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Identification of Proteins that Modify Cataract of the Eye Lens

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Abstract

The occurrence of a nuclear cataract in the eye lens due to disruption of the $\alpha 3$ Cx46 connexin gene, *Gja3*, is dependent on strain background in a mouse model, implicating factors that modify the pathology. The differences upon cataractogenesis in the urea soluble proteins of the lens of two mouse strains, C57BL/6J and 129/SvJ, were analyzed by a comparative proteomics approach. Determination of the complete proteome of an organ offers the opportunity to characterize at a molecular level, differences in gene expression and post-translational modifications occurring during pathology and between individuals. The abundance of 63 protein species was altered between the strains. A unique aspect of this study is the identification of chaperonin subunit 6A, mortalin, ERp29 and syntaxin binding protein 6 in the eye lens. DNA polymorphisms resulting in non-conservative amino acid changes that led to altered physicochemical properties of the proteins were detected for mortalin, chaperonin subunit 6A, annexin A1 and possibly gamma N crystallin. The results show HSP27/25 and/or ERp29 are the likely major modifying factors for cataractogenesis. Extension of the results suggests that small heat shock proteins have a major role for influencing cataract formation in humans.

Keywords

Cataract; Modifying Factors; Molecular Chaperones; Proteomics; Syntaxin binding protein

1. Introduction

The mature lens of the eye consists of two cell types; the abundant elongated fiber cells aligned with the general direction of light entry and a single layer of metabolically active cuboidal epithelial cells at the lens anterior surface. The innermost region of the lens, the lens nucleus consists of fiber cells that were formed prenatal, the primary fiber cells. The younger, more peripheral secondary fiber cells of the lens cortex are derived from the epithelial cells located at the lens equator which are renewed by continuous migration of cells from the anterior apex. These equatorial cells undergo terminal differentiation, losing organelles including the endoplasmic reticulum, elongate and are subsequently layered as the lens grows. The mature fiber cells contain predominately a class of proteins termed crystallins [1]. The high concentration of proteins and short-range order of the crystallin matrix, as well as the organization of cytoskeletal and membrane constituents are responsible for lens transparency [2]. This order can be disrupted by post-translational modifications resulting in unfolding, insolubilization and aggregation of lens proteins and

ultimately in a lens opacity that is termed cataract [3,4]. This protein conformational disease is the primary cause of visual impairment worldwide. It is linked to a number of risk factors and genetic predisposition [5,6]. Recent figures attest to some 600,000 new cases every year in the USA and the UK [7].

The development of cataracts in mice lacking the gap junction protein connexin $\alpha 3$ Cx46 was described previously [8,9,10]. In the absence of the $\alpha 3$ Cx46 gene *Gja 3* the C57BL/6J strain is less susceptible to formation of a cataract than the 129/SvJ strain [11]. Nuclear opacities are observable at day 11–12 in the lens of the homozygous *Gja3* knockout mutant in a 129/SvJ background while lenses remain transparent in the C57BL/6J background. Indeed, the severity of the nuclear cataract was significantly milder on a C57BL/6J compared to a 129/SvJ background even at two months of age [11]. This suggests the presence of strain specific factors that modify cataract incidence and development.

The origin of the specific factors may be genetic, epigenetic or environmental. Regardless, they are likely to involve changes in protein abundance and/or function. In the present study, the urea soluble proteins of the lens of ten day old mice of the wild type and *Gja3* knockout mutant of the *Mus musculus* C57BL/6J and 129/SvJ strains were compared by two-dimensional gel electrophoresis (2-DE), a technique capable of high-resolution separation of proteins by their physiochemical properties. Proteins that potentially differ by only one amino acid or post-translational modification migrate to discrete positions within the gel matrix. After staining, the intensity of these protein spots is proportional to the protein abundance in a dynamic range of four orders of magnitude [12]. Mass spectrometry (MS) was used for protein identification.

Lenses from 10 day old mice were examined because the process of cataractogenesis is likely to be initiated at this young age just prior to the actual formation of the cataract. All of the proteins that varied in their abundance by 2-fold or more between the wild type of both strains were identified (total 63 protein species, Table 1). Proteins whose abundance varied exclusively due to the lack of $\alpha 3$ Cx46 connexin were also identified. In the lens, most of the fiber cells lack organelles and possess low biosynthetic activity, including transcription and translation. Therefore, it is more suitable for analysis by proteomic rather than genomic methods. Hence, in this study, we place special emphasis on the proteomics approach. Based on this comprehensive assessment of differential protein abundance in the lenses of the C57BL/6J and 129/SvJ strains it is suggested that HSP27/25 and/or ERp29 are major genetic modifiers of cataractogenesis.

2. Materials and Methods

2. 1. Sample preparation

All animal experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the guidelines established by the Animal Care Committee of the University of Illinois at Chicago. The lenses from each three ten day old wild type and *Gja3* knockout mutant C57BL/6J and 129/SvJ animals were stripped of their capsule and adhering non-lenticular material by rubbing on tissue paper (three biological replicates). Subsequently they were ground down in a mortar and pestle under liquid nitrogen. The resulting powder was suspended in 5 times 10^{-3} weight per volume (w/v) buffer containing 9 M urea, 70 mM DTT, 50 mM potassium chloride, 25 mM Tris pH adjusted to 7.3, 3 mM EDTA, 2.9 mM benzamidine, 1 mM PMSF, 2.1 μ M leupeptin, 0.1 μ M pepstatin, 2% (w/v) Servalyte 2–4 and 2% (w/v) CHAPS. The suspension was agitated and centrifuged at 100000 g. The supernatant, containing the urea soluble fraction of the proteins of the lens was kept and stored at -80°C . The protein concentration was determined with a modified protocol according to Lowry [13,14]. For real time PCR

lenses were dissected in RNase-free medium, transferred immediately into extraction reagent (TRIzol; Invitrogen-Gibco, Rockville, MD) on ice, and stored at -80°C .

2. 2-Dimensional gel electrophoresis of lens proteins

The proteins from the three preparations of the C57BL/6J and 129/SvJ wild type and knockout lenses were each separated with 2-DE (23 cm $\times$ 30 cm $\times$ 0.25 cm gels), combining carrier ampholyte isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the protocol of Klose and Kobalz [12]. Sixty μg of proteins were loaded onto the IEF tube gels for silver staining [15], or 300 μg for Coomassie Brilliant Blue (CBB) G250 staining [16].

