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Cataract-causing allele in CRYAA (Y118D) proceeds through endoplasmic reticulum stress in mouse model

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ABSTRACT

As small heat shock proteins, α-crystallins function as molecular chaperones and inhibit the misfolding and aggregation of β/γ-crystallins. Genetic mutations of *CRYAA* are associated with protein aggregation and cataract occurrence. One possible process underlying cataract formation is that endoplasmic reticulum stress (ERS) induces the unfolded protein response (UPR), leading to apoptosis. However, the pathogenic mechanism related to this remains unexplored. Here, we successfully constructed a cataract-causing CRYAA (Y118D) mutant mouse model, in which the lenses of the CRYAA-Y118D mutant mice showed severe posterior rupture, abnormal morphological changes, and aberrant arrangement of crystallin fibers. Histological analysis was consistent with the clinical pathological characteristics. We also explored the pathogenic factors involved in cataract development through transcriptome analysis. In addition, based on key pathway analysis, up-regulated genes in CRYAA-Y118D mutant mice were implicated in the ERS-UPR pathway. This study showed that prolonged activation of the UPR pathway and severe stress response can cause proteotoxic and ERS-induced cell death in CRYAA-Y118D mutant mice.

INTRODUCTION

The eye lens is a key refractive medium composed of capsule and crystallin fibers (Banh et al., 2006). The unique short-range and orderly arrangement of highly concentrated crystallins in the eye lens is important for lens transparency (Delaye et al., 1983). Cataracts, which are the leading cause of blindness worldwide, are produced by protein misfolding and aggregation (Lee et al., 2017). Genetic mutation and environmental stress can affect the protein-folding process, resulting in protein misfolding and, in turn, disruption of lens protein stability, solubility, and interactions (Fu et al., 2021; Li et al., 2020; Xu et al., 2021; Yang et al., 2020b). Crystallins are the most important structural proteins in the lens and contain two superfamilies: i.e., α-crystallins and β/γ-crystallins. Poor crystallin stability results in protein aggregation and precipitation, leading to lens opacity (Moreau et al., 2012). Therefore, exploring the potential pathogenic mechanisms associated with cataracts and the regulatory pathways involved in protein homeostasis in the lens are important for the development of new cataract prevention and treatment strategies.

The small heat shock protein, α-crystallins, which consist of αA- and αB-crystallin, function as molecular chaperones to

inhibit the misfolding and aggregation of β/γ-crystallins (Roskamp et al., 2019). In addition, α-crystallin also protects the lens cells against apoptosis (Ahsan et al., 2021). Previous studies have reported that cataract pathogenesis is correlated with aberrant changes in the ratio of crystallin subunits and apoptosis of lens epithelial cells (Li et al., 2008). Furthermore, under environmental stress, α-crystallins act as chaperones to maintain protein stability and solubility and thus transparency of the eye lens (Derham, 1999). *CRYAA* gene mutant mice show the pathological features of cataracts with nuclear opacity (Graw et al., 2001). In addition, congenital cataracts caused by *CRYAA* mutation exhibit polymorphisms in different individuals (Yu et al., 2012). Therefore, mouse models with genetic mutation of the *CRYAA* gene could be applied to explore the pathogenic mechanism underlying cataract formation. Studies have shown that mutations in the β-strand of the conserved αA-crystallin domain (residues 83 to 120) can lead to autosomal dominant cataracts (Huang et al., 2009). Mutation sites in the β-strand have the same physiochemical properties. Furthermore, αA-crystallin position 118 Tyr (Y118) deletion in humans is known to lead to cataracts (Sun et al., 2011). Thus, we studied the effects of αA-crystallin on cataract pathogenesis using a Y118D mouse model.

Endoplasmic reticulum stress (ERS) caused by the excessive accumulation of misfolded proteins plays an important role in cataracts (Zhou et al., 2020) and can trigger the unfolded protein response (UPR) (Ikesugi et al., 2006). In mammals, the UPR pathway is mediated by three ER proteins: i.e., IRE1, PERK, and ATF6 (Bartoszewska et al., 2020). The UPR can alleviate ERS by mediating different signaling pathways at both the transcriptional and translational levels (Uddin et al., 2020). There are two main protein degradation pathways in cells: i.e., autophagy lysosome pathway (ALP) and ubiquitin proteasome system (UPS) (Al Mamun et al., 2020). In addition, pathogenic factors of cataracts can activate the ERS-UPR pathway. Under high levels of ERS, however, the UPR pathway cannot eliminate misfolded proteins, thereby resulting in cellular apoptosis (Lee et al., 2015). Excessive accumulation of unfolded or misfolded proteins can cause ERS-UPR, reactive oxygen species (ROS), and cellular apoptosis in epithelial cells of the lens, ultimately resulting in cataract development (Yang et al., 2015).

αA-crystallin plays a chaperone role in the eye lens and regulates intracellular protein homeostasis by modulating the ERS-UPR pathway (Andley et al., 2016). In this study, we successfully constructed a cataract-causing CRYAA (Y118D) mutant-related mouse model to explore the role of the ERS-UPR pathway and other potential pathogenic mechanisms in cataracts. This study should help enrich our understanding of cataract pathogenesis and clarifies the involvement of ERS in the occurrence and development of cataracts by affecting the biological processes of epithelial cells in the lens. Our results suggest that ERS may be a potential biological target for the treatment of cataracts, providing a theoretical and practical basis for the development of new therapeutic drugs and treatments, which has important clinical significance.

MATERIALS AND METHODS

Cataract mouse model
Mouse care and breeding were conducted following animal experimental protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Laboratory Animal Center, Zhejiang University. Global CRYAA-mutant (αA$^{Y118D/Y118D}$) mice were constructed at GemPharmatech Co., Ltd. (China) using CRISPR/Cas9 recombination to modify *CRYAA* (Shao et al., 2018). The αA$^{Y118D/+}$ mutant mice were intercrossed to produce homozygous mice. Genomic DNA was extracted from tails or toes for polymerase chain reaction (PCR) amplification and sequencing to identify mouse genotypes. Phenotypic analysis was performed on homozygous male mice and their lenses were harvested for genome-wide analysis. Mice were humanely sacrificed by carbon dioxide asphyxiation or cervical dislocation. Eyes were harvested and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for paraffin embedding and sectioning or placed in PBS for lens dissection.

Slit-lamp examination
A slit lamp was used to observe lens turbidity and evaluate cataract phenotype. A mixture of 10% phenylephrine hydrochloride and 1% topicaine (Santen Pharmaceutical, China) was used to dilate the pupils. Left eyes of the mice were examined and photographed by an ophthalmologist. Evaluation was performed based on the grading standard of the Ophthalmology Laboratory of Oxford University (Andley, 2009).

Histological lens analysis
Fixed eyes were washed several times with PBS. Histological examination was then performed sequentially using the following steps: dehydration-transparency-wax immersion-embedding. Trimmed wax blocks were sectioned, and the cornea and retina were simultaneously cut out in a direction parallel to the optic nerve. Sections (3 μm) were then stained using hematoxylin and eosin (H&E).

