Evidence of Highly Conserved $\beta$-Crystallin Disulfidome that Can be Mimicked by In Vitro Oxidation in Age-related Human Cataract and Glutathione Depleted Mouse Lens*§

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Low glutathione levels are associated with crystallin oxidation in age-related nuclear cataract. To understand the role of cysteine residue oxidation, we used the novel approach of comparing human cataracts with glutathione-depleted LEGSKO mouse lenses for intra- versus inter-molecular disulfide crosslinks using 2D-PAGE and proteomics, and then systematically identified in vivo and in vitro all disulfide forming sites using ICAT labeling method coupled with proteomics. Crystallins rich in intra-molecular disulfides were abundant at young age in human and WT mouse lens but shifted to multimeric inter-molecular disulfides at older age. The shift was 4x accelerated in LEGSKO mouse. Most cysteine disulfides in $\beta$-crystallins (except $\beta$A4 in human) were highly conserved in mouse and human and could be generated by oxidation with H$_2$O$_2$, whereas $\gamma$-crystallin oxidation selectively affected $\gamma$C23/42/79/80/154, $\gamma$D42/33, and $\gamma$S83/115/130 in human cataracts, and $\gamma$B79/80/110, $\gamma$D19/109, $\gamma$F19/79, $\gamma$E19, $\gamma$S83/130, and $\gamma$N26/128 in mouse. Analysis based on available crystal structure suggests that conformational changes are needed to expose Cys42, Cys33 in $\gamma$C; Cys42, Cys33 in $\gamma$D, and Cys83, Cys115, and Cys130 in $\gamma$S. In conclusion, the $\beta$-crystallin disulfidome is highly conserved in age-related nuclear cataract and LEGSKO mouse, and reproducible by in vitro oxidation, whereas some of the disulfide formation sites in $\gamma$-crystallins necessitate prior conformational changes. Overall, the LEGSKO mouse model is closely reminiscent of age-related nuclear cataract. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.050948, 3211–3223, 2015.

Aging lens crystallins accumulate post-synthetic modifications that can be broadly classified into three categories, namely (1) protein backbone changes, such as racemization and truncation (1–3), (2) conversion of one amino acid into another, such as deamidation of asparagine into aspartate or deguanidination of arginine into ornithine, deamination of lysine into allysine and 2-aminoacidipic acid (4–6), and (3) amino acid residue damage from reactive carbonyls and reactive oxygen species (7,8). Carbonyl damage results from the Maillard Reaction by glucose, methylglyoxal, or oxidation products of ascorbate, tryptophan or lipids which form adducts and crosslinks with nucleophilic group of lysine, arginine and cysteine. Examples include carboxymethyl-lysine, pentosidine, methylglyoxal hydroimidazolones, HNE-cysteine adducts and kynurenine (7,9–12). Oxidative damage results from reactive oxygen species that directly damage amino acid residues, e.g. oxidizing tryptophan into N-formyl kynurenine and kynurenine, methionine into its sulfoxide, and cysteine into cysteine disulfides or cysteic acid (13–15).

Because of their relevance to age-related cataract, the impact of each of these modifications on crystallin structure and stability is the subject of intense investigation. Importantly, Benedek proposed that high molecular weight (HMW)$^1$ crystallin aggregates the size of 50 million daltons are needed in order for lens opacification to be visible(16,17). Crystallin aggregation conceivably occurs by one of several mechanisms that include conformational changes as a consequence amino acid mutations (18) or physical-chemical protein modifications. Of the latter, one mechanism that is dominant in several

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* The abbreviations used are: HMW, high molecular weight; GSH, glutathione; GlcL, $\gamma$-glutamyl cysteine ligase catalytic subunit; LOCS, lens opacities classification system; TCA, trichloroacetic acid; IAM, iodoacetamide; TFA, trifluoric acid; TCEP, tris (2-carboxyethyl) phosphine; DTPA, diethylene triamine pentaacetic acid.
types of cataract involves oxidation of cysteines into protein disulfides (18) and formation of HMW aggregates that scatter light (19).

In order to mimic the oxidative process and formation of protein disulfides linked to low concentrations of glutathione (GSH) in the nucleus of the human lens, we recently created the LEGSKO mouse in which lenticular GSH was lowered by knocking out the γ-glutamyl cysteine ligase subunit (Gclc) knockout mouse were created as previously described (20). These mice develop full-blown nuclear cataract by about 9 months and represent an important model for the development of drugs that might block or reverse the oxidation of crystallin sulfhydryls and presumably protein aggregation. However, this assumption in part depends on whether the sites of disulfide bond formation are similar in mouse and human age-related cataract. To test this hypothesis we performed the first comparative analysis of the cataract prone LEGSKO mouse and human aging and cataractous lens crystallin disulfidome, and compared the results with the disulfidome from mouse lens homogenate oxidized in vitro with H₂O₂ as a model of crystallin aggregation and opacification.

**EXPERIMENTAL PROCEDURES**

**Animals**—All animal experiments were conducted in accordance with procedures approved by the Case Western Reserve University Animal Care Committee and conformed to the ARVO Statement for use of Animals in Ophthalmic and Vision Research. Animals were housed under diurnal lighting condition and allowed free access to food and water. Lens conditional γ-glutamyl cysteine ligase, catalytic subunit (Gclc) knockout mouse were created as previously described by us and named as LEGSKO mouse (20).

**Human and Mouse Lens Samples**—All human tissue used for this study was approved by Case Western Reserve University institutional ethics committee (IRB). Three young normal human eyes at 15, 7, and 3 years of age, and three old normal human eyes at 74, 72, and 68 years of age were collected from the Cleveland Eye Bank/Midwest Eye Bank within post-mortem interval of 2–8 h, (average = 4.1 h). The lenses were immediately processed. Three young and normal lens nuclei were pooled to serve as control for all other human lenses. Human cataract lenses were obtained from Huichang County People’s and Ganzhou City People’s Hospital in Jiangxi, China. Cataract was graded based on Lens Opacities Classification System (LOCS) III. The grade II-V lenses were collected and there were three lenses in each grade. The age of grade II group was 64, 67, and 60 years old, the grade III was 60, 59, and 68 years old, the grade IV was 56, 63, and 77 years old, and the grade V was 75, 80, and 78 years old. All lenses were immediately processed. Three young and normal lens nuclei were pooled to serve as control for all other human lenses. Human cataract lenses were processed in China, and then shipped to Cleveland for ICAT labeling and proteomics analysis. LEGSKO mouse cataract and age matched wild type control lenses were collected as previously described (21), and processed using the same procedure as for human lenses, except that the whole lens was used rather than the nucleus because of its small size. Three age-matched wild type lenses were pooled to serve as control for LEGSKO cataract lenses.