2. 3. Software supported 2-DE spot matching and image analysis

The proteins of each of the three replicate preparations of the lenses of the wild type and knockout mutant of both strains were separated into around 1000 protein spots each. 2-DE was performed at least twice for every one of the biological replicates (at least two technical replicates for each biological replicate). The silver stained 2-DE gels were scanned and the images of the protein spot patterns imported into the PDQuest 2-DE image analysis software version 7.1.0 (Bio-Rad, Hercules, CA, USA) where they were incorporated into a match set. The staining intensity of the individual protein spots was normalized to the total staining intensity of all protein spots on a gel. The protein spot patterns were calibrated with mathematical regression methods [17]. This was done using the GelCali© software in the publicly available Proteome 2-D PAGE Database from the Max Planck Institute for Infection Biology (Berlin, Germany) under the URL <http://web.mpiib-berlin.mpg.de:9080/gelcali/>. The correlation coefficient (R^2) of the protein spot patterns (meaning the position and intensity of every spot on the 2-DE gels) of the three biological replicates was 87% for the C57BL/6J wild type, 92% for the C57BL/6J *Gja3* knockout mutant and 90% for the 129/SvJ wild type and knockout mutant. Protein spots that had a 2-fold or greater increase or decrease of the mean staining intensity of the biological replicates were selected for analysis with mass spectrometry (MS). A 2-fold or greater change in staining intensity and hence protein abundance was chosen to eliminate any possibilities of errors or inaccuracies due to technical limitations and so ensure only biologically meaningful results. The coefficient of variance (CV) of the staining intensity of biological replicates of all of the selected spots was calculated and is shown in Supplementary table 1. The mean CV of all of the spots was determined to be 33%, the median 27%. All protein spots that were selected had clearly defined spot borders and little to no streaking or smearing.

2. 4. In-gel digestion of proteins

Protein spots were excised from CBB G250 stained 2-DE gels and the proteins were in-gel digested as described by Otto and coworkers [18] with some modifications. The proteins were destained in 200 mM NH_4HCO_3 in 50% acetonitrile (ACN) in water and digested with 0.4% (w/v) trypsin (Sequencing grade modified trypsin, Promega, Madison, WI, United States) in 50 mM NH_4HCO_3 in 5% ACN in water at 37°C overnight. The resulting peptides were extracted from the gel by passive diffusion in 0.5% trifluoro acid (TFA) in 60% ACN in water and in 100% ACN. The extracts were combined and the peptides were dried. The peptides were solubilized in 0.1% TFA in 33% ACN in water for matrix assisted laser desorption ionization (MALDI) MS or in 0.1% formic acid (FA) in water for electro spray (ESI) ion trap MS. Some proteins were digested with Asp-N in 50mM NH_4HCO_3 in 5% ACN in water at 37°C overnight otherwise according to the manufacturers instructions.

2. 5. Mass spectrometry of in-gel digested proteins

The solubilized peptides were mixed with an equal volume of 4% (w/v) 2,5-dihydroxybenzoic acid (DHB) (Bruker Daltonics, Billerica, MA, USA) in 0.5% TFA in 33% ACN in water for analysis with a Voyager Elite MALDI time of flight (TOF) mass spectrometer (Applied Biosystems, Foster City, CA, USA). Peptide mass fingerprint (PMF) mass spectra were recorded with a mass accuracy of better than 100 ppm with external calibration with standard peptides with the mass to charge ratios (m/z) 896.572, 1503.888 and 2154.168.

Alternatively the peptides were mixed with a four times volume of 0.5% (w/v) α -cyano-4-hydroxycinnamic acid (CHCA) (Sigma Aldrich, Munich, Germany) in 0.3% TFA in 50% ACN in water for analysis with a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems). PMF mass spectra were recorded with a mass accuracy of better than 30 ppm with internal calibration with three autolysis products of porcine trypsin which was used for in-gel digestion of proteins, calibration with an external standard, supplied by Applied Biosystems for the calibration of MALDI plates achieved a mass accuracy of better than 100 ppm. The five m/z peaks with the highest intensity that surpassed a signal to noise threshold (S/N) of 10 were selected for collision induced dissociation (CID) without collision gas. The resulting product ions were recorded in tandem mass spectrometry (MS/MS) mass spectra under stop conditions if at least 15 peaks in the spectrum surpassed (S/N) of 40.

Some solubilized peptides were loaded onto a C18 column (150 $\times$ 0.075 mm) (ZORBAX stable bond, Agilent Technologies, Palo Alto, CA, USA; C18 PepMap^{TM}, LC Packings, Sunnyvale, CA, USA) with a nano HPLC system (1100 series, Agilent Technologies; UltiMate^{TM}, LC Packings). The peptides were eluted from the column into an ion trap mass spectrometer (XCT, Agilent Technologies; LCQ Deca XP, Thermo Electron, Waltham, MA, USA) with an ESI ion source at a flow rate of 200 nl per minute with a linear gradient inclination of 3–30% 0.1% FA in ACN over a duration of 40 or 175 minutes. Data dependent acquisition of ion mass spectra was throughout chromatographic duration if the ion signal intensity exceeded 10000 counts and 2% of base intensity. The ions with the three highest intensities in a full scan of all ions stored in the trap were selected for CID and their product ions recorded in MS/MS spectra. The selected ions were excluded from further MS/MS analysis for 30 seconds (exclusion duration 30 s). Calibration with an external standard supplied by Agilent Technologies achieved a mass accuracy of 0.4 Da or better.

2. 6. Identification of lens proteins

The peptide and peptide fragment ion masses in the mass spectra were used to search the NCBI protein sequence database with the Mascot version 1.9 and 2.0 (Matrix Science, Boston, MA, USA) and the SEQUEST version 1.9 (Thermo Electron) mass spectrometry software suite. The set of ion masses from a mass spectrum used as an input for the software (peak list) from PMF mass spectra recorded with the Voyager Elite mass spectrometer were generated by manual selection of ion signals with the Grams/386^{TM} software version 3.03 (Galactic Industries, Salem, NH, USA).

The peak lists from PMF mass spectra recorded with the 4700 Proteomics Explorer mass spectrometer were generated automatically with the 4000 Series Explorer^{TM} software version 3.0 with a minimum S/N of 10 and a maximum of 100 peaks. The peak lists from MS/MS mass spectra were generated with a minimum S/N of 2, a minimum peak area of 5, a maximum number of 50 peaks per 200 Da and a maximum number of 100 peaks. The combined MS and MS/MS peak lists from mass spectra recorded with the XCT and the LCQ Deca XP mass spectrometer were generated automatically applying the DataAnalysis

software version 2.0 (Agilent Technologies) and the SEQUEST software respectively with a minimum absolute ion signal intensity threshold of 1×10^5 counts.

The database searches were performed with the software species controller set to *Mus musculus*. A maximum peptide mass accuracy error of 100 ppm was tolerated in searches with the data from the Voyager Elite mass spectrometer with one exception where an error of 150 ppm was tolerated. A maximum peptide mass accuracy error of 60 ppm and peptide fragment mass accuracy error of 0.4 Da was tolerated for searches with the data from the 4700 Proteomics Explorer mass spectrometer. A maximum peptide mass and peptide fragment mass accuracy error of 0.4 Da was tolerated in searches with the data from the XCT and the LCQ Deca XP mass spectrometers. A maximum of one trypsin missed cleavage was tolerated.

The protein identification was only considered conclusive if a Mascot Score indicating protein identity with a P-value less than 0.05 was achieved. Two MS/MS spectra of peptides with different primary structure which contained four consecutive mass peaks, indicative of three sequential amino acid residues, had to be achieved for database searches with MS/MS data. Peptides identified with the SEQUEST software were the top ranking suggestions for the respective MS/MS spectrum with an Xcorr of 2 or greater and an Rsp of less than 1. Proteins were also identified by matching congruent spots to identified spots in the protein spot patterns.