Lens RNA preparation and transcriptome analysis
Total RNA was extracted from lenses of αA$^{Y118D/Y118D}$ and αA$^{+/+}$ mice using Trizol reagent (#15596018, Invitrogen, USA). Two sets of RNA from six lenses were quantified using spectrophotometry. Samples with an RNA integrity number (RIN)>6.5 were selected for transcriptome sequencing according to the requirements of Novogene (China). Transcriptome data analysis was performed as follows: FPKM (fragments per kilobase per million mapped reads) values of each sample were used for normalization, and then log2 values were calculated using the ratio of the expression levels of the treatment and control groups. Gene Ontology (GO) (http://geneontology.org/) and protein-protein interaction (PPI) analyses were performed for differentially expressed genes (DEGs) with an expression difference fold-change≥2. Expression levels of ERS-UPR marker genes were determined through quantitative PCR (qPCR) using an Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (#A25742, Thermo, USA). Gene expression levels were normalized by β-actin.

Immunoblotting

Lens extracts were centrifuged into water-soluble and -insoluble fractions at 4 °C and 12 000 r/min for 30 min and immunoblotting was performed. Polyclonal antibodies against total α-crystallin (#sc-28 306, Santa Cruz, USA) and the ERS-UPR signaling pathway (ATF4, #11815S, CST; GRP78, #3177S, CST; p62, #66184, Protein Tech , USA) were used as primary antibodies. Anti-mouse/rabbit IgG (H+L) antibodies were used as secondary antibodies for an additional hour at room temperature. Image J software was used to analyze the band gray level for calculation.

Data analysis

Data were presented as mean±standard deviation (*SD*) in graphs generated by GraphPad Prism 8. Statistical analysis was performed by Student's two-tailed *t*-test, while one-way analysis of variance (ANOVA) was used when more than two groups were compared. Values of *P*<0.05 were considered statistically significant.

RESULTS

Cataract phenotype in αA$^{Y118D/Y118D}$ mouse lens

Mutations in the β-strand of the conserved domain of αA-crystallin (residues 83 to 120) can cause autosomal-dominant cataracts (Hejtmancik, 2008). In addition, the αA-Y118del mutation is correlated with congenital cataracts (Sun et al., 2011). As such, we analyzed amino acid sequence conservation between positions 83 to 120 in αA-crystallin. The αA-crystallin sequences of multiple species were retrieved from the UniProt database (http://www.uniprot.org/) and MEGA (http://www.megasoftware.net/) was used for protein sequence conservation analysis. Amino acids at positions 83 to 120 in the αA-crystallin sequence showed significantly high evolutionary conservation among the examined mammals (Figure 1A).

Previous reports have demonstrated that αA-Y118D mutant mice exhibit a cataract phenotype (Xia et al., 2006). Therefore, we explored the pathogenesis of human cataracts using the αA-Y118D mutant mouse model. Based on CRISPR/Cas9 technology, we constructed a αA$^{Y118D/Y118D}$ mutant mouse model (Figure 1B). Specifically, the *CRYAA* gene was first amplified using primers and sequenced to confirm successful development of the CRYAA-Y118D mutant model (Figure 1C). Mice with knock-in mutations survived and reproduced normally. Intercrossing of αA$^{Y118D/+}$ mice produced αA$^{Y118D/Y118D}$ homozygous offspring and wild-type (WT) mice, while a control group was also established without changes in the lens phenotype. Lenses were observed and analyzed using a slit lamp to determine the effects of αA$^{Y118D/Y118D}$. The anterior segment images in Figure 1D confirmed lens opacity in αA$^{Y118D/Y118D}$ mice compared with WT mice. Lenses harvested from two-month-old αA$^{Y118D/Y118D}$ mice showed completely opaque nuclear cataracts, including cortical fibers, whereas lenses from WT mice showed no abnormalities. These findings indicate that the Y118D mutation in the *CRYAA* gene can affect the correct folding of crystallins, resulting in nuclear cataracts in mice.

αA-Y118D mutation affects lens histology

To explore the mechanism underlying cataract formation, we analyzed histological changes during lens development in mice with the αA-Y118D protein mutation. Histological changes in the lenses of two-week-old WT and αA$^{Y118D/Y118D}$ mice are shown in Figure 2A. Analysis of the lens cortex in αA$^{Y118D/Y118D}$ mice showed that the lens matrix was arranged in layers, with disordered fiber lamellar arrangement, irregularly shaped anterior fiber cells, and enlarged intercellular spaces. In the lens nucleus, fibers were twisted and damaged, showing a honeycomb structure. Furthermore, excessive proliferation and irregular distribution of epithelia were observed in the equator of the lens, and uniform fiber disintegration and calcium deposition occurred. Histological analysis of WT lenses in the control group showed that the outer membrane was intact, epithelium was neatly arranged, and cell morphology was consistent. Morphology of the lens fiber layer was complete and tightly arranged with normal homogeneity, and the various layers of the lens were closely connected.

Detection of αA-crystallin soluble proteins showed that changes in the lens were partly caused by protein insolubility (Figure 2B). Lenses from WT and αA-Y118D mutant mice were centrifuged. Changes in the ratio of water-soluble protein and precipitate were determined using the supernatant and precipitate of the cortex and nucleus. Results showed that, compared with WT mice, the proportion of precipitate content in total lens protein content was significantly higher in mutant mice.

As a ubiquitin-binding protein encoded by *SQSTM1*, p62 mediates various biological functions, including intracellular protein renewal (McManus et al., 2012). In some neurodegenerative diseases, abnormal protein aggregates and inclusion bodies formed in cells are often accompanied by p62 (Ichimura et al., 2010). Here, compared with the extremely low expression of p62 found in the WT lenses, the αA-Y118D mutant mouse lenses, especially in the nuclear region, showed significantly increased expression of p62 (Figure 2C). Consistent with the phenotypic results, protein insolubility was proportional to the formation of nuclear cataracts. These findings imply that abnormal expression of the αA-crystallin mutant in the lens can cause protein aggregation, thus affecting lens development and protein homeostasis.

