A missense mutation in Pitx2 leads to early-onset glaucoma via NRF2-YAP1 axis

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Glaucoma is a leading cause of blindness, affecting 70 million people worldwide. Owing to the similarity in anatomy and physiology between human and mouse eyes and the ability to genetically manipulate mice, mouse models are an invaluable resource for studying mechanisms underlying disease phenotypes and for developing therapeutic strategies. Here, we report the discovery of a new mouse model of early-onset glaucoma that bears a transversion substitution c. G344T, which results in a missense mutation, p. R115L in PITX2. The mutation causes an elevation in intraocular pressure (IOP) and progressive death of retinal ganglion cells (RGC). These ocular phenotypes recapitulate features of pathologies observed in human glaucoma. Increased oxidative stress was evident in the inner retina. We demonstrate that the mutant PITX2 protein was not capable of binding to Nuclear factor-like 2 (NRF2), which regulates Pitx2 expression and nuclear localization, and to YAP1, which is necessary for co-initiation of transcription of downstream targets. PITX2-mediated transcription of several antioxidant genes were also impaired. Treatment with N-Acetyl-L-cysteine exerted a profound neuroprotective effect on glaucoma-associated neuropathies, presumably through inhibition of oxidative stress. Our study demonstrates that a disruption of PITX2 leads to glaucoma optic pathogenesis and provides a novel early-onset glaucoma model that will enable elucidation of mechanisms underlying the disease as well as to serve as a resource to test new therapeutic strategies.

**INTRODUCTION**

Glaucoma, affecting 70 million people worldwide, is a group of neurodegenerative diseases characterized by the progressive death of retinal ganglion cells (RGCs) and atrophic excavation of the optic nerve, ultimately resulting in irreversible loss of vision [1, 2]. Elevated intraocular pressure (IOP) is thought to be a major risk factor for glaucoma, and patients with high-pressure glaucoma generally present defects in outflow of aqueous humor [3, 4]. IOP is maintained through a balance between aqueous humor secretion, by the ciliary body (CB), and drainage through the trabecular meshwork (TM), a porous tissue located in the iridocorneal angle [5]. An increased resistance to aqueous humor drainage can arise from developmental malformations of ocular structures and ultimately lead to early-onset glaucoma.

Early-onset glaucoma cases generally have strong genetic contributions and many disease-causing mutations that lead to pathogenesis have been identified [6]. For example, mutations in CYP1B1, LTBP2, MYOC, FOXC1, GPATCH3, and TEK have been associated with Primary Congenital Glaucoma (PCG) [7]. Moreover, mutations in PAX6, PITX2, PITX3, FOXC1, FOXE3, EYA1, LMX1B, and MAF have been detected in patients with early-onset secondary glaucoma [8–12]. It should also be noted that glaucoma is a complex, heterogeneous disease likely to be the consequence of the interaction of multiple genes. This underlying complexity may hinder efforts to identify glaucoma-associated genes (or related mutations) and to uncover their pathogenic mechanisms. Identification of monogenic murine glaucoma models, may assist in addressing both of these issues. To this end, the Eye Mutant Resource screening program at The Jackson Laboratory (JAX) identified a glaucoma model caused by a mutation in Pitx2.