**In Vitro Modeling of Lens Crystallin Disulfide Crosslink Formation**—Three months old mouse lens protein homogenate (20 mg/ml), prepared in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTPA and free of insoluble proteins and small molecules by centrifugation at 26,000 × g for 20 min at 4 °C followed by dialysis against 4 liter 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTPA for 48 h, was incubated in a microtiter plate with or without 1 or 5 mM of hydrogen peroxide (H₂O₂) at 37 °C up to 12 h. Protein aggregation was determined by measuring the absorbance of each sample at 595 nm using a UV-visible spectrophotometer (Tecan Microlute Readers, Switzerland). At 2 h and 12 h incubation, 150 μl of sample were collected and mixed with equal volume of ice-cold 20%TCA with 200 mM IAM, the sample was centrifuged at 26,000 × g for 15 min after 20 min incubation on ice. The pellet was washed three times with 500 μl ethyl ether and dried at room temperature for 5 min. The pellet was then re-suspended in 500 μl of ICAT buffer with 100 mM IAM, and processed following the same procedures as described above for human and mouse lens samples.

**Quantitative Isotope-coded Affinity Tag labeling Experiments (ICAT)**—For ICAT-based quantification by mass spectrometry, 100 μg of protein aliquot of lens sample processed as above was mixed with equal volume of ice-cold 20% TCA, and kept at −20 °C for 2 h. After centrifugation at 26,000 × g for 15 min, the pellet was washed twice with 500 μl cold acetone, and then dried for 3 min in a Savant Speed Vac concentrator. The dried samples were resuspended in 80 μl freshly made ICAT buffer with 10 mM TCEP, and incubated at 30 °C for 30 min after 30 s sonication in water bath. The samples were then labeled with the cleavable ICAT reagent (AB Sciex, Foster City, CA) at 37 °C for 2 h in the dark as described in the instruction manual. All human aged normal, cataract and LEGSKO mouse cataract lenses were labeled with cleavable “heavy” ICAT reagent (C₁₂-ICAT), whereas the young human and age-matched wild type mouse lens control samples were labeled with the cleavable “light” ICAT reagent (C⁵₋ ICAT). After labeling, a 1:1 mixture of each aged or cataract lens sample with control sample was prepared, to which 800 μl fresh 10 mM calcium chloride was added. To digest the proteins, 4 μg of mass spectrometry grade lysisyl endopeptidase (Wako, Richmond, VA) was added into each sample. Samples were incubated at 37 °C for 2 h in the dark, followed by addition of 5 μg of sequence grade trypsin (Promega, Madison, WI) per sample and head-to-head rotation for 18 h at 37 °C in the dark. The peptides solution was adjusted to pH 3.0 by formic acid and then passed through Oasis HLB Plus cartridge, eluted with 5 ml 70% acetonitrile and 5% formic acid after washing with 5 ml 5% formic acid. The eluted peptides were further purified by high-resolution cation-exchange column using POROS 50HS beads (6 mm × 7 cm, column volume 1 ml, Thermo Fisher, Grand Island, NY), perfused with Pharmacia LKB peristaltic pump. The collected peptides were finally enriched and purified by Avidin affinity column, and eluted with 30% acetonitrile, 0.4% trifluoroacetic acid (TFA), after sequential washing with 2× PBS, 1× PBS, 50 mM ammonium bicarbonate/20% methanol (pH8.3), and Milli-Q water. The samples were dried with a SpeedVac concentrator, and the biotin was removed by cleaving the linker with TFA. The samples were then subjected to LC/MS analysis at the CWRU Center for Proteomics.

**Quantification of Proteins by Dimethyl Labeling**—The aliquots of samples subjected to the ICAT was resuspended in 100 μl 6 μm urea,
50 mM Tris-HCl (pH 7.5) buffer with 2 min water-bath sonication, then diluted by adding 800 μl digestion buffer (50 mM Tris-HCl (pH 7.5), 1 mM CaCl2) and digested by sequencing grade-modified trypsin (Promega). The peptide from digest was purified through LC-18 Cartridge (Supelclean LC-18 SPE, 100 mg, Sigma-Aldrich, St. Louis, MO), and eluted with 90% acetonitrile, 1% formic acid after washing with 1% formic acid. The trypptic peptides from control were labeled with normal and trypptic peptides from aged and cataract samples were labeled with deuterium labeled formaldehyde (Sigma-Aldrich, St. Louis, MO) respectively according to previous report(22). After labeling, a 1:1 mixture of each aged or cataract lens sample with control sample was prepared, and the peptide from mixture was again desalted and purified using LC-18 Cartridge as mentioned above.

Analysis of Dimethyl Labeled Proteins—In addition of carbamidomethylation of Cys and oxidation of Met residues, light and heavy dimethylation (with the mass shift of 28.0313 and 32.0475 Da, respectively) at peptide N-terminal and Lys residues were also set as variable modifications for Mascot Daemon searching of dimethyl labeling samples. The ratio of light to heavy dimethylation modification was calculated by manually extraction of each peptide on basis of peptide peak heights. Protein ratios were reported as the median of the measured peptide ratios for given protein.