2. 7. Real time PCR

Real time PCR was performed using a protocol that will be described in detail later. Briefly, 2 µg of total RNA was used for generation of cDNA by reverse transcription using a kit according to the manufacturer's protocol (cMaster RT, Eppendorf AG, Hamburg, Germany). Using a SmartCycler instrument (Cepheid: Sunnyvale, CA) real-time PCR was performed on the cDNA, using HotMaster Taq Polymerase, with the product detected by SYBR Green I dye staining. The cycling conditions consisted of 1 cycle at 95°C for 100s for denaturing followed by 32–40 3-step cycles for amplification (each cycle consisted of 95°C incubation for 20s, appropriate annealing temperature for 10s and product elongation and signal acquisition at 70°C incubation for 20 s). Primer pairs used for mouse HSP27/25 were 5'TCTCTCGGTGCTTCACCC3' and 5'ATGGCTTCTACTTGGCTCCA3'. An equivalent amount of lens cDNA from the different mice was used as normalized by their content of 18S rRNA.

3. Results

3.1 A comprehensive study of differential protein abundance in the lens of the eye

The urea soluble proteins of the lens whose abundance varies 2-fold or more between the wild types of the strains were completely determined. In total, these were 94 protein species, 63 of which were identified by MS (Table 1). The abundance of the unidentified protein species was too low for them to be identified. The analysis was then extended to the *Gja3* knockout mice to identify proteins that are altered exclusively due to the lack of $\alpha 3C \times 46$ connexin. Four additional protein species were identified, two alpha A crystallins (SSP 4103 and SSP4114) and two beta B1 crystallins (SSP3209 and SSP5312), leading to a total of 67 different protein species. All of these protein species are central to what is presently known about cataract development and/or the different lens phenotypes of the mouse strains or in case of novel findings they can be readily connected to pathogenesis. This indicates the high quality of the study and makes us certain we are observing genuine biological phenomena in the model system.

The proteins of the different lenses were separated into about 1000 protein spots each. The intensities of the spots were normalized to the total intensity of all spots on the 2-DE gel and then used for protein quantification. Spot intensities were compared between strains for wild types and *Gja3* knockout mutants as well as between the wild types and mutants in each strain. Because the lens contains a high concentration of crystallins and only urea soluble material was analyzed, the proteins detected are likely to represent cytoplasmic constituents rather than integral membrane proteins. Furthermore, the proteins are likely derived from the fiber cells that make up the bulk of the lens, which has only a single layer of epithelial cells.

The objective of this study was to determine the protein factors that initiate and/or influence the formation of a cataract. Thus, lenses from 10 day old mice were examined since this is an age at which it is assumed that the process of cataractogenesis is initiated, yet is just prior to the actual formation of a cataract phenotype in the *Gja3* knockout mice [11]. That this is a reasonable assumption is indicated by the slightly different protein profiles of knockout compared to wild type lenses especially in crystallin proteolytic fragments (Table 2). This greatly simplifies interpretation of the results because, in an observable cataract, changes in protein species that are due to the opacity rather than its initiation may be identified and hence provide minimal information on cataractogenesis.

The 2-DE protein spot patterns and mass spectrum peak lists can be found in the Proteome 2D-PAGE Database from the Max Planck Institute for Infection Biology (Berlin, Germany) under the URL <http://www.mpiib-berlin.mpg.de/2D-PAGE/>. The SSP numbers in the text and tables correspond to the protein spots on these patterns.

3. 2. The protein phakinin (CP49) and the beaded filament are completely absent from lenses of the 129/SvJ strain

Our investigation detected ten protein species of the protein phakinin (CP49) in lens of the wild type and the *Gja3* knockout mutant of the C57BL/6J strain. Most of the protein species likely represent truncation products because they migrated to a position of lower molecular weight than the full length protein on the 2-DE gel. All of the protein species are absent in the urea soluble proteins of the lens of the wild type and the mutant of the 129/SvJ strain. The abundance of the CP49 protein is not significantly affected by the *Gja3* knockout mutation (Figure 1).

CP49 is one of the two obligate assembly partners of the filamentous backbone of the major cytoskeleton element in lens fiber cells, the beaded filament [19]. Absence of the protein and hence the beaded filament itself in the 129/SvJ strain has been previously described and determined to be due to a DNA polymorphism that results in a premature stop codon [20,21]. Our findings corroborate these studies. Filensin, also named CP94, is an interaction partner of CP49 and was detected in both of the examined mouse strains but was significantly less abundant in 129/SvJ. Most likely this is due to destabilization of this protein due to lack of CP49.

3. 3. Non-conservative DNA Polymorphisms in C56BL/6J and 129/SvJ Mice

Four other proteins, specifically, mortalin (SSP 3804; SSP 3809), chaperonin subunit 6A (SSP 6717; SSP 6704), annexin A1 (SSP 6325; SSP 5409) and gamma N crystallin (SSP 6132; SSP 6122) migrated to different pI positions on the 2-DE gel in the C57BL/6J strain compared to the 129/SvJ strain but did not change in their relative abundance. This may be due to DNA polymorphisms that result in non-conservative amino acid changes. Indeed, it has been previously reported that mortalin differs in primary structure between the C57BL/6J and 129/Sv strains [22].

Comparison of the predicted protein sequences for chaperonin subunit 6A that are deposited in the Genbank database for the C57BL/6J (GI:74144061) and 129/SvJ (GI:468554) strains indicates an amino acid change of E348G. This would alter the pI of the protein and account for the shift that we observe. While the protein sequence for annexin A1 is not available for the 129/SvJ strain, comparison of the sequences for the C57BL/6J (GI:7418300) and the Balb/C strain (GI:198845) indicates an amino acid change, R212I, that would also result in a shift to a more basic pI position. The gamma N crystallin cDNA sequence from the 129/SvJ strain was determined and found to have a nonconservative amino acid change of Q33H compared to the C57BL/6J strain consistent with an altered migration position of the proteins on the 2-DE gels.

3. 4. Truncated crystallin proteins are differentially abundant in the wild type and the mutant of both strains

The crystallins constitute 80–90% of the entire lens protein mass. Twenty one of the protein species with a 2-fold or greater difference in abundance between the wild type of both strains were identified as crystallins. These crystallins were at positions of a lesser molecular weight on the 2-DE protein spot patterns than the mature proteins with complete primary structure indicating protein truncation. Truncation of crystallins is well known and accompanies visual deterioration and cataract during ageing and because of foreign or inherent insult [23,24,25,26].

The abundance of the 21 crystallin truncation products that were determined to be at least twice as abundant in the 129/SvJ compared to the C57BL/6J strain and were also at least minimally abundant in the C57BL/6J strain are listed in Table 2 and shown in Figure 2. A Student's t-test also determined the abundance of these truncation products to be significantly less in the *Gja3* knockout mutant of the C57BL/6J compared to the 129/SvJ strain ($\alpha=0.05$; P-value=0.006). This indicates an increase in proteolytic activity upon disruption of the *Gja3* gene which is more pronounced in the C57BL/6J strain, presumably because proteolysis is already elevated in 129/SvJ due to inherent factors. These observations underscore that C57BL/6J is more resistant to *Gja3*-induced cataract that is a result of crystallin truncation and show that loss of the gap junction protein has some effects similar to natural effects in both strains which are however stronger in 129/SvJ. These inherent effects and the effects of the mutation are not necessarily cumulative and are features of the healthy state of the lens in both strains.

