account for this effect. First, the change in isoelectric point and addition of a second cysteine residue in αA-R49C may disrupt the normal conformation of the chaperone, increase its potential for disulfide crosslinking and increase the co-precipitation of substrate proteins leading to insolubilization (8,41). An increase in inappropriate disulfide bond formation has also been reported for an arginine to cysteine mutation in γD-crystallin (42). Second, the αA-R49C mutation may increase the dissociation αA-crystallin oligomer into smaller multimers thereby gaining the capacity to bind substrate proteins. Our results demonstrate an increased interaction of αB-crystallin, β-crystallins, γ-crystallins and β-tubulin in αA-R49C heterozygous knockin lenses. Third, the chaperone activity of αA-R49C may decrease, and increase the concentration of destabilized substrate proteins. Studies in the literature suggest that the arginine 49 to serine mutation decreases the in vitro chaperone activity of αA-crystallin (18). While the loss of chaperone function of αA-crystallin may be one factor that contributes to underlying cataract development in the αA-R49C mutant lenses, the observed high interactions of αA-crystallin with other proteins in our heterozygous mutant lenses may be the result of a gain-of-function that increases the interaction of the mutant protein with some proteins. This gain-of-function mechanism is supported by the early onset of the cataract in the heterozygous knockin lenses, since it would take some time for proteins to partially denature in these lenses.

Analysis of interaction of αA-crystallin with αB-crystallin and β-tubulin in αA-R49C heterozygous lenses by co-immunoprecipitation demonstrated that αA-crystallin senses the presence of unstable β-crystallin, γ-crystallin and β-tubulin by binding to these substrate proteins at a higher level. Previous studies have shown that αA-crystallin is essential for maintaining unpolymerized tubulin in a assembly-competent conformation (43). Studies in the literature show that αA-crystallin forms co-aggregates with γ-crystallin (44,45). Our results are consistent with these studies and indicate an increase in the interaction between γ-crystallin and αA-R49C in the heterozygous lenses. Investigators have documented an interaction between the N-terminal domain of
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αB-crystallin with αA-crystallin (46). The current work demonstrated an increase in the interaction between αA-crystallin and αB-crystallin in heterozygous αA-R49C lenses. This observation is somewhat unexpected considering that αB-crystallin is a small heat shock protein and another chaperone, and is meant to survive stress and protect other proteins. Nonetheless, the increased interaction between αB-crystallin and mutant αA-crystallin has also been reported by other investigators using different methodologies (3,47).

It is well established that the expression of β-crystallin and γ-crystallin increases dramatically during lens fiber cell differentiation (48). Although no change in β-crystallin expression was observed in the αA-R49C lenses, γ-crystallin was upregulated as compared with wild type lenses. The mechanism of γ-crystallin upregulation appears to be an increase in gene transcription in the case of heterozygous lenses, suggesting that expression of the αA-R49C mutant protein may reduce the rate of mRNA turnover (49). Our results suggest that further analysis of mechanisms regulating γ-crystallin expression in the αA-R49C knockin lenses may be warranted.

The small eye phenotype that we observed in our knockin mice was due to increased cell death and not reduced proliferation. These results are consistent with our previous finding that gene disruption of αA-crystallin increases lens epithelial cell apoptosis, and that αA-crystallin arginine mutant expression is toxic to lens cells and enhances apoptosis (3,17,21). The small eye phenotype was also reported in hereditary human cataract caused by the R116C mutation in αA-crystallin but its mechanism has not been investigated (2). Although both the autosomal dominant mutations, αA-R49C and αA-R116C, have been shown to enhance apoptosis in lens epithelial cultures, αA-R49C has been found to be more toxic (3,17). Thus, it is plausible that the small eye phenotype in αA-R49C homozygous mice is due to cytotoxicity of the mutation, and the loss of normal anti-apoptotic function of αA-crystallin. αA-crystallin has been shown to act as an anti-apoptotic protein by preventing the activation of caspases (50). Investigators have shown that the lens is important for the proper growth of the anterior segment and the eye (51). The current work demonstrates that a decrease in lens weight led to a corresponding decrease in eye weight of the αA-R49C homozygous mice. Furthermore, since the αA-R49C heterozygous mice did not demonstrate the small eye phenotype, our results suggest that the absence of one wild type αA-crystallin allele in the mouse is not sufficient to cause enhanced cell death in vivo. These results further indicate that expression of αB-crystallin and one normal copy of αA-crystallin are sufficient to inhibit apoptosis in vivo, but insufficient to prevent protein insolubilization and lens opacity. These results are also consistent with the absence of the small lens phenotype in the heterozygous αA-crystallin gene knockout mice (52).