Activation of UPR in lens of αA-Y118D mutant mice

Increased insoluble protein content in the mutant mouse lens indicated that aggregated protein was highly expressed and accumulated in the lens. The UPR is a signal transduction mechanism in cells for regulation of misfolded proteins (Malhotra et al., 2007). Activation of the UPR increases the protein-folding ability of cells, however, the apoptosis pathway is activated when cell integrity cannot be restored (Kim et al., 2006). Volcano plots, which are enhanced scatter plots, contain two important boundaries. The first one is significance, whereby the *P*-value of two groups is obtained by a difference test, and the negative logarithm log10 (*P*-value) is taken as the ordinate. The second one is abscissa, which displays log2 fold-changes. Thus, based on the obtained volcano plots and
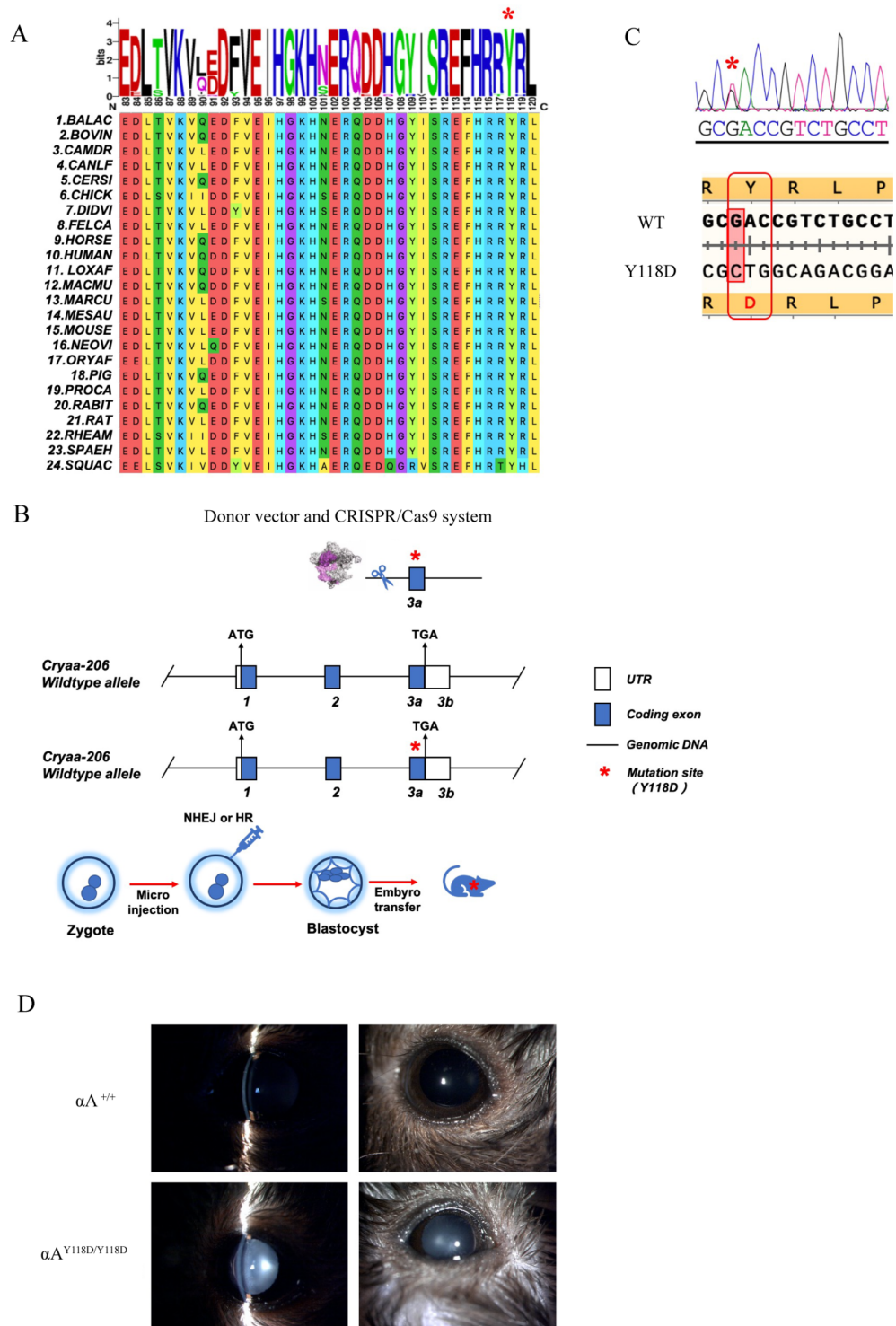
Figure 1 Construction of αA^Y118D/Y118D mice and evaluation of cataract phenotype

A: Conservation analysis of amino acids 83-120 of CRYAA protein. * represents the 118th amino acid of CRYAA protein. B: Flow diagram o construction process of αA^Y118D/Y118D mice. C: Sequencing results of αA^Y118D/Y118D heterozygous mice showing successful mutation of Y118D. * represents the gene mutation site corresponding to the 118th amino acid mutation. D: Anterior segment images of αA^Y118D/Y118D mice and wild-type (WT) mice. Lens of WT mice was transparent; lens of mutant mouse showed severe opacity.

screening criteria (fold-change>2, P≤0.05), significant DEGs can be identified for subsequent research. In the current study, transcriptome sequencing and analysis of the lenses identified 825 significant DEGs (>2-fold-change, P≤0.05), including 337 up-regulated genes and 488 down-regulated genes (Figure 3A). Interestingly, GO analysis indicated that
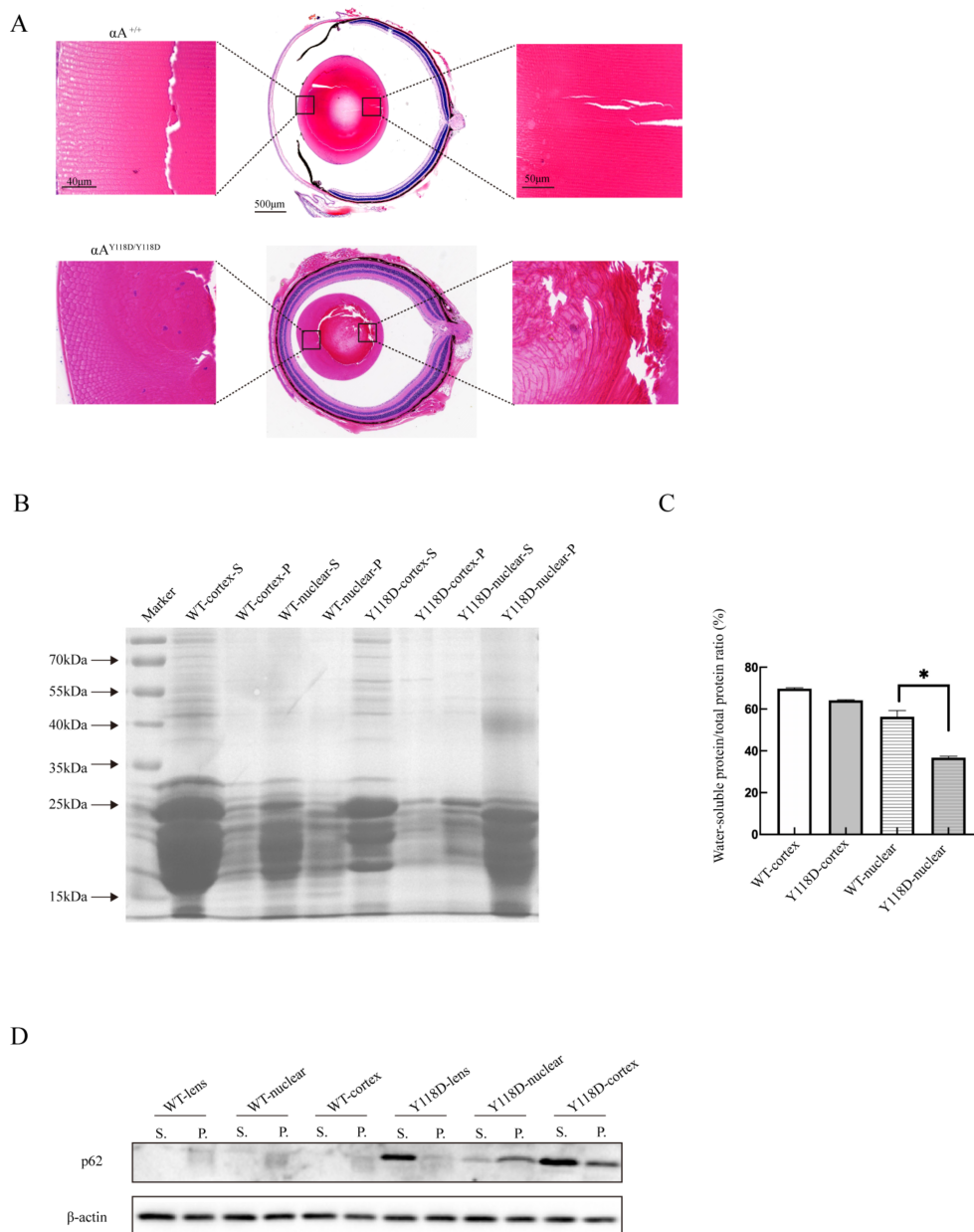
Figure 2 Detection of αA^{Y118D/Y118D} mouse lens properties