In humans, mutations in Paired-Like Homeodomain Transcription Factor 2 (PITX2) has been associated with Axenfeld-Rieger syndrome (ARS) (OMIM: 180500). PITX2, which encodes a member of the bicoid-like class of homeodomain (HD) transcription factors, play key roles in embryonic development and tissue morphogenesis. To date, a range of PITX2 mutations have been identified in ARS patients including missense, nonsense, splicing mutations, and copy number variations [13, 14]. The majority of ARS-causing mutations are missense mutations within the HD region, which may impair DNA binding and decreasing transcription activity [15]. ARS in humans is characterized by both systemic and ocular anomalies, such as corneal opacity, iris hypoplasia, corectopia, and
iridolenticular adhesions [12]. These developmental malformations of the anterior segment in ARS patients lead to severe forms of early-onset glaucoma in ~50% of affected individuals. Previous studies have suggested that Pitx2 deficiency in mice lead to an arrest in anterior segment development in structures derived from the periciliar mesenchyme, including the cornea, iris and outflow tract, which likely results from abnormal differentiation and migration of neural crest cells during the formation of anterior ocular structures [16–20]. Moreover, mice heterozygous for a Pitx2 null allele recapitulate the anterior segment dysplasia and developmental glaucoma observed in ARS patients [21]. However, the role of PITX2 in maintenance of normal optic function in adults and the precise mechanisms underlying glaucomatous pathologies when PITX2 is disrupted remain largely unknown.

In this study, we identified a novel missense mutation, c. G344T, in Pitx2. In the homozygous state, mice bearing this mutation presents with bulging and distended eyes, a striking feature of glaucoma. Moreover, increased oxidative stress was evident in mutant retinas, which further activated persistent glial activation in the inner retina and RGC apoptosis. Mechanistically, the missense mutation disrupts protein interaction of PITX2 with NRF2 (regulating Pitx2 expression and nuclear localization) and YAP1 (co-initiating transcription of downstream targets), leading to impaired PITX2-mediated transcription of several antioxidant genes, which activates the antioxidant response after ocular injury. Additionally, treatment with N-Acetyl-L-cysteine exhibited profound neuroprotective effect. Overall, this work describes a novel early-onset glaucoma model and sheds light on the pathogenesis of glaucoma in ARS.

MATERIALS AND METHODS

Mouse models

All animal experiments were approved by the Institutional Animal Care and Use Committee of Sichuan Provincial People’s Hospital (Chengdu, Sichuan, China) and The Jackson laboratory (JAX, Bar Harbor, Maine, USA) and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The original mutant was identified in a N-ethyl-N-nitrosourea (ENU) chemical mutagenesis screen in the Mouse Resource for Craniofacial Research (http://craniofacial.jax.org/). Briefly, C57BL/6J male mice, G0, were treated with ENU (weekly 80 mg/kg dosage for 3 weeks), and bred to normal C57BL/6J females after returning to fertility. G1 male offspring were backcrossed to unmutagenized C57BL/6J females and their G2 female offspring were backcrossed to their G1 sires to generate a population of G3 mice that were screened for visible phenotypes. A G3 mutant with a shortened face and enlarged eyes was identified. This mutant was backcrossed again to C57BL/6J and the short face phenotype segregated from the ocular phenotype. This mutant was named early-onset glaucoma (egl) and was bred to homozygosity and subsequently maintained by sibling intercrosses (C57BL/6J-Pitx2<sup>egl1</sup>/Boc; stock No. 004240). The egl1 mutant mice in this study were bred and maintained in standardized conditions of the Research Animal Facilities at The Jackson Laboratory (JAX). They were provided with a NIH31 6% fat chow diet and acidic water, in a pathogen-free vivarium environment with a 14-hour light/10-hour dark cycle.

Clinical evaluation and electroretinography

Eyes of all mice used in the characterization studies and linkage crosses were diluted with 1% atropine ophthalmic drops (Bausch and Lomb Pharmaceuticals Inc, Tampa, FL, USA) and were evaluated by indirect ophthalmoscopy with a 78-diopeter lens. Fundus photographs were taken with a Micron III in vivo bright field retinal imaging microscope equipped with image-guided OCT capabilities (Phoenix Laboratories, Inc, Phoenix, AZ, USA). The intraocular pressure (IOP) was measured by an induction–impact tonometer (TonoLab Colonial Medical Supply, London-derry, NH, USA). IOP was measured immediately after induction with an intraperitoneal injection of xylazine (80 mg/kg) and ketamine (16 mg/kg) in normal saline (~5 min). The mouse was gently restrained by hand on an adjustable platform, and the eye was oriented to align the probe tip with the optical axis of the eye at about 2-mm distance under dissecting scope. All tested animals with the same age were randomly divided into the experimental groups. Five consecutive IOP readings were averaged.