3. 5. Two alpha A- and two beta B1 crystallin truncation products are caused exclusively by the mutation and are involved in cataractogenesis

Two alpha A crystallin protein species (spots SSP 4103 and SSP 4114) are abundant only in the *Gja3* knockout mutant of both strains (Figure 3A). Assuming that their apparent molecular weights are accurate, one of these (SSP 4114) would correspond to residues 1-163 of alpha A crystallin since the C-terminal peptide was identified by ion trap tandem mass spectrometry (MS/MS) of an Asp-N in-gel digest. The other (SSP 4103) presumably corresponds to residues 1-157, as this was also identified as the C-terminal peptide by matrix assisted laser desorption ionization time of flight (MALDI/TOF) MS in a tryptic digest. However, since residue 157 is also a trypsin cleavage site this is not conclusive. Such fragments have been previously detected and characterized in a study using Lp82/85 [27, 28]. Since previous studies have determined that Lp82/85 is involved in cataractogenesis occurring in the lenses of *Gja3* knockout mice, this is also consistent with our assumption that initiation of this process occurs at or prior to 10 days of age.

Two beta B1 crystallin truncation products (SSP 3209, Figure 3B; SSP 5312, Figure 3C) are also highly abundant in the mutant and completely absent in the wild type of both strains.

The protein species in SSP 5312 was examined in more detail with ion trap MS/MS. The truncation site was determined to be near the beta B1 crystallin N-terminus between threonine residue 27 and glycine residue 28 and is probably a result of calpain protease activity confirming previous results [29] (Figure 4). It is known, that truncated beta B1 crystallin induces cataract [30]. The high abundance of these four crystallin truncation products exclusively in the *Gja3* knockout mutant of both strains can only be due to the gene knockout and subsequent effects. Possibly together with the previously described gamma crystallin truncation product [8] in the *Gja3* knockout 129/SvJ strain, they may aid in the formation of the cataract in this strain. All four protein species are more abundant in the knockout of the 129/SvJ than the C57BL/6J strain which is to be expected in the presence of modifying factors that suppress cataractogenesis in C57BL/6J.

3. 6. Chaperones prevent cataract development in the *Gja3* knockout mutant of the C57BL/6J strain

Three HSP27/25 protein species were detected in the wild type and the mutant of both strains (SSP 2315, SSP 3313 and SSP 4316). The protein spots, shown in Figure 5, have an observed molecular weight (Mw) of 26 kDa which is in disagreement with the theoretical molecular weight of 23 kDa. This discrepancy has also been observed by others [31]. The isoelectric point positions of two spots are more acidic than the calculated pI of 6.12. This suggests some form of modification. HSP27/25 phosphorylation at serine residues 15 and 86 is well documented [32]. In a previously published study we confirmed phosphorylation at serine residue 15 for the protein species in SSP 3313 with alkaline phosphatase treatment and MALDI-TOF mass spectrometry. This resulted in quenching of the m/z peak for the phosphorylated peptide and appearance and spiking of a peak that was absent before treatment with the enzyme that corresponded to the respective unphosphorylated peptide in the PMF mass spectra [33]. In addition, peptide mass fingerprint mass spectra acquired from spot SSP 2315 suggest protein phosphorylation at both sites, serine residues 15 and 86, which would be in agreement with the spots most acidic position (Figure 6).

The total abundance of HSP27/25 is 4-fold increased in both the wild type and the mutant of the C57BL/6J compared to the 129/SvJ strain and all three protein species are at least two times more abundant in the former strain (Table 3). There is no significant difference in protein phosphorylation states between the strains. The levels of *Hsp27/25* gene expression were determined by real time PCR (Figure 7). The expression of the gene is increased 4-fold in the wild type and 4.5-fold in the *Gja3* knockout mutant of the C57BL/6J compared to the 129/SvJ strain. The protein abundance and gene expression data correlate, suggesting that the increase in HSP27/25 is due to either transcriptional and/or post-transcriptional RNA regulation. The high abundance of HSP27/25 protein and RNA in the wild type and the mutant of C57BL/6J is consistent with its potential as a genetic modifier of cataractogenesis.

The endoplasmic reticulum protein 29 (ERp29) was detected in the lenses of the wild type and the mutant of the C57BL/6J strain. This protein species is not significantly present in the lens fiber cells from the 129/SvJ strain. Comparison of ERp29 sequences derived from C57BL/6J (GI:12836245), Balb/C (GI:74207676) and NOD (GI: 74181930) indicates no change in the protein sequence. However, we have determined the cDNA sequence of ERp29 from the 129/SvJ strain and find nonconservative amino acid change of N135S and Q187L. Whether this change influences the stability of ERp29 is not known.

3. 7. Syntaxin binding protein was uniquely identified as increased in abundance in the C57BL/6J *Gja3* knockout lens

In this study syntaxin binding protein 6 was found to be more than twice as abundant in the lens of the *Gja3* knockout mutant of both strains and the wild type 129/SvJ strain compared

to the wild type C57BL/6J strain (Figure 8). There is a discrepancy between the position on the 2-DE protein spot patterns and its molecular weight and pI, suggesting the protein species we detected could be a modified form of syntaxin binding protein 6 or an isoform. With the exception of the crystallins, this was the only protein whose abundance was increased to this degree in the mutant compared to the wild-type, suggesting that it was due to the lack of $\alpha 3\text{Cx}46$. However, its abundance is also elevated in the wild type 129/SvJ strain. This indicates the cause is genetic. Still, we think this protein is unlikely to be a genetic modifier for the cataract phenotype since it is approximately equally abundant in the C57BL/6J and 129/SvJ *Gja3* knockout lens. Its function as a regulator of ion channels however makes its presence in the lenses with perturbed ion flux intriguing and suggests it inhibits cataract development in the C57BL/6J strain.

4. Discussion

The avascularity of the lens and its metabolic demands require pathways to supply and remove metabolites and ions. These pathways are provided by gap junctions that contain the protein connexin. In the lens fiber cells there are two connexins, the $\alpha 3\text{Cx}46$ and the $\alpha 8\text{Cx}50$. In the mouse, the knockout of the $\alpha 3\text{Cx}46$ connexin gene, *Gja3*, results in a dense nuclear cataract beginning at about 12 days of age [8,9,10]. The disruption of gap junction pathways mediated by $\alpha 3\text{Cx}46$ leads to an increase in Ca^{++} concentration in the nuclear region of the lens [10,34]. This activates a lens specific isoform of calpain 3, Lp82/85 that is Ca^{++} -dependent [10,35]. The involvement of Lp82/85 is strongly supported by a study using calpain 3/*Gja3* double knockout mice [35]. The activation of Lp82/85 leads to cleavage of gamma crystallin between asparagine residue 73 and serine residue 74 resulting in generation of 9 and 11 kDa gamma crystallin truncation products [8,9,10,35].