A: Histological analysis of WT and αA^{Y118D/Y118D} mutant mouse eye lenses. Sagittal sections of αA^{Y118D/Y118D} mouse eyes stained with H&E showing degeneration of lens nucleus and cortex fiber cells and highly disrupted morphology. B: Cortex and nucleus were separated from mouse lens. After centrifugation, proteins were divided into water-soluble and insoluble fractions for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). C: Gray value analysis of electrophoresis lanes showed significant decrease in soluble components in αA^{Y118D/Y118D} lens nucleus. D: Immunoblot analysis of p62 in WT and αA^{Y118D/Y118D} mutant eye lenses. Expression of p62 in homozygous knock-in mutant lens was significantly higher than that in WT lens. Values are mean±*SEM*, *: *P*<0.05.

the DEGs in the CRYAA-Y118D lens were significantly enriched in two biological processes, i.e., "response to unfolded protein" and "intrinsic apoptotic signaling pathway in response to ERS" (Figure 3B). These findings indicate that the CRYAA-Y118D mutation caused protein structure disorder. Furthermore, the UPR was significantly up-regulated in the lens, resulting in cell apoptosis. Hub genes play important roles in biological processes and regulation of other genes in related pathways. We used the Cytoscape and STRING databases to analyze PPIs with a combined score >0.4. Through the calculation of network structure and weighted connections between nodes using the cytoHubba plug-in algorithm, we extracted the Top 10 key genes (highest degree in the software) and constructed a network graph according to network topology. The ATF4 branch in the UPR signaling pathway was a key gene in the PPI network (Figure 3C). In addition, the expression levels of 127 genes in the KEGG ERS-related pathways were determined, with 12 genes found
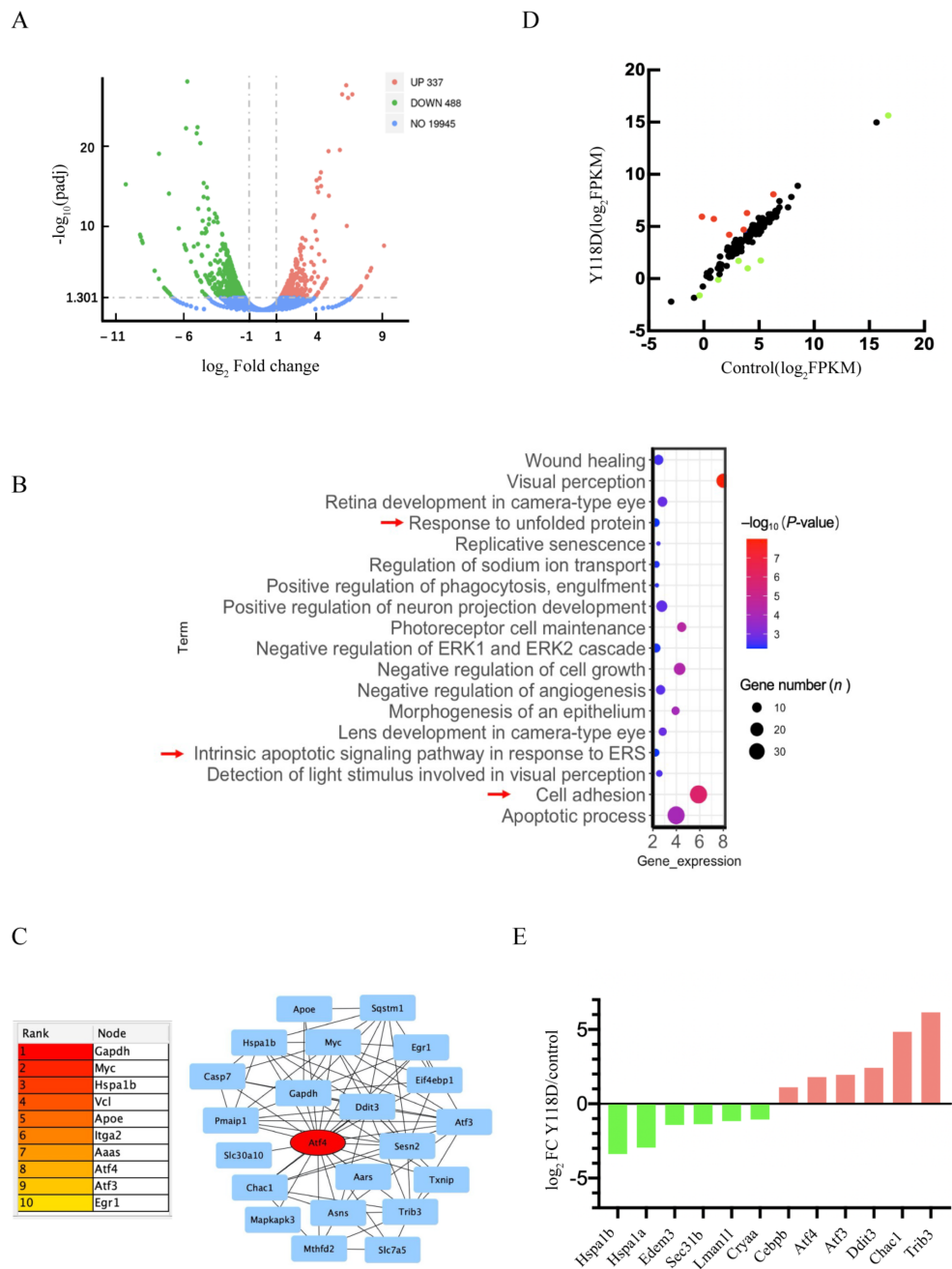
Figure 3 Bioinformatics analysis of αA$^{Y118D/Y118D}$ mouse lens transcriptome

A: Volcano map of all detected genes in transcriptome. B: Gene Ontology analysis of differentially expressed genes (DEGs). C: Protein-protein interaction analysis of DEGs and hub gene screening. D: Scatter plot of ERS-UPR-related genes in KEGG pathway analysis. E: DEGs related to ERS-UPR in transcriptome.