For electroretinographic evaluation of mutants, following an overnight dark adaptation, mice were anesthetized with an intraperitoneal injection of xylazine (80 mg/kg) and ketamine (16 mg/kg) in normal saline. Additional anesthetic was given, if akinesia was inadequate. The equipment and protocol used here have been previously described [22]. Briefly, dark-adapted, rod-mediated ERGs were recorded with the responses to short-wavelength flashes over 4.0-log units to the maximum intensity by a photopic stimulator. Cone-mediated ERGs were recorded with white flashes after 10 min of complete light adaptation. The signals were sampled at 0.8 ms intervals and averaged.

Whole-exome sequencing (WES)

DNA purification, library construction, deep Next Generation sequencing, and data quality control was performed by the Jackson Laboratory’s Genome Technologies service, and data analysis and annotation were performed by the Computational Sciences Biostatistics service. Purified genomic DNA from egl1/egl1 mice and C57BL/6J controls were used to create libraries for whole-exome sequence (WES) capture. High-quality reads were mapped to the genome with aligner (BWA) and the resulting alignment was sorted by coordinates and further converted to binary alignment map (BAM) format by Picard v1.95 SortSam utility (http://picard.sourceforge.net). Variants with total read depth >5X with the SNP quality score >50 were included and variants in non-coding regions and synonymous variants were excluded. The filtered candidate genes are shown in Table S1.

Gene mapping, sequencing and genotyping

To determine the chromosomal location of the egl1 mutation, we mated egl1/egl1 mice to DBA/2J mice. The resultant F1 mice, which did not exhibit retinal abnormalities, were backcrossed to B6-egl1/egl1 mice to produce N2 mice. Tail DNA was isolated as previously reported [23]. A genome-wide scan of pooled DNA from 12 affected and 12 unaffected mice was carried out using 48 microsatellite markers [24]. The egl1 phenotype cosegregated with markers on Chromosome 3. Subsequently, DNAs of 93 N2 offspring were genotyped using microsatellite markers to develop a fine structure map of the Chromosome 3 region. Microsatellite markers D3Mit106 and D3Mit291 were used to genotype individual DNA samples. The causative mutation was identified by comparing the whole-exome sequences from a homozygous egl1 mutant and control [25]. Between D3Mit106 and D3Mit291 on Chromosome 3, a unique point mutation c. G344T of Pitx2 was identified in the filtered data of WES (Table S2), in egl1/egl1 but not in wild-type DNA. Allele-specific PCR (AS-PCR) was used on genomic DNA to confirm the presence of the Pitx2<sup>egl1</sup> mutation. Three oligo primers were selected from exon 2 of the Pitx2 gene with [G/T] single base change (WT = G, egl1 mutant = T) using a web-based software "Web-based Allele-Specific Primer" (http://bioinfo.biotec.or.th/WASP): wild-type (W), mutant (M) and common (C) primers: W reverse primer: CGAAGTTGCATGTTCTGGAA; C forward primer: AGCAAGGAAAGATGAGGAT, and the PCR product size 145 bp (base pairs). The AS-PCR assay was conducted in two parallel experiments: (1) "mutant + common" primer experiment (MC) and (2) "wild + common" primer experiment (WC). The PCR assay for Pitx2<sup>egl1</sup> mutation was performed in 10 μl reactions. The PCR conditions were as followed: initial denaturation for 3 min at 94 °C followed by 36 cycles of denaturation for 15 s at 94 °C, annealing for 2 min at 55 °C, extension for 2 min at 72 °C, and a final extension step for 7 min at 72 °C. PCR products were run on 1.5% SeaKem agarose gel.