Targeted disruption of the CP49 gene in the C57BL/6J strain leads to radical changes in fiber cell morphology and plasma membrane organization that result in decreased lens optical quality and light scatter but no observable cataract [36,37]. This suggests that the loss of the beaded filament is compensated to some extent by other intermediate filaments containing vimentin that create an atrophied cytoskeleton, which nevertheless can maintain lens architecture as a whole and its transparency [37]. Clearly, CP49 and the beaded filament are central to healthy lens development and pathogenesis. The results of our proteomics approach however show that there are other additional factors which could play a crucial role in suppressing cataract development in the C57BL/6J strain and that lack of CP49 is not sufficient for making the 129/SvJ strain more susceptible to this disease.

Genes with non-conservative DNA polymorphisms, namely mortalin, chaperonin subunit 6A, annexin A1 and gamma N crystallin were detected by our differential analysis due to the resulting changes in the pI/Mw values in the mouse strains. It is not known how the proteins function is affected by these amino acid changes. Notably, the abundance of these proteins did not change significantly in the lens when one strain was compared with the other and many of these proteins also exist in other cell types. Together with the quality control mechanisms in cells that degrade malformed proteins, this is consistent with the mutations having minimal effects on protein function thereby disqualifying them as modifiers of cataract.

The proteins of the crystallin superfamily are by far the most abundant proteins in the lens of the eye. Based on our experience and others [18,20] we estimate that on a 2-DE gel of the water or urea soluble proteins of the young lens, which separates the proteome into a total of about 1000 protein spots, about 200 to 300 spots would be crystallin protein species. For the truncated crystallin proteins that were determined to be differentially abundant between the wild types of the examined strains we note that they do not cause cataract and may even be

necessary components of the healthy crystallin matrix, which is underscored by their presence in the wild type of C57BL/6J. The protein species approximate equal abundance in the knockout and the wild type of 129/SvJ indicates that factors specific to this strain, most notably lack of CP49 and the beaded filament and the loss of the α 3C α 46 protein due to the mutation have very similar effects. The reduced abundance in the knockout of C57BL/6J and the approximate equal abundance in the wild type and knockout in the 129/SvJ strain suggest that inhibitors of cataractogenesis are to be found in the former strain because the abundance of these protein species would be expected to be elevated further in the 129/SvJ knockout if cataractogenesis would be specifically promoted on this background.

The two alpha A- and two beta B1 crystallin truncation products we detected in our comparative analysis of the knockouts and the wild types, are highly abundant in the knockout and absent (or nearly absent) in the wild type of both strains. As discussed above, many other crystallin truncation products are encountered with a similar abundance in the wild type and knockout of 129/SvJ implicating similar effects between the gene knockout and the strain specific factors, particularly loss of CP49 and the beaded filament. Thus, the absence of specifically the two alpha A- and beta B1 crystallin protein species in the wild type of 129/SvJ means they can only be due to loss of α 3C α 46 and the accompanying increase of Ca⁺⁺ ions and protease activity in the lens nucleus. The cataract phenotype in both knockouts and absence of the truncation products in the wild types also strongly suggests they are a cause of cataract. Their lower abundance in C57BL/6J compared to 129/SvJ is consistent with the assumption of modifying factors that protect C57BL/6J from cataract development. As discussed, the absence of the protein species in the wild type of the 129/SvJ strain is evidence that CP49 is not the major genetic modifier for the disease, because, taking into consideration that we have performed a comprehensive analysis, we do not observe these pathological truncation products at all in the wild type of this strain despite the similar effects that accompany the loss of CP49 and the beaded filament and the *Gja3* knockout mutation.

Heat shock proteins act as chaperones to renature malformed proteins and prevent them from forming large aggregates if present at low concentrations [38,39]. At higher concentrations of malformed proteins, heat shock proteins may complex with the damaged proteins resulting in formation of large aggregates that can be degraded by the proteasome. Since the lens is continually exposed to potentially oxidative insults, such as exposure to sunlight and low protein biosynthesis capability, it contains an abundance of molecular chaperones for protection against detrimental protein modifications. In the lens fiber cells, the most prevalent are the small heat shock proteins (sHSP) which include HSP27/25. HSP27/25 can decrease reactive oxygen species (ROS) and increase reduced glutathione (GSH) levels, thereby providing antioxidant defense [40,41]. It can also affect cell growth by inhibiting caspases [42,43]. The different roles of HSP27/25 are modulated by its phosphorylation and oligomerization status. Non-phosphorylated oligomers can act as molecular chaperones while bi-phosphorylated forms can target aggregates to the proteasome and/or act as apoptotic factors.

HSP27/25 and alpha crystallins also associate with beaded filaments, vimentin and actin filaments and modulate assembly of the cytoskeleton network [44,45,46,47]. Thus, the absence of the beaded filaments in the 129/SvJ strain may have a role in the observed expression changes for HSP27/25 at both the RNA and protein levels and hence indirectly contribute to preventing cataract pathology in the C57BL/6J strain. Additionally, a modified form of gamma actin (SSP 1531) was only detected in the C57BL/6J and not the 129/SvJ strain. It may be of interest to determine if other mouse strains that have down-regulation of CP49 and/or HSP27/25 also have changes in the lens content for gamma actin.

In mature fiber cells the loss of organelles and lack of cell death suggest that the prime function of HSP27/25 in this region of the lens, that includes the nucleus, is to function as a chaperone by protecting and refolding proteins. The higher abundance of HSP27/25 in the C57BL/6J and its role as both a chaperone and antioxidant is highly suggestive that it may act as a modifying factor that prevents nuclear cataracts in this strain upon disruption of the *Gja3* gene. The approximate equal abundance of protein in the wild type and the knockout mutant indicates it is a genetic modifier not connected to the effects of the mutation.

ERp29 is ubiquitously found in the lumen of the endoplasmic reticulum in animals [48]. Since mature lens fiber cells lack an endoplasmic reticulum, it is not clear as to its function in these cells. Like HSP27/25, ERp29 may act as a molecular chaperone and provide additional defense against oxidative insult to the lens. It also may be involved in non-chaperone type folding with protein escort properties [49,50,51].

One of the most interesting questions raised by our study is why is the abundance of syntaxin binding protein elevated in the C57BL/6J *Gja3* knockout mutant? Syntaxin is an integral membrane protein that binds to the presynaptic calcium channels particularly of the N-type in other tissues [52,53,54,55]. It has an inhibitory effect keeping the channels in a closed state hence preventing calcium entry [56,57,58]. Syntaxin binding protein is not membrane associated and binds to syntaxin [59]. It sequesters syntaxin from the presynaptic calcium channels and G-protein thereby keeping the channels open and facilitating calcium ion current.