to be differentially expressed. Up-regulated UPR genes included Trib3>Chac1>Chop>Atf3>Atf4>Cebpb, and down-regulated genes included Hspa1b>Hspa1a>Edem3>Sec31b>kLman1l>Cryaa (Figure 3D, E). The PERK/eIF2α/ATF4/Ddit3 signal transduction pathway is a well-studied pathway in ERS (Ma et al., 2016). The UPR caused by continuous high-intensity ERS activates the apoptosis pathway through the apoptosis mediator, Ddit3 (Rozpedek et al., 2016). Transcriptome analysis showed that the expression levels of

ATF4 and Ddit3 are significantly up-regulated in the cataractous mouse lens compared with levels in the normal group, thus implying ERS response activation. Furthermore, Y118D mutation caused a significant down-regulation in the expression of heat shock proteins, such as Hspa1a and Hspa1b. Heat shock proteins are used as molecular chaperones to promote correct folding of proteins. These findings imply that ERS promotes the apoptosis of lens epithelial cells and the formation of cataracts.

αA-crystallin mutation affects ER function

Transcriptome analysis showed that progressive ERS during lens development in αA-Y118D mutant mice triggers significant transcriptional activation of UPR genes. UPR-related signaling pathways were further explored through western blotting and real-time qPCR. Results showed that the expression levels of ATF4, GRP78, and p62 increased in the lenses of the αA-Y118D mutant mice (Figure 4A), indicating that gene mutations caused aggregation of crystallins and activation of the ERS signaling pathway. Consistent with the transcriptome analysis, the qPCR results showed that protein aggregation stress in the cataractous lenses activated the PERK-ATF4-Ddit3 pathway. ATF4 and Ddit3 were highly expressed and showed significant differential expression in the nucleus of the cataractous lens compared with levels in WT mice (Figure 4B, C). Although the expression levels of ATF6 and IRE1 in the other two UPR signaling pathways were significantly up-regulated, western blotting indicated that the proteins in the two pathways were not significantly differentially expressed (Supplementary Figure S1).

In summary, the opacity of the crystallins caused by high-intensity stress was correlated with the activation and high expression of factors implicated in the ERS PERK-ATF4-Ddit3 pathway.

DISCUSSION

Congenital cataracts are often caused by genetic mutations, resulting in substantial damage to the structure and function of mutant crystallins. One of the most widely studied crystallins is γS-crystallin, which combines properties of β- and γ-crystallins. Mutations in γS-crystallin are associated with congenital and progressive juvenile cataracts (Bari et al., 2019b). Compared with WT, mutants exhibit higher aggregation and lower stability (Bari et al., 2019a). Furthermore, denatured γS-crystallins are usually bound by α-crystallin.

αA-crystallin is a member of the small heat shock protein family and plays a molecular chaperone function. αA-crystallin maintains the functions and transparency of the eye lens. A large number of *in vitro* experiments have shown that αA-crystallin can delay the aggregation of thermally or chemically denatured targets (Koteiche et al., 2015). αA-crystallin also plays an important role in lens development and transparency. The R49C mutant of αA-crystallin drives aggregation of γD-crystallin (Wu et al., 2018). Thus, αA-crystallin is an important target in the development of cataract drugs. Makley et al. (2015) reported that 25-dehydrocholesterol can reverse cataracts in mice by targeting αA-crystallin. Mutations in the β-strand of conserved αA-crystallin (residues 83 to 120) domains are known to cause autosomal dominant cataracts. Known mutations in this region include G98R, R116C, R116R, R117H, Y118del, and R119H (Hejtmancik, 2008; Sun et al., 2011; Zhuang et al., 2019). These mutations and the mouse Y118D mutation share common physicochemical properties and are implicated in the mechanism underlying cataract formation (Huang et al., 2009). Therefore, we explored a feasibility study on the pathogenesis of human cataract disease using the αA-Y118D mutation mouse model.

Previous studies have reported that the αA-Y118D mutation impairs the function of lens proteins (Xia et al., 2006). Increases in water-insoluble proteins in the lens can cause a cloudy appearance and the occurrence of dominant nuclear cataracts (Andley et al., 2016; Cheng et al., 2010). Previous studies have reported that αA-crystallin mutations can lead to the appearance of the cataract phenotype and an increase in the expression of certain factors in the ERS-UPR signaling pathway (Frankfater et al., 2020). However, systematic study on ER homeostasis is lacking. In this article, we analyzed ER homeostasis by chip data to identify the possible signaling pathway of αA-Y118D. Mutation of CRYAA-Y118D caused a down-regulation in the overall expression of molecular chaperone proteins, which promoted the misfolding of unfolded proteins. These misfolded proteins were not effectively restored or degraded, resulting in protein aggregation. As αA-crystallin plays a key role in protecting lens transparency, drugs developed to target its key sites may prevent cataract occurrence and development. *In vitro* studies have shown that αA-crystallin and chaperone peptides inhibit cell apoptosis caused by oxidative damage (Chothe et al., 2010). Exogenous αA-crystallin and cell-penetrating peptides introduced into cells show similar function to that of endogenous αA-crystallin (Christopher et al., 2014). Moreover, intramuscular injection of exogenous αA-crystallin in mice significantly inhibits the occurrence and development of cataracts (Nahomi et al., 2013). Thus, αA-crystallin is a promising target in the prevention of cataracts; however, its clinical application requires further exploration.

In this study, CRYAA-Y118D mice were established to explore the pathological changes in ERS in hereditary cataracts. The UPR signaling pathway is associated with the pathogenesis of protein aggregation diseases, including Alzheimer's disease, Parkinson's disease, and cataracts (Martinez et al., 2019). Here, DEG pathway analysis of the αA-Y118D lens showed UPR signaling pathway activation, ER protein processing, and pro-apoptotic activation. UPR genes related to ERS include Chac1 and Ddit3, which exert effects through PERK pathway activation and subsequent cell death (Galluzzi et al., 2012). These studies imply that the expression and accumulation of αA-Y118D mutants can trigger high-level ERS and UPR activation, leading to fibroblast death and cataract formation. Several studies have also reported that apoptosis is essential in cataract formation and progression (Tian et al., 2020; Yang et al., 2020a). Our findings showed that significant apoptosis occurred after induction of the ERS response in the αA-Y118D lens (Supplementary Figure S2). Thus, ERS may promote cataract occurrence by inducing cell apoptosis.