MS Analysis of ICAT Labeled Samples—Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the resulting peptides was performed with an Orbitrap Elite Hybrid Mass Spectrometer (Thermo Electron, San Jose, CA) coupled with a Waters nanoAcquity UPLC system (Waters, Taunton, MA). The spectra were acquired in the positive ionization mode by data dependent methods consisting of a full MS scan in high mass accuracy FT-MS mode at 120,000 resolution, and MS/MS on the twenty most abundant precursor ions in CID mode with the normalized collision energy of 35%. The identification of peptides was achieved with Mascot Daemon (version 2.4.0, Matrix Science) and the data were searched against SwissProt human database. The mass tolerance was set at 15 ppm and 0.8 Da, respectively for precursor ions and fragment ions. Carbamidomethylation of Cys, as well as the oxidation of Met residues was set as variable modifications. Both light and heavy ICAT labeled peptides with the mass shift of 227.1270 and 236.1571 Da, respectively, were first identified using Mascot Daemon searching algorithm, and then confirmed by manually examination, especially for the peptides having multiple Cys residues and mix-labeled with iodoacetamide (IAM) and ICAT. Quantification of ICAT labeling was conducted using Mascot Distiller (Matrix Science). The ratio of light ICAT to heavy ICAT was calculated and averaged on basis of peptide peak heights for each individual scan. The ratio for the same peptide but with different charge and methionine oxidation state were then averaged manually. For the peptide carrying multiple Cys residues but mix-labeled with IAM and ICAT, the ratio was manually calculated by the peptide peak heights extracted from the selected ion chromatogram. Each sample was run once in LC/MS analysis, the average ratio from multiple spectra covering same sites was calculated from each sample, and standard errors were calculated from three biological replicates. All reported ICAT ratios were identified with the standard error < 0.1 and correlation co-efficiency > 0.99. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (23) via the PRIDE partner repository with the dataset identifier PXD002658 and 10.6019/PXD002658.

Identification of Intra- and Intermolecular Disulfide Protein Crosslink Using 2-Dimensional SDS-PAGE—Two-dimensional SDS-PAGE analysis for intra- and intermolecular disulfide crosslink analysis was performed as previously described(24) with some modifications. In brief, the fresh lens was homogenized in ice-cold 10% trichloroacetic acid (TCA), and the precipitate was resolubilized in 100 mM Tris-HCl, pH 7.5, 5% SDS and 40 mM iodoacetamide on ice for 10 min with help of sonication of 20 s for three times. The protein content in supernatant was determined by BCA protein assay (Pierce, Rockford, IL). An equal volume of 2x SDS sample buffer, free of reducing agents, was added to protein extracts. Then, 400 μg protein was loaded to 12% SDS-PAGE gel and subjected to electrophoresis in the first dimension for 5 h at constant current (25 mA) using a Protean II Xi gel apparatus (Bio-Rad). After electrophoresis, the gel lanes containing the separated proteins were cut and immersed in 1x SDS sample buffer containing 100 mM DTT for 20 min at room temperature. Following a brief wash with SDS running buffer, the gel slices were further immersed in 1x SDS sample buffer containing 100 mM iodoacetamide for 10 min. Each gel strip was then applied horizontally to another gel (12% acrylamide, 1.5 mm thickness), and electrophoresis was performed in the second dimension for 20 h at constant current (9 mA/gel). Gels were fixed in 50% methanol, 5% acetic acid for 20 min and stained with Bio Safe Coomassie according to the manufacturer (Bio-Rad). Three replicate gels were run in each sample, and three biological replicates were performed in each age group.

Protein Spot Identification Using Proteomic Methodology—Two dimensional gel spots (manually cut) were first destained with 50% acetonitrile in 100 mM ammonium bicarbonate, and 100% acetonitrile. Then, the protein was reduced with 20 mM DTT at room temperature for 60 min followed by the alkylation using 50 mM iodoacetamide for 30 min in the dark. The reaction reagents were removed and the gel pieces were washed with 100 mM ammonium bicarbonate (pH7.0) and dehydrated in 60% acetonitrile in water. The dried gel pieces were then re-swelled in sequencing grade-modified trypsin (Promega) in 50 mM ammonium bicarbonate for overnight digestion. Proteolytic peptides extracted from the gel with 50% acetonitrile in 5% formic acid were completely dried in Savant Speedvac concentrator, and then resuspended in 0.1% formic acid. Tryptic peptide analysis was performed by using a LTQ Orbitrap XL linear ion trap mass spectrometer (Thermo Fisher Scientific) coupled with an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA). The spectra were acquired by data dependent methods consisting of a full scan and MS/MS on the five most abundant precursor ions at the collision energy of 30%. Proteins were identified by peptide sequencing on tandem mass spectrometry with Mascot search engine (Matrix Science, Boston, MA). A maximum of two missed cleavages were permitted. Mass tolerance for precursor ions and fragment ions was set at 15 ppm and 0.8 Da, respectively. SwissProt (May, 2010) database (516603 sequences; 181919312 residues) was used for searching against the taxonomy of mouse (16302 sequences). The significance threshold p value was set to < 0.05. Proteins hits with at least two unique peptides at Mascot score > 20 were considered to be identified.

Statistical Methods—All values were expressed as means ± S.E. Statistical analysis was performed according to methods previously described in detail (25). In brief, statistical significance of differences in mean values was assessed by repeated-measures ANOVA or Student’s t test. p values of <0.05 were considered statistically significant.

RESULTS

Using 2D gel electrophoresis and proteomics analysis we first asked the question of which proteins are associated with intra- versus intermolecular disulfide-bond in old normal versus young human lens, and determined how these compared with those detected in oxidatively stressed, precataractous LEGSKO mouse lens. Using the ICAT labeling technique to probe all existing disulfides and their quantitative change relative to an appropriate control, we then asked the novel question of how do crystallin disulfides found in human cat-

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aracts compare with those detected in glutathione-depleted LEGSKO mouse cataracts and mouse lens proteins undergoing in vitro oxidation.

Evidence of Age-related Shift From Intra- to Intermolecular Disulfide Formation in Human and LEGSKO Mouse Lens Crystals—The overall protein disulfide bond formation status in young and aged human lenses was first assessed using two-dimensional nonreducing then reducing SDS-PAGE. The schematic view of the conceptual strategy of this assay is shown in supplemental Fig. S1. The Coomassie stained gels show the pattern of intra-versus intermolecular disulfide bonds in fresh lens extract from human, WT and homozygous LEGSKO mouse analyzed by two-dimensional diagonal SDS gel electrophoresis. Proteins in the diagonal axis do not contain disulfides. Proteins with intramolecular disulfides migrate left whereas proteins with intermolecular disulfides migrate right of the diagonal axis. The MW of the proteins increases both from left to right and from top to bottom of the gel. Overall results indicate that a shift occurred with age from intramolecular disulfide cross-links into intermolecular disulfide HMW crosslinks in both normal human and WT mouse lens, and that these changes occur much faster in the homozygous LEGSKO mouse lens. A, 4-year-old human lens. B, 67-year-old human lens. C, 1-month-old wild type mouse lens. D, 20-month-old wild type mouse lens. E, 1-month-old LEGSKO mouse lens. F, 5-month-old LEGSKO mouse lens. G, 5-month-old wild type lens with protein identification spots. H, 5-month-old LEGSKO mouse lens with protein identification spots. These are representative data from three replicates for each sample, and at least three biological replicates for each age group. We manually created five bands labeled from I to V based on protein molecular weight clusters. Red circles and numbers are the spots chosen for protein identification by mass spectrometry.
Fig. 1) allow us to rapidly visualize the overall protein pattern which uncovered an age-related shift of protein spots associated with intramolecular disulfides (left of the diagonal axis) to proteins associated with intermolecular disulfide crosslinks (right of the diagonal axis), respectively. Nondisulfide-linked proteins are responsible for the diagonal axis. The typical result of negative control done in both dimensions under reduced conditions by DTT is shown in supplemental Fig. S2.