A network of L-type voltage gated calcium ion channels is present in the adult lens [60], yet evidence attests that syntaxin does not associate with L-type channels [61,62]. However, syntaxin is known to interact with many other ion channels [63]. An intriguing possibility is that syntaxin may interact with gap junctions and inhibit their activity. We speculate that the syntaxin binding protein is maximally synthesized in the 129/SvJ strain. In the absence of $\alpha 3\text{Cx}46$, the efflux of Ca^{++} is decreased resulting in higher concentration of this ion in the cells leading to an increase in the abundance of syntaxin binding protein specifically in the C57BL/6J mutant. Furthermore, the higher Ca^{++} concentration is speculated to result in sequestration of syntaxin by syntaxin binding protein in an attempt to prevent its inhibition of the remaining lens $\alpha 8\text{Cx}50$ containing gap junctions and hence Ca^{++} efflux. This may be sufficient in the C57BL/6J *Gja3* knockout mutant to prevent formation of a cataract due to the presence of other genetic factors, such as HSP27/25 and/or ERp29 that are absent from the 129/SvJ strain. In this model, the small heat shock proteins would have a major role in preventing cataractogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Sections of the 2-DE protein spot patterns showing the phakinin protein species. Phakinin is an abundant protein in the C57BL/6J strain and absent in the 129/SvJ strain, both in the wild type and the *Gja3* knockout mutant.

Figure 2.

Plot of the abundance of truncated crystallin proteins in the urea soluble proteins of the lens whose abundance varies by a factor of 2 or more between the wild type of the two mouse strains normalized to the wild type of the C57BL/6J strain.

Figure 3.

Two alpha A and two beta B1 crystallin truncation products are found exclusively in the *Gja3* knockout mutant of both strains. The abundance of the protein species (indicated by arrowheads a, c, d and e) is increased in the 129/SvJ compared to the C57BL/6J strain. A) Section of the 2-DE protein spot patterns showing the alpha A crystallins truncated between a) amino acid residues 163 and 164 (SSP 4114) and c) between amino acid residues 157 and 158 (SSP 4103); another alpha A crystallin truncation product b) (SSP 4106) whose abundance is increased more than 2-fold in the wild type and the knockout mutant of the 129/SvJ compared to the C57BL/6J strain is also localized in this section. B) Section showing d) the truncated beta B1 crystallin (SSP 3209). C) Section showing e) beta B1 crystallin truncated between amino acid residues 27 and 28 (SSP 5312).

Figure 4.

Mass spectrometric analysis indicating truncation of beta B1 crystallin between threonine residue 27 and glycine residue 28 (SSP 5312). A) ESI-ion trap-MS/MS spectrum of the peptide ion from amino acid residues 28 to 44 (peptide sequence: (T)GPAPAPGPTPVPASVPR) of beta B1 crystallin with the mass to charge ratio 784.75 Da carrying a double charge. B) Mass spectrum of all ions in the ion trap immediately before the MS/MS spectrum was recorded. The signal of the fragmented precursor ion is indicated.

Figure 5.

HSP27/25 is in total more than 4 times more abundant in the C57BL/6J than in the 129/SvJ strain. The doubly phosphorylated (SSP 2315), singly phosphorylated (SSP 3313) and unphosphorylated (SSP 4316) HSP27/25 proteins are indicated by arrowheads (a, b, c respectively). For the relative abundance of these proteins see table III. The abundance of the protein species is approximately equal in the wild type and the *Gja3* knockout mutant of the C57BL/6J strain.

Figure 6.

Peptide mass fingerprint mass spectra acquired from the singly and doubly phosphorylated HSP27/25 proteins. A) Singly phosphorylated HSP27/25 (SSP 3313); B) Doubly phosphorylated HSP27/25 (SSP 2315). All peaks are annotated, indicated by a star. 1031.52/1031.50 Da = AA 21–28; 1075.59 Da = AA 84–93; 1085.46 Da = AA 13–20, serine 15 phosphorylated; 1099.48 Da = AA 13–20, serine 15 phosphorylated, glutamic acid 17 methylated; 1104.52/1104.54 Da = AA 132–140; 1149.63 Da = AA 29–38; 1155.55 Da = AA 84–93, serine 86 phosphorylated.

Figure 7.

Real time PCR analysis of HSP27/25 in lens RNA from 10 day old mice. A) RNA from mouse lenses of the different genotypes indicated on top of each lane were subjected to real-time PCR analysis using specific HSP27/25 primers. To normalize the amount of input RNA, 18S rRNA primers were used. At least a four fold increase in HSP27/25 transcripts was observed when lens RNA from C57BL/6J was compared to that from 129/SvJ. B) Linear response of the real-time PCR assay to increasing mass of input template DNA. The intensity of the amplified DNA fragment is proportional to the mass of the HSP27/25-containing plasmid used as template.

Figure 8.

Syntaxin binding protein is more than twice as abundant in the *Gja3* knockout mutant of the C57BL/6J and the wild type and the mutant of the 129/SvJ strain than in the C57BL/6J wild type. Syntaxin binding protein (SSP 7106) is indicated by arrowhead a, a truncated alpha A crystallin (SSP 7001) is more than twice as abundant in the wild type of 129/SvJ than C57BL/6J and is indicated by arrowhead b.

Urea soluble proteins of the lens with difference in abundance of 2 or more in the wild type of the *Mus musculus* C57BL/6J and 129/SvJ strains at ten days of age