ATF4 is an important transcription target of the UPR under continuous ERS, providing a key link between ERS and apoptosis (Fusakio et al., 2016). *Ddit3* encodes a pro-apoptotic factor. In addition, *Chac1* is highly expressed in the lens and is a new apoptotic gene of the UPR, playing a downstream role in ATF4-ATF3-Ddit3 (Pendergrass et al., 2005; Mungrue et al., 2009). Here, Ddit3 expression was significantly up-regulated in the αA-Y118D mutant lens, indicating that cataract occurrence may be correlated with
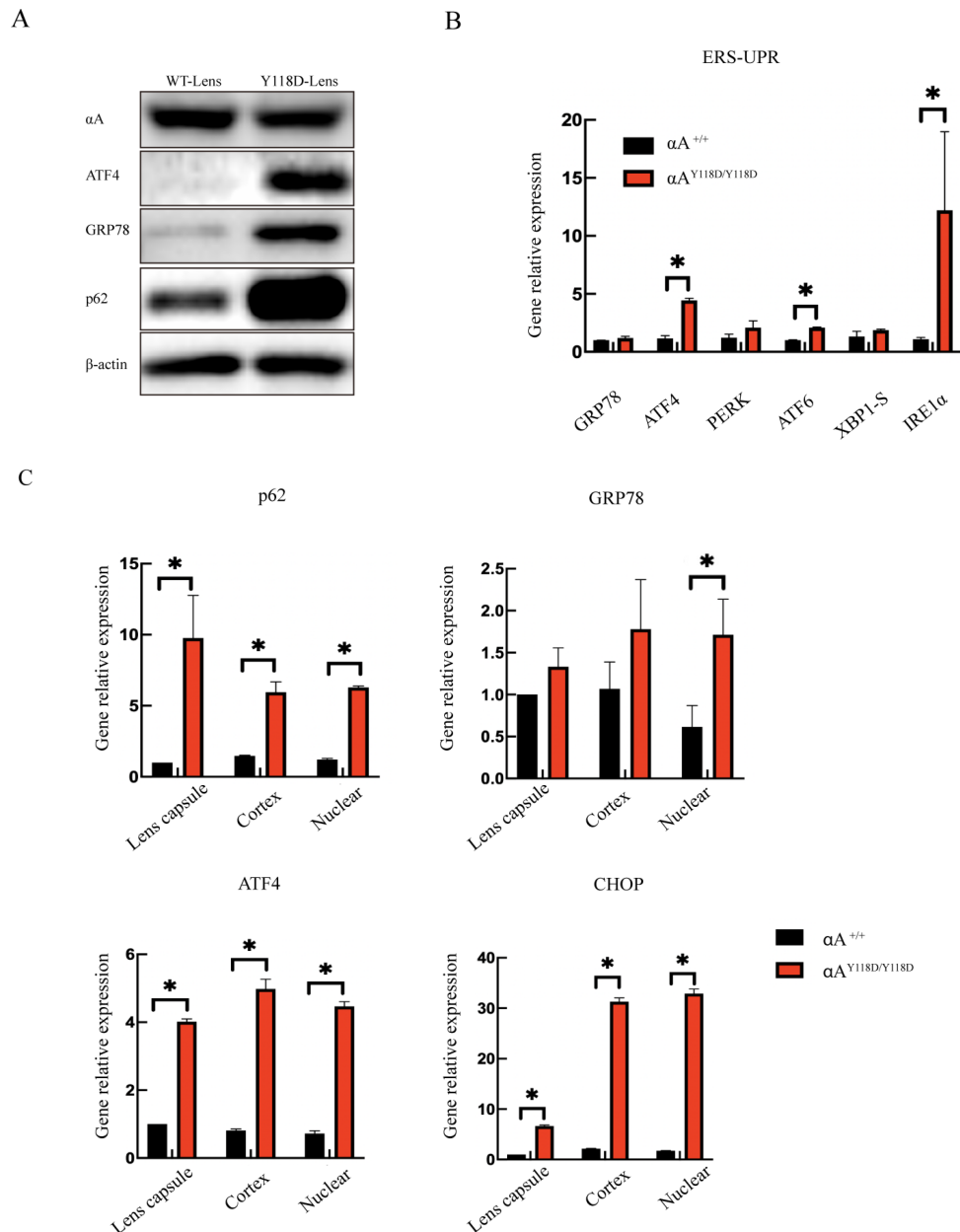
Figure 4 Verification of expression of ERS-UPR-related genes

A: Western blotting of genes related to ERS-UPR signaling pathway. B: qPCR results of genes related to ERS-UPR signaling pathway. C: Expression levels of ERS-UPR signaling pathway-related genes in lens capsule, cortex, and nucleus. Values are mean±*SEM*, *: *P*<0.05.

programmed cell death. Pro-apoptotic target genes, including *ATF4*, *ATF3*, *Cebpb*, and *Trib3*, were significantly up-regulated. However, the upstream ER sensor *perk* was not significantly up-regulated. *Cebpb* encodes the bZip transcription factor in response to ERS and participates in regulation of Chac1 transcription together with *ATF4* and *ATF3* (Crawford et al., 2015). KEGG analysis also showed that the p53 and HIF-1 signaling pathways play important functions in cataract formation. Phosphorylation and dephosphorylation of p53 modulate protein stability and transcription factor activity (Inoue et al., 2016). HIF-1α, an important regulator of the response to hypoxia, plays a

regulatory role in oxidative stress (Li et al., 2019).

In summary, we explored the role of αA-crystallin *in vivo* using the αA-Y118D mutant mouse model. This study provides information on the mechanism underlying congenital cataracts caused by αA-crystallin mutation and the pathogenesis of age-related cataracts. Transcriptome analysis of the αA-Y118D lens showed that continuous activation of the UPR ATF4-Ddit3-Chac1 branch caused cell death in the lens, resulting in cataract development (Figure 5). In addition, several other non-UPR-related genes were differentially expressed. Our findings imply that the development of targeted cataract drugs via the lens UPR should consider
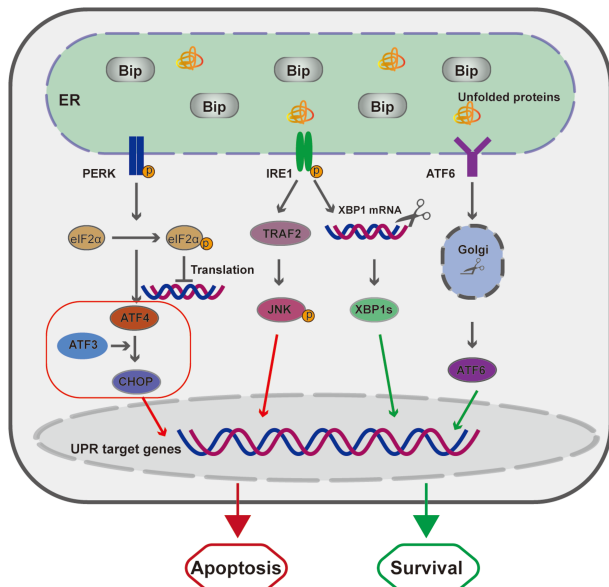
Figure 5 Unfolded protein response (UPR) signaling pathways
There are three distinct UPR signaling pathways in mammalian cells: i.e., PERK, ATF6, and IRE1 pathways. Under normal conditions, the endoplasmic reticulum (ER) molecular chaperone, BiP, directly interacts with PERK, ATF6, and IRE1. PERK phosphorylates eIF2α to attenuate ER burden by inhibiting its translation initiation activity. PERK-phosphorylated eIF2α also activates ATF4, which induces expression of ER chaperone-related genes and autophagy-related proteins. When BiP is separated from ATF6, the latter moves to the Golgi apparatus, where it is cleaved into its active form by S1P and S2P. Active ATF6 translocates to the nucleus and induces transcription of ERS-response genes. IRE1 endoribonuclease is activated through dimerization and transphosphorylation. This leads to removal of a 26-nucleotide intron from the premature unspliced XBP1 (XBP1-u) gene form to produce the spliced XBP1 (XBP1-s) form. XBP1-s moves to the nucleus and induces UPR-responsive genes.

stress response pathways.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Z.K.J. acquired and analyzed the data and drafted the manuscript. Z.K.J., C.X.F., and A.L.W. performed the experiments and revised the manuscript. K.Y. and X.J.C. conceived and designed the study, interpreted and analyzed the data, and revised the manuscript. All authors read and approved the final version of the manuscript.