Representative data from 4 and 67 years old normal lens are shown in Fig. 1A–1B. At four years of age, protein disulfides are almost completely present as intramolecular disulfides (Fig. 1A, spots at up left of the diagonal region). Over 25 intramolecular disulfide bond protein spots and less than six intermolecular disulfide protein spots were identified, whereas in 67 year old human lens, less than four intramolecular disulfide and more than 20 intermolecular disulfide proteins spots were identified. As expected, proteomic analysis using in-gel tryptic digestion and mass spectrometry confirmed that the majority of proteins in the disulfide crosslinks region of the gel were crystallins. Overall a similar shift in spot pattern was observed in the old versus young WT mouse (Fig. 1C and 1D). In contrast, the intra- to intermolecular disulfide shift had already occurred at 5mos in the precataractous, GSH suppressed, LEGSKO mouse lens (Fig. 1F) when compared with the 5 months old WT lens (Fig. 1G). In quantitative terms, the 1-month-old LEGSKO lens had only five to six intermolecular disulfide bond protein spots and over 28 intramolecular disulfide bond protein spots. In contrast, only one to two intramolecular disulfide bond proteins and more than >22 spots were present as intermolecular disulfide crosslinks at 5 months (Fig. 1F). Thus, GSH depletion greatly accelerated the aging of the lens proteins as reflected by the shift from intra- to intermolecular disulfide spots revealed by 2D-PAGE.

Proteomic analysis was carried out to identify the composition of each spot marked in red circle in Fig. 1. These segregated into two regions, A and B (Fig. 1), depending on whether they are highly enriched with gamma- or beta crystallins, respectively. The detailed composition of each spot is listed in supplemental Table S1 and S2. Almost all known human crystallins, except αB, were detected in the intramolecular disulfide region of the gel. They include human αA, βA1/3, βA4, βB1, βB2, βB3, and γA, γB, γC, γD, γE, and γS (spots 1–16). Similarly, normal (wild type) mouse lenses included αA, βA1/3, βB2 and βB3, and γA, γB, γC, γD, γE, and γS, whereby γF was not detected (spots 17–22).

For faster overview and mouse to human comparison, the data on the intermolecular disulfide clusters of 67 year old human and 5 month old LEGSKO lens based on the molecular weight (HMW) bands I–V (as illustrated in Fig. 1B and 1H, which we manually created based on protein clusters) are summarized in Table I. Three gamma crystallins, γC, γD, γS were detected in all band regions, and especially γS, which was present in high frequency in HMW crosslinks regions, i.e. from III to V. Four beta crystallins, βA1/3, βA4, βB1, and βB2 were present starting from band II, and βA1/3 and βB1 were detected with higher frequency in the HMW band regions. Some crystallin spots were present in higher frequency than others in certain spots, perhaps indicating they are more prone to forming protein-protein disulfides. In LEGSKO mouse, γC, γD, γE were present in all band regions, and both γC, γD had higher detection frequency in HMW band regions. Beside βA1/3, βA4, βB1, and βB2 beta crystallins, βB3 was also detected in band III to V, and similar to human lens, βA1/3 was present in high frequency in HMW regions. Interestingly, in band region V, we started to see noncrystallin protein, i.e. PRDX6 among intermolecular disulfide crosslinks. Thus, the disulfide pattern of the precataractous LEGSKO mouse and old human lens share high similarities.

| Band | Human* | LEGSKO Mouse* |
|------|--------|---------------|
| I    | αA, γC, γD, γS | αA, γC, γD, γE |
| II   | αA, βA1/3, βA4, βB1, βB2, βB3, γA, γB, γC, γD, γE, γS (spots 1–16) | αA, βA1/3, βB2 and βB3, γA, γB, γC, γD, γE, and γS, whereby γF was not detected (spots 17–22) |
| III  | αA, βA1/3, βA4, γC, γD, γE, γS (spots 1–16) | αA, βA1/3, βB2 and βB3, γA, γB, γC, γD, γE, and γS, whereby γF was not detected (spots 17–22) |
| IV   | αA, βA1/3, βA4, γC, γD, γE, γS (spots 1–16) | αA, βA1/3, βB2 and βB3, γA, γB, γC, γD, γE, and γS, whereby γF was not detected (spots 17–22) |
| V    | βA1/3, βA4, γC, γD, γE, γS (spots 1–16) | αA, βA1/3, βB2 and βB3, γA, γB, γC, γD, γE, and γS, whereby γF was not detected (spots 17–22) |

*Proteins are commonly presented in higher molecular weight band regions in italic.
Disulfidome of the Aging Mouse and Human Lens

Fig. 2. Cysteine residues that are mildly oxidized into disulfides in aged noncataractous human lenses, and not further oxidized in cataracts. A, Cysteine residues that are oxidized less than fivefold with no major change with presence or severity of cataract grade. B, Cysteine residue Cys166 in βA4 is in intradisulfide bond form in both young and aged normal and cataract lenses. C, Cys185 in βA1/3 is oxidized to relatively high level in aged noncataract lens but was not further oxidized in cataract lenses. Cat-II: grade II cataract, Cat-III: grade III cataract, Cat-IV: grade IV cataract, Cat-V: grade V cataract. D, Human γD crystallin NMR structure indicates that residue Cys19 is exposed to surface. Original structure annotation data was obtained from PUBMED, and further analyzed by Vector NTI 3D structure view program (Invitrogen). Cysteine atoms are in yellow color. All ICAT data is expressed as the ratio to surface. Original structure annotation data was obtained from PUBMED, and further analyzed by Vector NTI 3D structure view program (Invitrogen).

analysis are shown in supplemental Fig. S3, and the typical protein ratio calculation based on peptide dimethyl labeling is shown in supplemental Fig. S4.