Small heat shock proteins are physiological regulators that have co-evolved as integral components of signal transduction networks (53,54). Our studies have demonstrated an association of αA-crystallin with α6-integrin in membranes of lens fiber cells, consistent with the linkage of this chaperone with integrin survival signaling (55). Changes in abundance and relative levels of chaperones can change intracellular signaling pathways with some pathways becoming favored and others being suppressed or constitutively activated. Examination of the mechanism of cell death in αA-R49C homozygous knockin lenses showed that the pro-survival protein Bcl-2 was strongly upregulated in the cortical fiber cells of these lenses. Bcl-2 has been shown to be upregulated in lens epithelial cells exposed to
oxidative stress, and members of the Bel-2 family have been shown to be sequestered by αA-crystallin (56,57). In order for Bel-2 to have pro-survival functions, it must be released from the protein Bad, a central protein which in its phosphorylated form is necessary for survival of many cells (55,58). In the lens fiber cells of αA-R49C knockin heterozygous and homozygous lenses, both p-Bad (Ser112) and p-Bad (Ser136) were strongly down-regulated. These findings indicate that the mechanism of cell death that we are seeing in the αA-R49C mutant lenses is likely to be the result of dephosphorylation of Bad. Since Bad is phosphorylated by Akt and ERK, the fact that both Akt and ERK were enhanced in αA-R49C lens fiber cells suggests that p-Bad is also being targeted by other kinases. Other kinases that have been implicated in survival signaling have been proposed to mediate Bad phosphorylation, including Rsk, PAK, p70S6K, and PKA (59,60). Moreover, p38 and phospho-p38, known to be important for cell survival or apoptosis depending on the cell type, were enhanced in the αA-R49C knockin lens fiber cells (61,62). Activation of p38 has been shown to lead to induction of cataract (61).

In contrast to its low expression in the wild type lens fiber cells, the pro-survival protein Bel-2 was expressed at high levels in lens epithelial cells, and its levels were maintained in the αA-R49C heterozygous as well as homozygous knockin lenses, however, phosphorylated Bad was expressed at very low levels in lens epithelial cells of each of the genotypes. These data show that although lens cells in the αA-R49C lenses express elevated levels of some survival factors, the cells still die. The results are consistent with our studies demonstrating that numerous cell survival pathways are induced in lens cells undergoing apoptosis (35). Studies in the literature demonstrate that cells continually integrate signals from apoptosis-inducing and apoptosis-suppressing pathways as a means to coordinate their response to stressful conditions (35,63,64). Taken together, our in vivo cell proliferation, TUNEL and cell signaling and survival studies show that the loss of αA-crystallin function, due to expression of αA-R49C mutant protein in vivo is cytotoxic to the lens epithelial and fiber cells.

Investigators have shown that in addition to its more typical cytoplasmic distribution, αA-crystallin is also associated with lens epithelial and fiber cell membranes (65,66). Loss of αA-crystallin function by mutation or gene-deletion may lead loss of normal membrane morphology (17). Cell-cell adhesions are known to stabilize the packing arrangement of lens fibers (67-70). Changes in adhesion complexes occur at the stage between completion of fiber cell elongation and degradation of membrane-bound organelles (71). The dramatic fragility of the αA-R49C homozygous lenses suggests a destabilization of these adhesions. Although lens fiber elongation per se appears to occur in the αA-R49C knockin homozygous lenses, the current work demonstrated dramatic structural alterations in fiber cell morphology, and is consistent with the reported interactions between membranes and αA-crystallin in lens fiber cells (72,73).