Table 1

Increased in the C57BL/6J Wild Type				Mw and pI		Protein Fold Changes ^a				
Protein Spot Denominator	Protein Name	Accession Number NCBI	Mascot Score ^b	Protein Identification Method	Theoretical	2-DE	C57BL/6J wt	C57BL/6J <i>Gja3</i> KO	129/SvJ wt	129/SvJ <i>Gja3</i> KO
SSP 1422	CP49, Phakimin	50400333	193	MALDI/TOF/TOF-PMF	45.71/5.2	43.30/5.1	1	0.3	0	0
SSP 1528	CP49, Phakimin	50400333	94	MALDI/TOF/TOF-PMF	45.71/5.2	49.21/5.1	1	0.4	0	0
SSP 1531	Gamma-actin	74187644	98	MALDI/TOF/TOF-PMF	41.71/5.2	46.67/5.2	1	1.1	0	0
SSP 1536	CP49, Phakimin	50400333	78	MALDI/TOF/TOF-PMF	45.71/5.2	45.44/5.0	1	0.2	0	0
SSP 1537	CP49, Phakimin	50400333	66	MALDI/TOF/TOF-PMF	45.71/5.2	45.53/4.9	1	0	0	0
SSP 1538	CP49, Phakimin	50400333	129	MALDI/TOF/TOF-PMF	45.71/5.2	49.44/5.2	1	0.7	0	0
SSP 2315	HSP27/25	547679	126	MALDI/TOF-PMF	23.01/6.1	29.17/5.4	1	0.8	0.3	0.2
SSP 2521	CP49, Phakimin	50400333	230	MALDI/TOF/TOF-PMF	45.71/5.2	49.51/5.4	1	1.1	0	0
SSP 2522	CP49, Phakimin	50400333	177	MALDI/TOF/TOF-PMF	45.71/5.2	49.45/5.2	1	0.8	0	0
SSP 2523	CP49, Phakimin	50400333	170	MALDI/TOF/TOF-PMF	45.71/5.2	49.41/5.3	1	0.8	0	0
SSP 2527	CP49, Phakimin	50400333	201	MALDI/TOF/TOF-PMF	45.71/5.2	47.07/5.2	1	1.1	0	0
SSP 2617	CP49, Phakimin	50400333	111	MALDI/TOF/TOF-PMF	45.71/5.2	51.22/5.4	1	0.7	0	0
SSP 3101	Alpha A crystallin	62001857	103	MALDI/TOF/TOF-PMF	19.78/5.8	19.42/5.5	1	0.4	0.4	0.3
SSP 3313	HSP27/25	547679	186	MALDI/TOF-PMF	23.01/6.1	29.24/5.7	1	0.9	0.2	0.2
SSP 3617	CP94, Filensin	26343715	80	MALDI/TOF/TOF-PMF	71.46/5.6	50.71/5.7	1	0.7	0.3	0.2
SSP 3804	Mortalin	74205924	67	MALDI/TOF/TOF-PMF	63.92/6.1	61.91/5.5	1	1.1	0.1	0.1
SSP 3827	CP94, Filensin	26343715	70	MALDI/TOF/TOF-PMF	71.46/5.6	67.97/5.8	1	1.2	0.2	0.2
SSP 4316	HSP27/25	547679	138	MALDI/TOF/TOF-PMF	23.01/6.1	29.30/6.1	1	0.9	0.3	0.3
SSP 4326	CP94, Filensin	26343715	250	MALDI/TOF/TOF-MS/MS	71.46/5.6	39.86/5.8	1	0.2	0	0
SSP 4329	Endoplasmic reticulum protein ERp29, precursor	16877776	87	MALDI/TOF/TOF-MS/MS	28.81/5.9	31.60/5.9	1	1.2	0	0
SSP 4501	CP94, Filensin	26343715	139	MALDI/TOF/TOF-PMF	71.46/5.6	50.57/5.8	1	0.9	0.2	0.2
SSP 4806	CP94, Filensin	26343715	77	MALDI/TOF/TOF-PMF	71.46/5.6	67.48/5.9	1	1.2	0.2	0.2
SSP 4811	CP94, Filensin	26343715	95	MALDI/TOF/TOF-PMF	71.46/5.6	67.63/5.9	1	0.8	0.2	0.2
SSP 4812	CP94, Filensin	26343715	143	MALDI/TOF/TOF-PMF	71.46/5.6	67.40/5.9	1	0.9	0.4	0.4
SSP 4818	CP94, Filensin	26343715	195	MALDI/TOF/TOF-MS/MS	71.46/5.6	67.36/6.0	1	1.3	0.2	0.2
SSP 5522	Beta B1 crystallin	12963789	74	MALDI/TOF/TOF-PMF	27.99/6.8	48.30/6.2	1	0.5	0	0

Increased in the C57BL/6J Wild Type										Protein Fold Changes ^d			
Protein Spot Denominator	Protein Name	Accession Number NCBI	Mascot Score ^b	Protein Identification Method	Mw and pI		Theoretical	2-DE	C57BL/6J wt	C57BL/6J <i>Gja3</i> KO	129/SvJ wt	129/SvJ <i>Gja3</i> KO	
SSP 6125	Gamma B crystallin	42733606	132	MALDI/TOF/TOF-MS/MS	21.07/6.9	23.96/6.7	1	0.5	0.4	0.4			
SSP 6132	Gamma N crystallin	23346485	118	MALDI/TOF/TOF-PMF	21.39/6.3	23.38/6.6	1	1.1	0	0			
SSP 6325	Annexin A1	NE	187	MALDI/TOF-PMF	38.71/7.7	40.06/6.5	1	1.4	0	0			
SSP 6717	Chaperonin subunit 6A	74204595	195	MALDI/TOF/TOF-MS/MS	58.01/6.5	58.46/6.5	1	1.0	0	0			
SSP 7223	Gamma S crystallin	6753532	76	MALDI/TOF/TOF-PMF	20.84/6.9	27.00/6.8	1	0.8	0.5	0.4			

Increased in the 129/SvJ Wild Type										Protein Fold Changes ^d			
Protein Spot Denominator	Protein Name	Accession Number NCBI	Mascot Score ^b	Protein Identification Method	Mw and pI		Theoretical	2-DE	C57BL/6J wt	C57BL/6J <i>Gja3</i> KO	129/SvJ wt	129/SvJ <i>Gja3</i> KO	
SSP 1013	Beta A3 crystallin	20304089	76	MALDI/TOF/TOF-PMF	25.19/6.0	14.62/5.1	1	2.1	4.3	3.7			
SSP 1018	Beta B3 crystallin	10946674	114	MALDI/TOF/TOF-PMF	24.27/6.7	14.91/5.2	1	2.0	2.8	2.2			
SSP 2010	Alpha A crystallin	62201857	83	MALDI/TOF/TOF-PMF	19.78/5.8	17.56/5.4	1	3.2	4.8	4.4			
SSP 2104	Beta A3 crystallin	20304089	91	MALDI/TOF/TOF-PMF	25.19/6.0	19.54/5.3	1	1.5	4.9	2.9			
SSP 3004	Alpha A crystallin	62201857	102	MALDI/TOF/TOF-PMF	19.78/5.8	17.18/5.6	1	1.9	2.8	2.1			
SSP 3007	Alpha A crystallin	62201857	130	MALDI/TOF/TOF-PMF	19.78/5.8	17.48/5.6	1	3.3	3.3	6.3			
SSP 3010	Beta B3 crystallin	10946674	95	MALDI/TOF/TOF-PMF	24.27/6.7	16.12/5.8	1	2.2	2.5	2.1			
SSP 3014	Alpha A crystallin	62201857	105	MALDI/TOF/TOF-PMF	19.78/5.8	18.23/5.6	1	1.4	2.4	1.1			
SSP 3109	Alpha A crystallin	62201857	126	MALDI/TOF/TOF-PMF	19.78/5.8	20.89/5.7	0	+	+	+			
SSP 3809	Mortalin	74205924	95	MALDI/TOF/TOF-MS/MS	63.92/6.1	66.16/5.6	0	0	+	+			
SSP 4005	Alpha A crystallin	62201857	103	MALDI/TOF/TOF-PMF	19.78/5.8	16.96/5.9	1	1.8	2.0	3.1			
SSP 4008	Alpha A crystallin	62201857	102	MALDI/TOF/TOF-PMF	19.78/5.8	16.15/5.9	1	2.3	2.7	3.1			
SSP 4106	Alpha A crystallin	62201857	102	MALDI/TOF/TOF-PMF	19.78/5.8	18.97/6.0	1	2.2	3.6	3.1			
SSP 5002	Alpha A crystallin	62201857	84	MALDI/TOF/TOF-PMF	19.78/5.8	16.77/6.2	1	1.7	2.3	2.1			
SSP 5015	Beta A3/A1 crystallin	20304089	314	MALDI/TOF/TOF-MS/MS	25.19/6.0	17.02/6.5	0	+	+	+			
SSP 5409	Annexin A1	NE	104	MALDI/TOF/TOF-PMF	38.71/7.7	37.86/6.4	1	1.5	6.7	6.8			
SSP 6003	Alpha A crystallin	62201857	200	MALDI/TOF/TOF-MS/MS	19.78/5.8	16.55/6.6	1	2.0	2.8	2.7			
SSP 6010	Gamma C crystallin	6681037	113	MALDI/TOF/TOF-PMF	20.97/6	16.03/6.6	1	1.6	3.3	3.2			
SSP 6011	Alpha A crystallin	62201857	77	ESI-MS/MS	19.78/5.8	16.57/6.6	0	+	++	++			
SSP 6107	Alpha B crystallin	6753530	76	MALDI/TOF/TOF-PMF	20.06/6.8	18.30/6.6	1	2.6	2.9	2.4			
SSP 6122	Gamma N crystallin	23346485	117	MALDI/TOF/TOF-PMF	21.39/6.3	20.83/6.7	0	0	+	+			