1. Cysteine Residues that are Oxidized or Mildly Oxidized into Disulfides in Old Noncataractous Lenses and not Further Oxidized in Cataracts—This category includes cysteine residues, such as Cys67 in βB2, Cys19 in γD, Cys109 in γC and Cys99 in βA4, with mild oxidation, i.e. less than fivefold elevation in both normal and cataract lenses compared with young (Fig. 2A). Some of cysteine residue, such as Cys166 in βA4, had less disulfide formation compared with young control lenses (Fig. 2B). This might because of high level of intradisulfide bond between Cys166 and Cys99 in young lenses, which is suggested from the crystal structure (supplemental Fig. S2). Finally Cys185 of βA1/3 was more than 10 fold oxidized in old normal lens but no further increase was found in cataract lenses (Fig 2C). Human γD crystal structure (Fig. 2D) clearly indicates that Cys19 is relatively exposed to the surface, one of the key factors for intermolecular disulfide bond formation.

2. Cysteine Residues with Mild Oxidation in Normal Old Lenses but Massively Increased Oxidation in All Cataract Lenses Regardless of Severity—Several cysteine residues, such as Cys42 in γD, Cys52 and Cys170 in βA1/3, Cys38 in βB2 and Cys83 in γS are relatively mildly oxidized in old normal lenses, but are massively elevated in cataracts, regardless of severity (Fig. 3A and 3B). For some cysteine residues, such as Cys170 in βA1/3 and Cys83 in γS, oxidation jumped over hundred times in level II cataract lenses. Interestingly, these cysteine residues had significant oxidation in level II cataract lenses with no further increase in level III to V lenses. Human NMR γS structure clearly indicates that Cys83 is deeply buried (Fig. 3D). We believe that these cysteine residues are having much less surface exposure, i.e. buried, and significant protein unfolding is required for disulfide bond formation. More will be described in the discussion section.

If we extrapolate into percentile of cysteine residues (Cys170 and Cys83) oxidized, then at least 80% of this two cysteine residues have been oxidized. This indicates that these cysteine residues are likely playing an important role in early phase of ARNC.

3. Cysteine Residues that are Oxidized in Old Normal Lenses and Further Increased in Cataract Lenses—Three cysteine residues, Cys33 in βA4, Cys80 in βB1, and Cys23 in γC underwent oxidation during aging (>fivefolds) but this was increased significantly in cataract and was also associated with degree of cataract (Fig. 3C). We believe that these cysteine residues are having relative high surface exposure and accessibility for disulfide bond formation, and enhanced accessibility may be achieved during cataract formation, i.e. conformational change.

4. Cysteine Residues that are Barely Oxidized in Old Normal Lenses but Significantly Correlated With Cataract or Degree of Cataract Severity—Most interestingly, we found seven cysteine residues, whose oxidation was barely detectable in normal human lens but significantly increased in cataract. Cys154 in γC was almost completely oxidized in level II cataract (Fig. 4A) but was barely detected in aged noncatarac-
In the β-crystallin family, Cys52, Cys117, and Cys185 in βA1/3 were found oxidized eight-, six-, and two fold, respectively, compared with age-matched wild type lenses (Fig. 5). Similar to human cataract lenses, mouse Cys52 was more susceptible to oxidation, followed by Cys117, although we found no Cys170 oxidation in LEGSKO cataract lenses. Mouse βA1/3 has an extra cysteine residue Cys165, which is close to Cys170, and this may have helped to stabilize...
Cys170 via an intradisulfide bond. For βA crystallin, Cys33 and Cys99 were found oxidized two- and fourfold respectively (Fig. 5). Interestingly, Cys99 in human cataract lenses was only very mildly oxidized because of intradisulfide bond with Cys166 as mentioned above. Mouse βA does not have Cys166, and this may explain the finding of higher oxidation of Cys99 in LEGSKO than human cataract lenses. Both human and mouse βA crystallin have Cys151, but no oxidation was found in either cases. Apparently, Cys151 was highly resistant to oxidation during aging as well as cataractogenesis. For βB, Cys78, Cys150, and Cys240 were found oxidized 15-, 12-, and fourfold, respectively, relative to age matched wild type lenses (Fig. 5). Mouse βB1 Cys78 has a similar amino acid sequence as human βB1 at Cys80, except that only human has a single cysteine residue in βB1. For βB2, Cys38, and Cys67 were found oxidized six- and fourfold, respectively. This closely resembles the conditions of the human cataractous lens.

In the γ-crystallin family, mouse γD Cys19 and Cys109 were oxidized two- and tenfold respectively (Fig. 5) and Cys19 was oxidized both in old human and LEGSKO lens, whereas no Cys109 oxidation peptide was found in human lens. Instead, Cys33 and Cys42 oxidation was found in human cataract lenses. For γS, Cys83 and Cys130 were found oxidized four- and 12-fold, respectively (Fig. 5), which closely mimicked the human scenario together with Cys115 which was also oxidized in human γS. Oxidized Cys154 in αA, Cys33, Cys119 in βB2, and Cys19, Cys79 in γF were discovered in LEGSKO cataract lenses as illustrated in Fig. 5. Thus, overall there is strong agreement among oxidation sites of γ-crystallins between mouse and human, and some correlation among γ-crystallins.