In summary, this study clearly establishes the first mouse knockin model for hereditary cataract caused by a point mutation in αA-crystallin. αA-R49C heterozygosity demonstrated protein insolubility and lens opacities that were apparent at an early postnatal age whereas homozygosity exhibited a small eye and lens phenotype in addition to severe cataracts. These data show that the presence of mutant αA-R49C is detrimental to the development and emergence of the transparent phenotype, and convincingly show that αA-crystallin has non-refractive functions modulated by the genetics of lens epithelial and fiber cells (74). The dramatic effects that we are seeing in cell structure as a result of
αA-R49C mutation are the subject of future studies. Using our knockin approach, it would be interesting to determine whether other αA-crystallin mutations will have the same or other effects on the eye.
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Table 1. Cataract severity in wild type, αA-R49C heterozygous and αA-R49C homozygous knockin mice by in vivo slit lamp biomicroscopy*

| Age          | Genotype          | Total Number of Mice | Cataract Stage | p Value |
|--------------|------------------|----------------------|----------------|---------|
| 3-9 weeks    | Wild type        | 12                   | 0.5 ± 0.1      |         |
|              | αA-R49C heterozygous | 15               | 1.5 ± 0.1     | 0.011   |
|              | αA-R49C homozygous | 19               | 3.0 ± 0.0     | <0.001  |
| 16-24 weeks  | Wild type        | 18                   | 0.5 ± 0.2      |         |
|              | αA-R49C heterozygous | 16               | 2.0 ± 0.1     | 0.003   |
|              | αA-R49C homozygous | 14               | 4.0 ± 0.0     | <0.001  |

*Note the progression of lens opacities in the αA-R49C heterozygous and homozygous knockin lenses. The heterozygous knockin lenses showed an opacity greater than the wild type in each age group, but the stage (severity) of the opacity was lower than that of the αA-R49C homozygous knockin mice. In homozygous mice, the lens opacities were at stage 3 even in three week old mice and stabilized at an early age. Wild type lenses showed either mild or no significant abnormality at each age by slit lamp analysis.
Table 2. Quantitative Reverse Transcriptase-Polymerase Chain reaction (qRT-PCR) Analysis of γ-Crystallin Transcripts (Crygb, Crygc and Crygd) in Mouse Lenses

| Relative Intensity (Cryg/GAPDH) | Wild type | αA-R49C | Fold Increase (Wild type/αA-R49C) |
|---------------------------------|-----------|---------|----------------------------------|
| Crygb                           | 2.19 ± 0.30 | 5.35 ± 0.42 | 2.4                             |
| Crygc                           | 3.65 ± 0.27  | 10.23 ± 0.64 | 2.8                             |
| Crygd                           | 0.56 ± 0.28  | 4.55 ± 0.34  | 8.1                             |
Knockin model for αA-Crystallin R49C mutation

FIGURE LEGENDS

Figure 1. Gene targeting strategy and genotype analysis. (A) Diagram illustrating the gene targeting strategy used to produce the αA-R49C gene knockin mice. The numbered rectangles represent the exons. The starred exon 1 represents the mutated exon. Restriction sites relevant to Southern blot analysis are shown along with the size of the restriction fragments. Lox P sites are represented as closed triangles. The 5’ probe that was used for the Southern blot analysis is shown below the wild type and mutated alleles. Neo represents the neomycin cassette. Neomycin was excised by breeding the αA-R49C gene knockin mice with Cre EIIa expressing transgenic mice. Bold arrows indicate the PCR primers (pcr1 and pcr2) used to detect the wild type and mutated alleles. Light arrows indicate PCR genotyping primers (pcr3 and pcr4) to detect the neomycin cassette. (B) Genomic DNA from ES clones was sequenced to verify the C to T mutation in the mouse αA-crystallin gene. (C) Clones 8, 10, 23 and 85 are the ES clones demonstrating insertion of the plasmid (red arrow). WT represents a wild type ES clone with no insertion. The upper band (14.5 kb) represents the correctly targeted ES clones. Native αA-crystallin gene (12.5 kb) was present in each positive clone, and the appropriate knockin was the ES clones with the insertion. (D) PCR screening of genomic tail DNA confirmed recombination in mice. At the 5’ end a sense flanking primer (pcr1) was paired with an antisense αA-crystallin gene intronic primer (pcr2). Primers amplified a 500 bp band from wild type αA-crystallin chromosomes, whereas they amplified a 550 bp band from neomycin-deleted knockin chromosomes. Heterozygous mice amplified both the 500 and 550 bp bands. Absence of the 500 bp band and detection of only the 550 bp band showed homozygosity for the αA-R49C mutation.