Increased in the 129/SvJ Wild Type									
Mw and pI					Protein Fold Changes ^a				
Protein Spot Denominator	Protein Name	Accession Number NCBI	Mascot Score ^b	Protein Identification Method	Theoretical	2-DE	C57BL/6J wt	C57BL/6J <i>Gja3</i> KO	129/SvJ wt 129/SvJ <i>Gja3</i> KO
SSP 6127	Gamma D crystallin; Alpha A crystallin	34784220; 62201857	102; 67	MALDI/TOF/TOF-PMF	21.17/7.0; 19.78/5.8	20.38/6.8	0	+	+
SSP 6704	Chaperonin subunit 6A	6753324	66	MALDI/TOF/TOF-PMF	57.97/6.6	59.56/6.6	1	0.7	4.9
SSP 7001	Alpha A crystallin	62201857	337	MALDI/TOF/TOF-MS/MS	19.78/5.8	17.37/6.8	1	1.4	1.9
SSP 7017	Beta A3/A1 crystallin gene product	26350979	76	MALDI/TOF/TOF-PMF	12.43/6.5	13.85/7.0	1	2.2	3.4
SSP 7106	Syntaxin binding protein	19353298	72	MALDI/TOF/TOF-MS/MS	23.66/9.3	18.02/6.9	1	2.1	2.7
SSP 8006	Alpha B crystallin	6753530	128	MALDI/TOF/TOF-PMF	20.06/6.8	16.86/7.2	1	1	2.4
SSP 8014	Gamma E crystallin	34978370	96	MALDI/TOF/TOF-PMF	21.18/7.1	17.15/7.3	1	2.4	2.8
SSP 9013	Beta B3 crystallin	10946674	72	MALDI/TOF/TOF-PMF	24.27/6.7	15.25/8.0	1	2.0	2.5
SSP 9014	Beta B3 crystallin	10946674	208	MALDI/TOF/TOF-MS/MS	24.27/6.7	15.18/8.0	1	1.4	1.6
SSP 9017	Gamma B crystallin	2507570	79	MALDI/TOF/TOF-PMF	21.12/7.55	14.23/8.2	1	1.2	2.2

^aThe mean staining intensity of the three independent preparations of the urea soluble proteins of the lens is listed. If the protein is absent in the C57BL/6J strain, the abundance is listed as increased (+) or decreased (−).

^bA Mascot score of 63 or more indicates protein identity with a P-value of less than 0.05.

MALDI/TOF/TOF-PMF refers to PMF data recorded with the 4700 Proteomics Explorer, MALDI/TOF/TOF-MS/MS refers to additional MS/MS data recorded when identification with PMF data alone was not conclusive.

NE, Not entered because the protein primary structure could not be unambiguously determined.

Table 2

Truncated crystallin proteins detected in the *Mus musculus* in the C57BL/6J strain

Protein	Protein Species ^a	Protein Spot Denominator	Mw 2-DE	pI 2-DE	% SC ^b	Protein Fold Changes			
						C57BL/6J wt	C57BL/6J Gja3 KO	129/SvJ wt	129/SvJ Gja3 KO
Alpha A crystallin	Alpha A_A crystallin	SSP 4008	16.15	5.9	35	1	2.3	2.7	3.1
	Alpha A_B crystallin	SSP 6003	16.55	6.6	50	1	2.0	2.8	2.7
	Alpha A_D crystallin	SSP 5002	16.77	6.2	35	1	1.7	2.3	2.1
	Alpha A_E crystallin	SSP 4005	16.96	5.9	42	1	1.8	2.0	3.1
	Alpha A_F crystallin	SSP 3004	17.18	5.6	40	1	1.9	2.8	2.1
	Alpha A_G crystallin	SSP 7001	17.37	6.8	24	1	1.4	2.3	1.9
	Alpha A_H crystallin	SSP 3007	17.48	5.6	73	1	3.3	3.3	6.3
Alpha B crystallin	Alpha A_I crystallin	SSP 2010	17.56	5.4	35	1	3.2	4.8	4.4
	Alpha A_J crystallin	SSP 3014	18.23	5.6	35	1	1.4	2.4	1.1
	Alpha A_M crystallin	SSP 4106	18.97	6.0	36	1	2.2	3.6	3.1
	Alpha B_A crystallin	SSP 8006	16.86	7.2	53	1	1	2.9	2.4
	Alpha B_B crystallin	SSP 6107	18.3	6.6	29	1	2.6	2.9	2.4
	Beta A3_A crystallin	SSP 1013	14.62	5.1	29	1	2.1	4.3	3.7
	Beta A3_A crystallin	SSP 2104	19.54	5.3	35	1	1.5	4.9	2.9
Beta B3 crystallin	Beta B3_A crystallin	SSP 1018	14.91	5.2	34	1	2.0	2.8	2.2
	Beta B3_B crystallin	SSP 9014	15.18	8.0	32	1	1.4	2.8	1.6
	Beta B3_C crystallin	SSP 9013	15.25	8.0	41	1	2.0	4.5	2.5
	Beta B3_D crystallin	SSP 3010	16.12	5.8	32	1	2.2	2.5	2.1
	Gamma B_A crystallin	SSP 9017	14.23	8.2	49	1	1.2	2.5	2.2
	Gamma C_A crystallin	SSP 6010	16.03	6.6	51	1	1.6	3.3	2.3
	Gamma E_A crystallin	SSP 8014	17.15	7.3	47	1	2.4	2.9	2.8

^aProposed protein species nomenclature; Alpha A_C (SSP 6011), Alpha A_K (SSP 4103) and Alpha A_L (SSP 4114) crystallin are absent in the C57BL/6J wild type.

^bSC, sequence coverage

Table 3

Relative and absolute abundance of the detected HSP27/25 protein species in the C57BL/6J and 129/SvJ strains.

Protein	Protein Spot Denominator	Modification	Protein fold changes in C57BL/6J relative to 129/SvJ		
			Wild type		Gja3 KO
HSP27/25	SSP 2315	2x phosphorylated	3.1		3.6
	SSP 3313	1x phosphorylated	5.1		5.9
	SSP 4316	unphosphorylated	3.0		3.1
	Total		3.7		4.2

Protein Abundance in Staining Intensity Counts							
Protein Spot Denominator	C57BL/6J wt		C57BL/6J Gja3 KO		129/SvJ wt		129/SvJ Gja3 KO
	MEAN	CV	MEAN	CV	MEAN	CV	CV
SSP 2315	1199	23.9%	994	4%	383	10%	277.3 26%
SSP 3313	2007	11.5%	1896	5%	391	3.5%	320 13%
SSP 4316	1488	15%	1397	13%	490	31%	446 13%