In Vitro Modeling of Crystallin Cysteine Residue Oxidation
With Hydrogen Peroxide Reveals Selectivity of Oxidation Sites—To help define the relationship between the disulfide crosslink formation and crystallin aggregation, we continuously exposed mouse lens homogenate to 1 and 5 mM H₂O₂ at 37 °C for 12 h, and turbidity was measured using absorbance at 595 nm. The fraction at 2 h and 12 h were collected and subjected to ICAT analysis. A, Curve A: incubation without H₂O₂. Curve B: incubation with 1 mM H₂O₂. Curve C: incubation with 5 mM H₂O₂. The arrows and labels indicate the time points and sample ID of sample collection respectively for ICAT analysis. T2A: 2 h incubation w/o H₂O₂; T2B: 2 h incubation w/1 mM H₂O₂; T2C: 2 h incubation w/5 mM H₂O₂; T12A: 12 h incubation w/o H₂O₂; T12B: 12 h incubation w/1 mM H₂O₂; T12C: 12 h incubation w/5 mM H₂O₂. ICAT results (B–D): B, Cysteine residues found oxidized only in 5 mM H₂O₂ for 12 h, and no detectable oxidation was found in 2 h incubation at both 1 mM and 5 mM H₂O₂ condition, as well as 12 h incubation at 1 mM H₂O₂. C, Cysteine residues found oxidized at both 1 and 5 mM H₂O₂ at 2 h and 12 h time points (black bar), but not significantly oxidized over 12 h in 1 mM H₂O₂ or 2 h in 5 mM H₂O₂ (white bars). D, Cysteine residue oxidation that is associated with both dose and time of H₂O₂ incubation. All ICAT data was expressed as the ratio versus age-matched wild type control lens pool.
tion during the first 90 min, and aggregation/opacification appeared around 2 h and increased with time at both 1 mM and 5 mM H$_2$O$_2$, whereby 5 mM H$_2$O$_2$ clearly triggered more opacification. No protein opacification was found in lens homogenate without H$_2$O$_2$ present. We selected the 2 h time point, named as T2B and T2C, which was incubated with 1 mM and 5 mM H$_2$O$_2$, respectively, and 12 h time point, named as T12B and T12C, which was incubated with 1 mM and 5 mM H$_2$O$_2$, respectively. We determined the protein disulfide cross-link site using the ICAT method after pairing with same time points of the incubations without H$_2$O$_2$, named as T2A and T12A (Fig. 6A).

ICAT experiments revealed 30 cysteine residues involved in disulfide crosslinks in 12 types of crystallins (Fig. 6B–6D). Intriguingly, only selected cysteine residues in lens crystallins were oxidized into disulfide bond even under extreme oxidation condition by 5 mM H$_2$O$_2$ for 12 h (T12C). As expected, significantly more disulfide crosslinks were found in highly oxidized lens homogenate (T12C) compared with relative mild oxidation conditions (Fig. 6B–6D). Disulfide bonds were detected at Cys23 in $\gamma$A, Cys110/112 and Cys23 in $\gamma$C, Cys23 in $\gamma$B but only under extreme oxidation condition (T12C) (Fig. 6B). Tryptic peptide containing Cys23 from $\gamma$A, $\gamma$C and $\gamma$B has same sequence motif, and it could be assigned into each crystallin in random fashion. Nevertheless, this indicates that Cys23 in all three gamma crystallins are quite resistant to mild oxidation condition, but will be oxidized under strong oxidative stress environment. Interestingly, at 2 h oxidation (T2B and T2C) as well as 12 h under milder oxidation (T12B) conditions, several of the identified cysteine residues were oxidized at least twofold relative to lens homogenate incubated without oxidant, but no major difference was found between various oxidation conditions except T12C (Fig 6C). This indicates that these cysteine residues are susceptible to relatively milder oxidative stress but to a limited degree. It should be here mentioned that although 1 mM H$_2$O$_2$ is supraphysiological, this concentration was needed because kinetics of oxidation was too slow at 100 $\mu$M. We did find some cysteine residue oxidation and disulfide crosslink formation sites were associated with oxidation time and H$_2$O$_2$ concentrations, such as Cys33, Cys119 in $\beta$A2, Cys131 in $\gamma$C, Cys78 in $\beta$B1, Cys119 in $\gamma$F, and Cys185 in $\beta$A1/3 crystallins (Fig 6D).

**DISCUSSION**

The role of cysteine oxidation into disulfides in the pathogenesis of age-related cataract has been a topic of intense research for many years (29, 30). It is now a well accepted participant mechanism in the pathogenesis of age-related cataracts, particularly in nuclear cataractogenesis (31–33) despite an earlier report claiming, based on Raman spectroscopy, that crystallin disulfide crosslinks are not associated with aging and cataract formation in human lens (34). Subsequently, Lou et al. (35,36) also discovered a positive association of protein-thiol mixed disulfides with aging and cataract.

The question of site selectivity of cysteine oxidation into disulfides was first addressed by Smith J et al. (37,38) and Take moto L(39,40) who reported presence of an intramolecular disulfide bond in human $\alpha$A using mass spectrometry. More recently, Truscott et al. (18) reported an analysis of oxidized cysteine residues using N-ethylmaleimide (NEM) and iodoacetic acid (IAA) sequential alkylation method in each three young, old normal, and cataractous human lenses. Several oxidized cysteine residues involving disulfide crosslinks were discovered in grade III-IV cataracts. Yet, despite this progress, Truscott concluded that the absence of a suitable animal model was an impediment to further understanding of the role of oxidant stress in cataractogenesis (33). For this reason we generated the LEGSKO mouse whose depressed GSH levels are associated with lens opacification at 6 months(20) and are now asking the question of whether the changes observed in LEGSKO mouse are identical with those of human ARNC, and whether these can be simply mimicked with H$_2$O$_2$.

As shown in the Results section, an enormous amount of data was generated in all three systems, making their significance unwittingly difficult to grasp. For this reason we provide below a comparative synthesis which leads to the overall conclusion that the LEGSKO mouse mimics most human disulfide formation sites in beta $\beta$-crystallins and several of these also in gamma $\gamma$-crystallin.

The comparative summary of crystallin oxidation sites is presented in Table II. In this table, sites in black fonts were not found oxidized whereas those in red color were oxidized.