Figure 2. NanoLC-FTMS analysis of a combined LysC, GluC and tryptic digest of wild type and αA-R49C knockin mouse lenses. (A) Tandem MS spectrum of target peptide from wild type lens fiber cells. Wild type lenses showed the expected peptide LLPFLSSTISPYYR, with m/z value of 828.9537. The inset shows the MS spectrum with the expected signal at m/z = 828.9537 for the [M + 2H]^2 ion. (B) Tandem MS spectrum of target peptide from αA-R49C knockin lenses. Heterozygous knockin lenses showed a new peptide LLPFLSSTISPYYCQSLFR with an expected mass of 1146.591 due to loss of the arginine residue at amino acid 49 in the αA-crystallin sequence and its replacement by a cysteine. The inset shows the MS spectrum with the expected signal at m/z = 1146.591 for the [M + 2H]^2 ion. Note that in addition to accurate mass measurements LC-MS/MS experiments also verified the individual peptide sequences, thus confirming the expression of the mutant protein in the knockin lenses. The heterozygous mouse lenses also expressed the wild type peptide. The same mass spectra results were also obtained for proteins extracted from lens epithelial cells of wild type and αA-R49C mutant mice. The observed peptide masses were ± 2 ppm of the expected masses. (C) Amino acid sequences of wild type αA-crystallin and αA-R49C mutant proteins. The underlined region represents the α-crystallin domain of small heat shock proteins. The left arrow indicates the position at which LysC cleaves the proteins. The second arrow in the αA-R49C protein sequence indicates the R49C mutation that lies outside the conserved α-crystallin domain. The short bold arrow indicates the tryptic cleavage site in the αA-R49C mutant protein sequence.
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Figure 3. Eye and lens phenotypes of αA-R49C knockin mice. Eyes were dilated and examined by slit lamp in non-anesthetized mice. The dotted line shows the outline of the eye. (A-C) 5-week old mice. (A) Wild type mice at age 5 weeks had clear lenses. (B) Heterozygous αA-R49C knockin mice at 5 weeks displayed low level opacities in the nuclear and posterior regions of the lens. (C) Homozygous αA-R49C knockin mice at 5 weeks displayed a severe opacity in the lens nucleus (stage 3) and the small eye phenotype. Note that the opacity lies in the center of the lens while the cortical fibers are relatively clear. The opacities were digitally quantified using the Image J program. The light intensity at the cornea was normalized to 100. The plot profiles shown below the slit lamp images demonstrate a gradual increase in light scattering from the wild type to αA-R49C heterozygous to αA-R49C homozygous mice. (D-F) 16 week old mice. (D) Wild type lens at 16 weeks. (E) A 16 week old heterozygous mouse lens had stage 2 lens opacity. (F) A 16 week old αA-R49C homozygous mouse displayed micro-ophthalmia with severe (stage 4) lens opacities. Note the dotted line showing that the αA-R49C homozygous knockin eye is significantly smaller than the wild type and heterozygous littermates (D and E). The opacities were digitally quantified using the Image J program. The light intensity at the cornea was normalized to 100. The plot profiles shown below the slit lamp images demonstrate a gradual increase in light scattering from the wild type to αA-R49C heterozygous to αA-R49C homozygous mice.