Generation of knock-in mouse model by CRISPR/Cas9

The CRISPR/Cas9 oligonucleotide directed approach was used to generate the Pitx2<sup>p.R115L</sup> knock-in (KI) mice. The gRNT (CCTGCGAGTTGGA-CATGTCGGA) designed to mouse Pitx2 gene together with Cas9 mRNA and a donor oligo (CAGAGGAGACTTTCTACTAGGACGACGGAGGCTCTTCATCTCTCCAGAACGAAAAACAGTGTCGGA) was co-injected into C57BL/6J mouse zygotes to generate targeted knock-in offspring. F0 founder mice were mated to C57BL/6J wildtype mice and subject to sequence analysis. Positive F1 offspring (Pitx2<sup>p.R115L</sup>, strain name C57BL/6J-Pitx2<sup>p.R115L</sup>, hereafter named...
Pitx2β) were backcrossed to C57BL/6j for four generations and then intercrossed to generate heterozygous and homozygous offspring. After PCR amplification of the targeted region, homozygotes (Pitx2β) heterozygotes (Pitx2β/−) and wild-type (Pitx2β+/+) could be determined by Sanger sequencing analysis.

Genotyping of Pitx2β mice
Genomic DNA was isolated either from tail tips or from blood using the QiAamp DNA Blood kit (Qiagen) according to the manufacturer’s instructions. WES results or genotypes were confirmed by Sanger sequencing with the following primers (Sangon Biotech, Shanghai, China): Pitx2-Forward primer (F1): 5' CGGTAGAGGGTTCAGGTTTCTC-3'; Pitx2-Reverse primer (R1): 5' GGCAAGAGCCGCTGAGGTGTA-3'. All PCR amplification was performed using a master mix (Invitrogen, USA). The first cycle consisted of 95 °C for 5 min, followed by 32 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The PCR products were purified by FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific Fermentas), and directly sequenced using BigDye version 3.1 and an ABI 3730 automated sequencer (Applied Biosystems) according to the manufacturer’s instructions.

Plasmids and site-directed mutagenesis
The recombinant expression plasmids, pcDNA3.1-PITX2-Flag, pcDNA3.1-Nrf2-HA tag, and pcDNA3.1-YAP1-HA tag were purchased from Youbio Inc. (Youlou, Jiangsu, China). Point mutation c. G344T was introduced into the WT Pitx2 cDNA by site-directed mutagenesis using a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with a complementary pair of primers: F: 5' CCACCTTCCGAGAAGACTTCACCATCAGATG-3' and R: 5' CATTGCTGTGGTAGGTTTCTCTGTGGAAGTGG-3'. The recombinant plasmids containing PITX2 (R151L)-Flag fusion constructs were sequenced to confirm the desired mutation and to exclude any other sequence variations.

Cell culture and transfection
HEK293T and COS-7 cells were purchased from National Infrastructure of cell line Resource (Wuhan, China) and were recently authenticated by STR profiling. They were cultured in DMEM with high glucose (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 100 U/ml penicillin/streptomycin (Invitrogen, Waltham, MA, USA) in an incubator set to 37 °C with 5% CO2. For transfection, cells were seeded in plates (Corning, NY, USA) and transiently transfected with Flag-tagged PITX2WT/PITX2R115L or HA-tagged NRF2/YAP1 plasmid using Lipofectamine 3000 (Invitrogen, CA, USA) according to the manufacturer’s instructions, and the cell lysis were harvested after 48 h.

Immunocytochemistry
COS-7 cells were seeded in 24-well plates (Corning, Corning, NY, USA) and transfected at 70% confluence with constructs vectors or empty vectors using Lipofectamine 3000 (Invitrogen, CA, USA) according to the manufacturer’s instructions. Cells were harvested after 48 h and fixed in 4% paraformaldehyde for 15 min at room temperature. After blocking with 1× PBS containing 5% normal goat serum and 0.2% Triton X-100, cells were incubated with specific antibodies at 4 °C overnight. The primary antibodies used are shown in Table S3. AlexaFluor 594/488-conjugated goat anti-mouse/rabbit secondary antibody (Cat# A11005 and A11008, Invitrogen, Waltham, MA, USA, 1:500 dilution) was applied and nuclei were counter-stained with DAPI (Cat# D8417, Sigma, St Louis, MO, USA).