Four types of cysteine residues were found, i.e., (1) cysteine residues that are not prone to oxidation with age and cataract, (2) cysteine residues that are relative susceptible to oxidation with age and more significantly increased in cataract lenses regardless of severity, (3) cysteine residues that are susceptible to oxidation with age and more significantly associated with degree of cataract, and (4) cysteine residues that are barely oxidized with age but are significantly, if not only, found oxidized in cataract lenses and sometimes correlate with the severity of cataract. Our results are overall in good agreement with Truscott’s study(18). Both our studies found similar cysteine residues in various crystallins that are involved in disulfide bond formation with some differences. For example we did not find any oxidized cysteine residues in $\beta$B3, or Cys23/Cys25/Cys27 in $\gamma$S, but we found 13 more cysteine residues in $\beta$ and $\gamma$ crystallin families that were significantly involved in disulfide formation. The best example was Cys52 in $\beta$A1/3, which demonstrated high oxidation with aging and was significantly elevated in cataract in both human and LEGSKO cataract lenses. Another interesting example is Cys78/Cys79 and Cys42 in $\gamma$C crystallin. In Truscott’s study, Cys78/Cys79 was not significantly oxidized with age but was oxidized in level III-IV cataract, whereas Cys42 was oxidized neither in aged nor cataractous human lenses. In our study, we found Cys78/Cys79 oxidation only present in
level IV and V cataract lenses and Cys42 only present in level V cataract lenses. This indicates that both Cys78/Cys79 and Cys42 residues are quite resistant to oxidation except under strong oxidative stress conditions.

Our results also differ from those of Truscott's in a number of ways besides variations in cysteine residues coverage, in that we have also analyzed human cataract lens from various severity grade (II-V). For example, we found Cys170 in αA and Cys33 in αB were both significantly oxidized with age and with cataract, whereas in Truscott's study these were only associated with cataract. Also, we found Cys23 in γC was mildly oxidized in aged noncataractous lens and below level III cataractous lenses, but significantly oxidized in level IV and V cataracts, whereas Truscott's study concluded it is only associated with cataract. This may explain the discrepancy between the two studies because only level III-IV lenses were used in Truscott's study. Other potential reasons, i.e. different methodology, could also be the factor for some of the variation between the two studies. In that regard, the ICAT method is highly sensitive and specific because it allows selective enrichment of modified peptides (41).

**Table II**

Comparative summary of cysteine oxidation sites (red color) into disulfides in human old normal lens, human cataractous lens, LEGSKO mouse lens, and LEGSKO mouse lens homogenate oxidized with hydrogen peroxide

| Crystallin  | Human Lens (old normal) | Human Cataract | LEGSKO Cataract | Mouse (In Vitro Oxidized) |
|------------|-------------------------|----------------|-----------------|--------------------------|
| αA         | 131, 142                | 154            | 154             |                          |
| αB         | No cysteine             | No cysteine    |                 |                          |
| βA1/3      | 52**, 82, 117, 170, 185** | 52**, 117, 170, 185** | 52**, 117, 142, 185** | 52, 70, 82, 117, 142, 165, 170, 185 |
| βA2        | 27, 33, 37, 100, 119, 175 | 33**, 119**    | 13, 27, 33, 77, 100, 119 | 33**, 99**, 151 |
| βA4        | 5, 33**, 99**, 151, 166** | 5, 33**, 99**, 151 | 5, 33**, 99**, 151 | 5, 33**, 99**, 151 |
| βB1        | 80**, 80**              | 78**, 150**, 240 | 78, 150, 178, 240 | 78, 150, 178, 240 |
| βB2        | 38**, 67**              | 38**, 67**     | 38**, 67**      | 38**, 67** |
| βB3        | 39, 45                  | 207            | 39, 45, 207     |                          |
| γA         | 16, 19, 23, 33, 42, 79, 109, 111, 130 | 16, 19, 23, 33, 42, 79, 110, 112, 131 | 16, 19, 23, 33, 42, 79, 110, 112, 131 | 16, 19, 23, 33, 42, 79, 110, 112, 131 |
| γB         | 19, 23, 33, 42, 79, 110 | 79/80**, 110** | 16, 19, 23, 33, 42, 79, 110, 112, 131 | 16, 19, 23, 33, 42, 79, 110, 112, 131 |
| γC         | 23**, 42**, 79, 109, 130, 154 | 23**, 42**, 79/109, 154** | 16, 19, 23, 42, 79, 109, 111, 130 | 16, 19, 23, 42, 79, 109, 111, 130 |
| γD         | 19, 42**, 79, 109, 111 | 19, 42**, 109** | 19, 33, 42, 54, 79, 109, 110, 120 | 19, 33, 42, 54, 79, 109, 110, 120 |
| γE         | Does not exist          | 19**           | 19, 33, 42, 54, 79, 109 | 19, 33, 42, 54, 79, 109 |
| γF         | Does not exist          | 19**, 79**     | 19, 33, 42, 54, 79, 119 | 19, 33, 42, 54, 79, 119 |
| γS         | 23, 25, 27, 37, 83, 115, 130 | 83**, 115, 130** | 23, 25, 27, 37, 83, 115, 130 | 23, 25, 27, 37, 83, 115, 130 |
| γN         | 27, 51, 85, 103, 128, 128 | 116**, 128**   | 27, 51, 85, 103, 109, 116, 128, 162 | 27, 51, 85, 103, 109, 116, 128, 162 |

*a All sequences are displayed using MET for residue #1. Residues in black color have not been found oxidized, and blue color means less oxidized than in young lenses. A slash bar between the numbers indicates that precise oxidation assignment was not possible. The oxidation scale was relatively labeled as: "superscript indicates the fold change is < 5; "**" superscript indicates the fold change is between 5–20; "***" superscript indicates the fold change is between 20–50 and "****" superscript indicates the fold change is > 50.

Both in vitro and in vivo findings indicate that only selective cysteine residues are oxidized during the aging process, cataractogenesis or via in vitro oxidation. The formation of intermolecular disulfide bonds involves bringing the two-cysteine residues into very close proximity and several influencing factors are playing the role in cysteine oxidation, such as secondary/tertiary structure, solvent accessibility, the polarity and hydrophobicity of neighboring amino acids. With available crystal structure information, we can depict some potential mechanisms of individual cysteine oxidation discovered from this study. We believe that the first key criterion is surface exposure/accessibility, good examples are Cys38 and Cys67 in βB2 (supplemental Fig. S6), Cys19 in γD (Fig. 2D), which all are relatively well exposed to the surface and much more accessible for intermolecular disulfide bond formation. The second key criterion would be the degree of crystallin unfolding. Examples are Cys79 in γC (supplemental Fig. S7), Cys33 in γD (supplemental Fig. S8), Cys83 in γS (Fig. 3D) and Cys130 in γS (supplemental Fig. S9), in which cysteine is deeply buried. Only major conformational change will expose these cysteine residues in order to form intermolecu-
lar disulfide bond. This is well supported by Cys115 in γS, which is partially buried (Fig. 4D), and small degree conformational change will produce significant cysteine oxidation. We can then speculate that selectivity of cysteine residues oxidation in aging and cataractous lens is determined by (1) amino acids in the microenvironment and the secondary, tertiary and quaternary (chaperone effect) structure, (2) relative exposure and accessibility of cysteine residues relative to the surface, and (3) conformational changes/unfolding and exposure of buried residues because of age-related chemical-physical processes that may involve oxidation itself or any other damaging process.