Figure 4. Eye and lens mass in wild type and αA-R49C knockin lenses. Freshly dissected eyes and lenses were weighed. Data are shown for 11 week old wild type, αA-R49C heterozygous and homozygous mice. Note that the difference between the masses of the heterozygous and homozygous eyes was 12 mg, while that between their lenses was 11 mg, indicating that the small eye is almost entirely due to a small lens.

Figure 5. Cell proliferation and death in αA-R49C knockin eyes. 5 days old lenses mice were injected with BrdU and the labeling index was determined. (A-C) Cell proliferation as measured by in vivo BrdU labeling index shows that cell proliferation is qualitatively unchanged in wild type and knockin lenses. (A) Wild type lens; (B) αA-R49C heterozygous knockin lens; (C) αA-R49C homozygous knockin lens. The labeling index was determined by dividing the number of BrdU-positive cells with the total number of cells in each section. A total of 3 sections per lens, and 6 lenses per genotype were analyzed. Note that the BrdU labeling index was 0.06 ± 0.01 in the wild type, 0.05 ± 0.03 in αA-R49C heterozygous knockin lenses, and 0.06 ± 0.02 in αA-R49C homozygous knockin lenses. (D-F) TUNEL staining in αA-R49C knockin lens epithelial and fiber cells. (D) Wild type mouse lens; (E) αA-R49C homozygous knockin mouse lens. (F) Quantitative analysis of TUNEL staining in wild type, αA-R49C heterozygous and αA-R49C homozygous lenses.

Figure 6. Cell death in αA-R49C lens epithelial wholemounts. Wild type or αA-R49C homozygous lenses were dissected and whole mounts of lens epithelial cells were prepared, and analyzed by propidium iodide (PI) labeling (red) and fluorescein conjugated-TUNEL reagent (green). (A) A wild type lens epithelial wholemount stained with PI and viewed in the rhodamine channel of the confocal microscope. (B) The sample in (A) viewed in the fluorescein channel of the confocal microscope. Note that the wild type sample stained with TUNEL shows no TUNEL-labeled nuclei. (C) A merged image of the wild type specimen shown in (A) and (B).
Figure 7. Change in expression of cell survival and signaling proteins in αA-R49C knockin lens epithelial and cortical fiber cells. Lenses epithelial and fiber cell fractions were dissected from 16-20 wild type (WT), αA-R49C heterozygous and αA-R49C homozygous lenses. Cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting with specific antibodies to Bcl-2, p-Bad (Ser 112), p-Bad (Ser 136), p-Akt (308), p-Akt (473), total Akt, p-p38, total p38, p-ERK and ERK1/2. Note that WT lens epithelial cells expressed high levels of Bcl-2 which was maintained in the αA-R49C heterozygous and homozygous lenses. WT lens fiber cells expressed low levels of Bcl-2 which increased >4-fold in αA-R49C homozygous lenses, but not in αA-R49C heterozygous lenses. Note also that the high expression of p-Bad (Ser 112) and p-Bad (Ser 136) in lens fiber cells decreased gradually with decrease of WT αA-crystallin, and decreased 5.3- and 2.4-fold respectively, in the αA-R49C homozygous lenses. Expression of p-Akt (308) and p-Akt (473) in lens epithelial cells increased with the αA-R49C mutation. Although p-Akt (308) and p-Akt (473) and total Akt expression was hardly detectable in WT lens fiber cells, p-Akt (308) and p-Akt (473) increased 3- and 12-fold, respectively in the αA-R49C lens fiber cells, while total Akt increased only 1.2-fold. WT lens epithelial cells expressed high levels of p38 and p-p38, which were slightly reduced (<2-fold) in the αA-R49C homozygous lenses. However, the expression of p38 and p-p38 increased 2-4 fold in the lens fiber cells of αA-R49C homozygous lenses. Note that the antibody to p-p38 identifies the lower p38 band (arrow). Immunoblotting for p-ERK and ERK demonstrated sustained expression and activation of p-ERK and ERK1/2 in lens epithelial cells of WT, αA-R49C heterozygous and αA-R49C homozygous lenses. Note that WT lens fiber cells expressed one-tenth the level of p-ERK and ERK1/2 of lens epithelial cells. Note also that p-ERK expression was enhanced 3-fold in lens fiber cells of the αA-R49C homozygous lenses.