REFERENCES

Ahsan SM, Bakthisaran R, Tangirala R, Rao CM. 2021. Nucleosomal association and altered interactome underlie the mechanism of cataract caused by the R54C mutation of αA-crystallin. *Biochimica Et Biophysica*

Acta. General Subjects, **1865**(5): 129846.

Al Mamun A, Rahman MM, Zaman S, Munira MS, Uddin MS, Rauf A, et al. 2020. Molecular Insight into the Crosstalk of UPS Components and Alzheimer's Disease. *Current Protein & Peptide Science*, **21**(12): 1193−1201.

Andley UP. 2009. αA-crystallin R49Cneomutation influences the architecture of lens fiber cell membranes and causes posterior and nuclear cataracts in mice. *BMC Ophthalmology*, **9**(1): 4.

Andley UP, Goldman JW. 2016. Autophagy and UPR in alpha-crystallin mutant knock-in mouse models of hereditary cataracts. *Biochimica et Biophysica Acta (BBA) - General Subjects*, **1860**: 234−239.

Banh A, Bantseev V, Choh V, Moran KL, Sivak JG. 2006. The lens of the eye as a focusing device and its response to stress. *Progress in Retinal and Eye Research*, **25**(2): 189−206.

Bari KJ, Sharma S, Chary KVR. 2019a. Structure of G57W mutant of human γS-crystallin and its involvement in cataract formation. *Journal of Structural Biology*, **205**(3): 72−78.

Bari KJ, Sharma S, Chary KVR. 2019b. Conformational dynamics study on human γS-crystallin as an efficient route to childhood blindness. *Biochemical and Biophysical Research Communications*, **511**(3): 679−684.

Bartoszewska S, Collawn JF. 2020. Unfolded protein response (UPR) integrated signaling networks determine cell fate during hypoxia. *Cellular & Molecular Biology Letters*, **25**: 18.

Cheng C, Xia C hong, Huang Q, Ding L, Horwitz J, Gong X. 2010. Altered Chaperone-like Activity of α-Crystallins Promotes Cataractogenesis. *Journal of Biological Chemistry*, **285**(52): 41187−41193.

Chothe PP, Thakkar SV, Gnana-Prakasam JP, Ananth S, Hinton DR, Kannan R, et al. 2010. Identification of a Novel Sodium-Coupled Oligopeptide Transporter (SOPT2) in Mouse and Human Retinal Pigment Epithelial Cells. *Investigative Opthalmology & Visual Science*, **51**(1): 413.

Christopher KL, Pedler MG, Shieh B, Ammar DA, Petrash JM, Mueller NH. 2014. Alpha-crystallin-mediated protection of lens cells against heat and oxidative stress-induced cell death. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, **1843**(2): 309−315.

Crawford RR, Prescott ET, Sylvester CF, Higdon AN, Shan J, Kilberg MS, et al. 2015. Human CHAC1 Protein Degrades Glutathione, and mRNA Induction Is Regulated by the Transcription Factors ATF4 and ATF3 and a Bipartite ATF/CRE Regulatory Element. *Journal of Biological Chemistry*, **290**(25): 15878−15891.

Delaye M, Tardieu A. 1983. Short-range order of crystallin proteins accounts for eye lens transparency. *Nature*, **302**(5907): 415−417.

Derham B. 1999. Alpha-Crystallin as a molecular chaperone. *Progress in Retinal and Eye Research*, **18**(4): 463−509.

Frankfater C, Bozeman SL, Hsu FF, Andley UP. 2020. Alpha-crystallin mutations alter lens metabolites in mouse models of human cataracts. *PloS One*, **15**(8): e0238081.

Fu C, Xu J, Jia Z, Yao K, Chen X. 2021. Cataract-causing mutations L45P and Y46D promote γC-crystallin aggregation by disturbing hydrogen bonds network in the second Greek key motif. *International Journal of Biological Macromolecules*, **167**: 470−478.

Fusakio ME, Willy JA, Wang Y, Mirek ET, Al Baghdadi RJT, Adams CM, et al. 2016. Transcription factor ATF4 directs basal and stress-induced gene expression in the unfolded protein response and cholesterol metabolism in the liver (S Wolin, Ed.). *Molecular Biology of the Cell*, **27**(9): 1536−1551.

Galluzzi L, De Santi M, Crinelli R, De Marco C, Zaffaroni N, Duranti A, et al. 2012. Induction of Endoplasmic Reticulum Stress Response by the Indole-3-Carbinol Cyclic Tetrameric Derivative CTet in Human Breast Cancer Cell

Lines (A Ahmad, Ed.). *PLoS ONE*, **7**(8): e43249.

Graw J, Löster J, Soewarto D, Fuchs H, Meyer B, Reis A, et al. 2001. Characterization of a new, dominant V124E mutation in the mouse alphaA-crystallin-encoding gene. *Investigative Ophthalmology & Visual Science*, **42**(12): 2909−2915.

Hejtmancik JF. 2008. Congenital cataracts and their molecular genetics. *Seminars in Cell & Developmental Biology*, **19**(2): 134−149.

Huang Q, Ding L, Phan KB, Cheng C, Xia CH, Gong X, et al. 2009. Mechanism of Cataract Formation in αA-crystallin Y118D Mutation. *Investigative Opthalmology & Visual Science*, **50**(6): 2919.

Ichimura Y, Komatsu M. 2010. Selective degradation of p62 by autophagy. *Seminars in Immunopathology*, **32**(4): 431−436.

Ikesugi K, Yamamoto R, Mulhern ML, Shinohara T. 2006. Role of the unfolded protein response (UPR) in cataract formation. *Experimental Eye Research*, **54**(83): 508−516.

Inoue K, Fry EA, Frazier DP. 2016. Transcription factors that interact with p53 and Mdm2. *International Journal of Cancer*, **138**(7): 1577−1585.

Kim R, Emi M, Tanabe K, Murakami S. 2006. Role of the unfolded protein response in cell death. *Apoptosis*, **11**(1): 5−13.

Koteiche HA, Claxton DP, Mishra S, Stein RA, McDonald ET, Mchaourab HS. 2015. Species-Specific Structural and Functional Divergence of α-Crystallins: Zebrafish αBa- and Rodent αA(ins)-Crystallin Encode Activated Chaperones. *Biochemistry*, **54**(38): 5949−5958.