The lens evolved as an anaerobic biological system with millimolar concentrations of both glutathione (GSH) and ascorbic acid (ASA). However, both GSH and ASA protective systems are impaired in aged human lens, particularly in the lens nucleus (42,43). Our recently developed GSH biosynthesis conditional knockout mouse (LEGSKO) demonstrated similar phenotypical characteristics with human in age-related nuclear cataractogenesis. This study provides new evidence that the oxidatively stressed mouse crystallins form disulfide crosslinks similar to human lens aging and cataract formation. In LEGSKO cataract lenses, Cys52, Cys117, and Cys185 in βA1/3, Cys33 and Cys99 in βA4, Cys88 and Cys130 in γS, Cys19 and Cys109 in γD, Cys38 and Cys67 in βB2, and Cys80 in βB1 were all in full agreement with the findings from human cataract lenses. In LEGSKO cataract lenses, we started to see Cys154 oxidation in αA crystallin, whereas we did not find it in human lenses. Because mouse has a single cysteine residue (Cys154), and human has two cysteine residues (Cys131 and Cys142), our speculation is that Cys131 and Cys142 are mainly present as intramolecular disulfides. This may explain why Cys154 is prone to oxidation in the mouse, because ICAT approach is more sensitive to intermolecular disulfide bond determination. Indeed, Takemoto (39) reported a high level of Cys131 and Cys154 intradisulfide bond formation. Similar reasoning can be used to explain Cys170 (βA1/3) oxidation that was found in human but not LEGSKO lenses, because mouse has extra cysteine Cys165 close to Cys170. For crystallin βB3, we did not find any cysteine oxidation in human lenses, but Cys207 was found oxidized in mouse lenses. Both Cys39 and Cys45 are conserved between human and mouse; Cys207 is not present in human and chimpanzee as shown in supplemental Fig. S10. Similarly, two extra-oxidized cysteine residues (Cys150 and Cys240) were found in mouse βB1 crystallin, but it has all been replaced by valine in chimpanzee and human (supplemental Fig. S11). Quite opposite, Cys166 in βA4 was found in human but not in LEGSKO mouse lenses. One of βA4 crystallin isoform (isoform2) does not have Cys166 as shown in supplemental Fig. S12. We speculate that βA4 isoform2 is the dominant form in mouse lenses. Some cysteine residues oxidation were also discovered in γF, γB and γN crystallins but not detected in human lenses. Cys78-Cys79 sequence motif in γB is very close to γC, one type of human γN isoform has only 125 amino acids but without Cys116 and Cys128 included, and γF crystallin was not present in human lens.

Finally, two important points need to be addressed, i.e. whether the reported formation of disulfides is important for the opacification process per se, and two, whether the concept of disulfidome is justifiable in the context of the ICAT procedure used in this study.

First, the relevance of individual cysteine oxidation sites for crystalline aggregation and opacification is currently the subject of intense research in our laboratory whereby we are systematically mutating each of the cysteine oxidation sites described above in order to clarify their relative roles in comparison to all other modifications found in the aging and cataractous human lens. However, the strongest argument for their importance in lens opacification is that both the LEGSKO mouse lens and the H2O2 treated crystallins became opaque in just 6 months and 12 h, respectively, together with massive evidence of cysteine disulfide formation. We are aware that tryptophan oxidation will also have to be systematically studied, as suggested by Truscott (11), if we are to understand whether it is more important to the opacification process than cysteine oxidation.

Second, whether the term disulfidome is justified impinges on whether the tris(2-carboxyethyl) phosphine reagent (TCEP) used in the ICAT labeling procedure also reduced other oxidative modifications prior to derivatization with the ICAT reagent. TCEP is said to be highly selective for disulfides, however, as suggested by Truscott (11), if we are to understand whether the tris(2-carboxyethyl) phosphine reagent (TCEP) used in the ICAT labeling procedure also reduced other oxidative modifications prior to derivatization with the ICAT reagent. TCEP is said to be highly selective for disulfides. Obviously our data likely include not only protein-protein but also some of the protein-GSH disulfides, the presence of which is well documented in the lens (Lou et al. (45)). No reducing activity of TCEP, GSH and DTT toward stable oxidative modifications of SH groups (sulfenic, sulfone, or sulfonate) was reported to our knowledge (46, 47). However, TCEP was found to reduce sulfenic acids, the precursor to disulfides, S-nitroso groups (48, 49), as well as S-sulfo peptides (50). Several reasons however suggest that these are highly unlikely to be significant contributors to the species assayed by the ICAT procedure used in this study. First, protein nitrrosylation in lens, while studied in vitro (51), has not been reported in vivo. Second, although sulfenic acid is an important biological modification in cell signaling, the consensus is that it would be labile to the acidic conditions created by the TCA that we used for the ICAT procedure (49,52,53). Finally, the S-sulfo modification is an important by-product during in vitro peptide synthesis or protein oxidation via mixing with inorganic sulfate (50,54), but to our knowledge, it has not been described in biological proteins. In sum, most if not all cysteine oxidation sites identified by the ICAT procedure can be safely attributed to disulfides, thus warranting the term “cysteine disulfidome” in the context of this study.

In summary, our comprehensive and comparative disulfide crosslink analysis first revealed evidence of a similar age-related shift from intra- to intermolecular disulfide bond
formation in both mouse and human lens, and second, it revealed that most oxidation sites with exception of some sites in γ-crystallins are identical between mouse and human. Most importantly, many of these sites can be easily reproduced by in vitro oxidation. Obviously, the precise relationship between the formation of these disulfides and opacification process per se still needs to be further clarified. In that regard, this study clearly validates the LEGSKO mouse as a humanized mouse model for further investigation of the relationship between cysteine oxidation and cataractogenesis.

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