Figure 8. Expression of α, β, γ-crystallins and MIP in wild type and αA-R49C knockin lenses. 8 weeks old lenses were divided into soluble and insoluble fractions and analyzed by immunoblot analysis with antibodies to αA-crystallin, αB-crystallin, total β-crystallin, total γ-crystallin and MIP. The expression of αA-crystallin and αB-crystallin increased significantly in the insoluble fractions of the R49C knockin lenses. γ-Crystallin but not αB-crystallin or β-crystallin expression was upregulated in soluble fractions of αA-R49C heterozygous and homozygous lenses. MIP was detected as a 27 kDa band. The expression of MIP was the same in wild type, αA-R49C heterozygous and homozygous lenses when corrected for protein loading in the αA-R49C homozygous lane.

Figure 9. Immunofluorescence analysis of MIP expression in wild type and αA-R49C homozygous knockin lenses. Lens slices were cut in the equatorial plane and stained with an antibody to lens major intrinsic protein MIP (AQP0) to visualize fiber cell membranes. Sections of wild type (A, C, E and G) and homozygous (B, D, F and H) lenses are shown. Low
magnification images of (A) wild type and (B) αA-R49C homozygous lenses shows the dramatic decrease in lens size in the mutant. Note that the αA-R49C homozygous lenses were significantly more fragile and susceptible to tearing during processing. (C, D) Visualization of fiber cell membranes in the onset of differentiation (cell elongation) region of wild type (C) and αA-R49C homozygous (D) lenses. Nuclei are indicated by bold arrows. The organization of the fiber cells in these equatorial sections in different, with the neat packing of membranes of the wild type lenses (small arrows). Vacuoles were observed in the αA-R49C mutant lenses (small arrowheads). (E,F) In contrast to the neat parallel organization of lens fiber cells in the wild type lens (E), fiber cells of the αA-R49C homozygous lenses (F) were highly disorganized. The distance from the center of the lens was 400 µm. (G, H) Posterior region of the lens. Lens fiber cells demonstrate a neat hexagonally packed arrangement in this section of the wild type lens (G), in contrast to the highly disorganized pattern in the αA-R49C homozygous lens (H).

**Figure 10. Interaction of αA-crystallin with substrate proteins in wild type and αA-R49C knockin lenses.** Six week old lenses were extracted in lysis buffer and proteins were immunoprecipitated with a monoclonal antibody to αA-crystallin. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with antibodies specific to αB-crystallin, β-crystallin, γ-crystallin and β-tubulin. Quantitative analysis of the interaction by densitometric scanning (bar graphs) showed that the interaction of αA-crystallin in the αA-R49C heterozygous knockin lenses with αB-crystallin, β-crystallin, γ-crystallin and β-tubulin was strongly enhanced. In the αA-R49C homozygous knockin lenses the interaction between αA-crystallin and αB-crystallin, β-crystallin, γ-crystallin and β-tubulin diminished.
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FIGURE 1

A

Knockin construct

5' Arm
2.9 kb

1

XhoI

2

3

3' Arm
4.0 kb

Wild type allele

XhoI

pcr1

5' Probe

500 bp

Homologous recombination

XhoI

pcr1

pcr2

5' Probe

2.5 kb

Cre recombination (deletes Neo' cassette)

Targeted knockin allele without Neo'

Targeted knockin allele with Neo'

B

WT
Arginine

R49C
Cysteine

C

kb

bp

550

8 10 23 85 WT

D

WT
R49C heterozygous homozygous
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FIGURE 2