Lee CM, Afshari NA. 2017. The global state of cataract blindness. *Current Opinion in Ophthalmology*, **28**(1): 98−103.

Lee WS, Yoo WH, Chae HJ. 2015. ER Stress and Autophagy. *Current Molecular Medicine*, **15**(8): 735−745.

Li J, Chen X, Yan Y, Yao K. 2020. Molecular genetics of congenital cataracts. *Experimental Eye Research*, **191**: 107872.

Li H, Li C, Lu Q, Su T, Ke T, Li DWC, Yuan M, Liu J, Ren X, Zhang Z, Zeng S, Wang QK, Liu M. 2008. Cataract mutation P20S of alphaB-crystallin impairs chaperone activity of alphaA-crystallin and induces apoptosis of human lens epithelial cells. *Biochimica Et Biophysica Acta*, **1782**(5): 303−309.

Li HS, Zhou YN, Li L, Li SF, Long D, Chen XL, et al. 2019. HIF-1α protects against oxidative stress by directly targeting mitochondria. *Redox Biology*, **25**: 101109.

Ma J, Yang YR, Chen W, Chen MH, Wang H, Wang XD, et al. 2016. Fluoxetine synergizes with temozolomide to induce the CHOP-dependent endoplasmic reticulum stress-related apoptosis pathway in glioma cells. *Oncology Reports*, **36**(2): 676−684.

Malhotra JD, Kaufman RJ. 2007. The endoplasmic reticulum and the unfolded protein response. *Seminars in Cell & Developmental Biology*, **18**(6): 716−731.

Martinez A, Lopez N, Gonzalez C, Hetz C. 2019. Targeting of the unfolded protein response (UPR) as therapy for Parkinson's disease. *Biology of the Cell*, **111**(6): 161−168.

McManus S, Roux S. 2012. The adaptor protein p62/SQSTM1 in osteoclast signaling pathways. *Journal of Molecular Signaling*, **7**: 1.

Moreau KL, King JA. 2012. Protein misfolding and aggregation in cataract disease and prospects for prevention. *Trends in Molecular Medicine*, **18**(5): 273−282.

Mungrue IN, Pagnon J, Kohannim O, Gargalovic PS, Lusis AJ. 2009. CHAC1/MGC4504 Is a Novel Proapoptotic Component of the Unfolded Protein Response, Downstream of the ATF4-ATF3-CHOP Cascade. *The Journal of Immunology*, **182**(1): 466−476.

Nahomi RB, Wang B, Raghavan CT, Voss O, Doseff AI, Santhoshkumar P,

et al. 2013. Chaperone Peptides of α-Crystallin Inhibit Epithelial Cell Apoptosis, Protein Insolubilization, and Opacification in Experimental Cataracts. *Journal of Biological Chemistry*, **288**(18): 13022−13035.

Pendergrass W, Penn P, Possin D, Wolf N. 2005. Accumulation of DNA, Nuclear and Mitochondrial Debris, and ROS at Sites of Age-Related Cortical Cataract in Mice. *Investigative Opthalmology & Visual Science*, **46**(12): 4661.

Roskamp KW, Kozlyuk N, Sengupta S, Bierma JC, Martin RW. 2019. Divalent Cations and the Divergence of βγ-Crystallin Function. *Biochemistry*, **58**(45): 4505−4518.

Rozpedek W, Pytel D, Mucha B, Leszczynska H, Diehl JA, Majsterek I. 2016. The Role of the PERK/eIF2α/ATF4/CHOP Signaling Pathway in Tumor Progression During Endoplasmic Reticulum Stress. *Current Molecular Medicine*, **16**(6): 533−544.

Shao Y, Wang L, Guo N, Wang S, Yang L, Li Y, et al. 2018. Cas9-nickase–mediated genome editing corrects hereditary tyrosinemia in rats. *Journal of Biological Chemistry*, **293**(18): 6883−6892.

Sun W, Xiao X, Li S, Guo X, Zhang Q. 2011. Mutation analysis of 12 genes in Chinese families with congenital cataracts. *Molecular Vision*, **17**: 2197−2206.

Tian F, Zhao J, Bu S, Teng H, Yang J, Zhang X, Li X, Dong L. 2020. KLF6 Induces Apoptosis in Human Lens Epithelial Cells Through the ATF4-ATF3-CHOP Axis. *Drug Design, Development and Therapy*, **14**: 1041−1055.

Uddin MdS, Tewari D, Sharma G, Kabir MdT, Barreto GE, Bin-Jumah MN, et al. 2020. Molecular Mechanisms of ER Stress and UPR in the Pathogenesis of Alzheimer's Disease. *Molecular Neurobiology*, **57**(7): 2902−2919.

Wu SY, Zou P, Mishra S, Mchaourab HS. 2018. Transgenic zebrafish models reveal distinct molecular mechanisms for cataract-linked αA-crystallin mutants. *PloS One*, **13**(11): e0207540.

Xia CH, Liu H, Chang B, Cheng C, Cheung D, Wang M, et al. 2006. Arginine 54 and Tyrosine 118 Residues of αA-Crystallin Are Crucial for Lens Formation and Transparency. *Investigative Opthalmology & Visual Science*, **47**(7): 3004.

Xu J, Wang H, Wang A, Xu J, Fu C, Jia Z, et al. 2021. βB2 W151R mutant is prone to degradation, aggregation and exposes the hydrophobic side chains in the fourth Greek Key motif. *Biochimica Et Biophysica Acta. Molecular Basis of Disease*, **1867**(2): 166018.

Yang J, Liu J, Zhao S, Tian F. 2020a. N6-Methyladenosine METTL3 Modulates the Proliferation and Apoptosis of Lens Epithelial Cells in Diabetic Cataract. *Molecular Therapy. Nucleic Acids*, **20**: 111−116.

Yang X, Xu J, Fu C, Jia Z, Yao K, Chen X. 2020b. The cataract-related S39C variant increases γS-crystallin sensitivity to environmental stress by destroying the intermolecular disulfide cross-links. *Biochemical and Biophysical Research Communications*, **526**(2): 459−465.

Yang J, Zhou S, Gu J, Wang Y, Guo M, Liu Y. 2015. Differences in Unfolded Protein Response Pathway Activation in the Lenses of Three Types of Cataracts. *PloS One*, **10**(6): e0130705.

Yu Y, Li J, Xu J, Wang Q, Yu Y, Yao K. 2012. Congenital polymorphic cataract associated with a G to A splice site mutation in the human beta-crystallin gene CRYβA3/A1. *Molecular Vision*, **18**: 2213−2220.

Zhou S, Yang J, Wang M, Zheng D, Liu Y. 2020. Endoplasmic reticulum stress regulates epithelial-mesenchymal transition in human lens epithelial cells. *Molecular Medicine Reports*, **21**(1): 173−180.

Zhuang J, Cao Z, Zhu Y, Liu L, Tong Y, Chen X, et al. 2019. Mutation screening of crystallin genes in Chinese families with congenital cataracts. *Molecular Vision*, **25**: 427−437.