Luciferase assay
The luciferase assays to detect the transcriptional activity of PITX2 were performed as described previously [26, 27]. The human LEF-1 promoters were constructed in the luciferase vector as previously described [28, 29]. SV-40 β-galactosidase reporter plasmid was used as a control for transfection efficiency. 293T cells were prepared and mixed with 2.5 µg of expression plasmids, 5 µg of reporter plasmid and 0.5 µg of SV-40 β-galactosidase plasmid in 60 mm culture dishes. After incubating for 48 h, transfected cells were lysed and assayed for reporter activities. Luciferase activity was measured using reagents from Promega (Cat# E1500, Madison, WI, USA), β-galactosidase was measured using Galacto-Plus reagents (Cat# T2118, Invitrogen, Waltham, MA, USA). All luciferase activities were normalized to β-galactosidase activity and are shown as mean-fold differences relative to empty luciferase plasmids.

RNA-sequencing analysis
RNA-sequencing analysis was performed on four independent biological replicates from four wild-type (WT) and four Pitx2 mutant retina at 2 months of age. After harvesting, both retinas for each animal were collected and immediately frozen. RNA was extracted using RNeasy Mini kits (Qiagen). RNA integrity and concentration were evaluated using a Bioanalyzer 2100 with RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). For library preparation, a total of 2 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEB Next, Ultra RNA Library Prep Kit for Illumina® (Cat# E7530L, NEB, USA), following the manufacturer’s recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature conditions in NEBNext First-Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized using random hexamer primers and RNase H. Second-strand cDNA synthesis was subsequently performed using dNTPs, DNA polymerase I and RNase H. The library fragments were purified with QiaQuick PCR kits and eluted with EB buffer, and then terminal repair, A-tailing and addition of an adapter were implemented. The RNA concentration of the library was measured using a Qubit RNA Assay Kit in Qubit 3.0 and the library was diluted to 1 ng/µl. Insert sizes were assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies), and the qualified insert size was accurately quantified using a StepOnePlus™ Real-Time PCR System (library valid content ranging from 10 ng to 1 µg). The clustering of the indexed samples was performed on a cBot cluster generation system using a HiSeq PE Cluster Kit v4-CBot-HS (Illumina, CA, USA) according to the manufacturer’s instructions. After cluster generation, the libraries were sequenced on an Illumina HiSeq 2500 platform, and 150 bp paired-end reads were generated. All gene expression values from RNA-seq were converted to a log2 value and analyzed further. Then, a P value of less than or equal to 0.05 was considered significant. The raw sequence data have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number CRA005112 that are publicly accessible at "https://ngdc.cnbc.ac.cn/gsa".

RNA extraction and quantitative PCR
Optic nerve total RNA was extracted using TRIzol reagent (Sigma, Saint Louis, MO, USA) as recommended by the manufacturer. First-strand cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA). Quantitative PCR was carried out using a StepOnePlus™ Real-Time PCR System (library valid content ranging from 10 ng to 1 µg). The clustering of the indexed samples was performed on a cBot cluster generation system using a HiSeq PE Cluster Kit v4-CBot-HS (Illumina, CA, USA) according to the manufacturer’s instructions. After cluster generation, the libraries were sequenced on an Illumina HiSeq 2500 platform, and 150 bp paired-end reads were generated. All gene expression values from RNA-seq were converted to a log2 value and analyzed further. Then, a P value of less than or equal to 0.05 was considered significant. The raw sequence data have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number CRA005112 that are publicly accessible at "https://ngdc.cnbc.ac.cn/gsa".