A. MS/MS of LLPFLSSTSPYYR

B. MS/MS of LLPFLSSTSPYYQSLFR

C. Wild type mouse αA-crystallin

1. MDVTQQHPFKRALGPFPYP5RLFDQFFGEGLFYDLPFLSSTSPYYRQSLFRVTLDG

61. ISFVRSDRDKFVEIFDKHFSFEDLTQYKLDFVIEHGKHNFRQDDHGYIERSFHRYYRLP

121. SNVDQGASLCGLSADGMLFSGPKVQSGGLDAHGSAIPVSREEKPSAFSS

R49C mouse αA-crystallin

1. MDVTQQHPFKRALGPFPYP5RLFDQFFGEGLFYDLPFLSSTSPYYQSLFRVTLDG

61. ISFVRSDRDKFVEIFDKHFSFEDLTQYKLDFVIEHGKHNFRQDDHGYIERSFHRYYRLP

121. SNVDQGASLCGLSADGMLFSGPKVQSGGLDAHGSAIPVSREEKPSAFSS
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FIGURE 3
Knockin model for αA-Crystallin R49C mutation

FIGURE 4

![Graph showing weight comparison between Wild Type, αA-R49C Heterozygous, and αA-R49C Homozygous mice.](http://www.jbc.org)
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FIGURE 5
Knockin model for αA-Crystallin R49C mutation

FIGURE 7

| Lens Epithelium | Lens Fibers |
|-----------------|-------------|
| WT              | Bcl-2       |
| αA-R49C         | p-Bad (112) |
| Homozygous      | p-Bad (136) |
|                 | p-Akt (308) |
|                 | p-Akt (473) |
|                 | Akt         |
|                 | p-p38       |
|                 | p38         |
|                 | p-ERK       |
|                 | ERK1/2      |
| αA-R49C         | Heterozygous|
| Homozygous      |             |
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FIGURE 8

[Image of a gel showing expression levels for soluble and insoluble fractions of αA-crystallin, αB-crystallin, β-crystallin, γ-crystallin, and MIP under different conditions.]
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FIGURE 10

[Diagram showing immunoblot results for αB-crystallin, β-crystallin, γ-crystallin, and β-tubulin with relative intensity levels indicated.]
Mechanism of small heat shock protein function in vivo: A knockin mouse model demonstrates that the R49C mutation in \( \alpha \)-crystallin enhances protein insolubility and cell death

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Mechanism of small heat shock protein function in vivo. A knock-in mouse model demonstrates that the R49C mutation in αA-crystallin enhances protein insolubility and cell death.

Jing-hua Xi, Fang Bai, Julia Gross, R. Reid Townsend, A. Sue Menko, and Usha P. Andley

On page 5802, in the right column, lines 22–24 from the top, the corrected sentence is as follows: “First generation offspring that inherited the targeted allele with neomycin were subsequently backcrossed into C57BL/6J (for at least three generations).” In the right column, line 19 from the bottom, the corrected sentence is as follows: “Some of these mice were further genotyped to exclude the presence of a deletion mutation in the gene for lens phakinin (CP49), which is characteristic of the 129 strain from which the ES cells were derived.”

On page 5807, in the left column, lines 8–10 from the top, the corrected sentence is as follows: “Fig. 2 shows the MS and fragmentation spectra of the predicted peptide from a combined endopeptidase digest (LysC + GluC + trypsin) of the wild type αA-crystallin from lenses derived from mice prior to Cre mating.” In the left column, lines 8–11 from the bottom, the corrected sentence is as follows: “αA-R49C homozygous eyes weighed 60–70% less than eyes of wild type and heterozygous littermates.”

On page 5809, Fig. 4 is being withdrawn because it contained erroneous data.

These additions and corrections do not result in any change in the conclusions of the article.

The metabolome of Chlamydomonas reinhardtii following induction of anaerobic H₂ production by sulfur depletion.

Matthew Timmins, Wenzu Zhou, Jens Rupprecht, Lysha Lim, Skye R. Thomas-Hall, Anja Doebbe, Olaf Kruse, Ben Hankamer, Ute C. Marx, Steven M. Smith, and Peer M. Schenk

On page 23415, Dr. Timmins’ first and last names were reversed. His correct name is shown above.

These additions and corrections do not result in any change in the conclusions of the article.