Histological analysis
For haematoxylin and eosin staining (H&E), enucleated eyes from control and mutant mice were fixed overnight in 1.22% glutaraldehyde and 0.8% paraformaldehyde in 0.08 M phosphate buffer, embedded in paraffin and then cut in 5 µm sections. To ensure sections used for quantification came from the same eccentricity, the globe was embedded in the same orientation. Sections that encompassed the optic nerve (ON) were selected for staining with haematoxylin and eosin according to standard protocol.

Immunohistochemistry
For immunohistochemistry, eyes were removed from euthanized mice by intraperitoneal injection of pentobarbital (75 mg/kg), and by cervical dislocation and fixed in 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4) for 1 h at 4 °C, followed by cryoprotection in 30% sucrose for 2 h. Lens were removed and eyes were embedded in optimal cutting temperature solution (OCT) and sectioned at 10 µm thickness. After blocking and permeabilization with 10% normal donkey serum and 0.2% Triton X-100 in phosphate buffer for 1 h, the sections were labeled with the primary antibody at 4 °C overnight. The primary antibodies used are shown in Table S3. The sections were rinsed in PBS three times and Alexa Fluor 594/488-conjugated goat anti-mouse/rabbit secondary antibody (Cat# A11005 and A11008, Invitrogen, Waltham, MA, USA, 1:500 dilution) was applied and nuclei were counter-stained with DAPI (Cat# D8417, Sigma, St Louis, MO, USA).
conducted, and one representative blot is presented. The density of the protein. At least three independent western blots were shown in Table S3. Primary antibodies were detected with either an anti-

ImageJ was used to calculate the intensity of the targeted protein. The relative binding capacity was quantified by the normalized IP-HA intensity/normalized IP-Flag intensity. At least three independent co-IP experiments were conducted, and one typical blot is presented.

Measurement of superoxide production

Superoxide production was evaluated in retinal cryosections using dihydroethidium (DHE) as described previously [31]. Briefly, frozen sections were incubated with DHE (2 μM) for 30 min at 37 °C. DHE is oxidized upon reaction with superoxide to ethidium bromide, which binds to DNA in the nucleus and fluorescence red. Excessive reactive oxygen species (ROS) production is a hallmark of oxidative stress. Thus, retinal levels of superoxide, as determined by DHE staining with subsequent quantification of fluorescence intensity, was measured. Images were captured on a Zeiss LSM 800 confocal scanning microscope. The relative fluorescence intensity within the images obtained was determined via automated image analysis of ZEISS ZEN Intellisys or ImageJ software.

Measurement of the MDA level and SOD, GSH-Px activity

Retinas were immediately extracted from enucleated eyes. The weighed retinal samples were prepared as a 10% homogenate in 0.9% saline. After homogenization on ice, the homogenate was sedimented at 2000 × g for 10 min, and the supernatant was collected and diluted. The GSH-Px, SOD activity, and MDA levels of the retinal lysates were determined using ELISA kits from the Nanjing Jiangcheng Bio-Company (Cat#A005, A001, A003, respectively, Nanjing, China). All procedures were carried out according to the manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. The data sets were tested for normal distribution using Shapiro–Wilk test. For normally distributed data, statistical significance was determined by Student’s t-test or ANOVA. If the data was not normally distributed, non-parametric statistic was used. p-values were calculated by Student’s t-test or ANOVA followed by a, Tukey, Dunnett or Sidak’s multiple comparisons test as appropriate. P < 0.05 was considered statistically significant.

RESULTS

A new model of early-onset glaucoma identified in an ENU screening program

The egl1 mutant, with bulging eyes, was discovered while screening an ENU treated cohort for craniofacial phenotypes. Affected mice were distinguished from C57BL/6J controls by a high IOP phenotype (Fig. 1A). Optic neuropathy characterized by optic nerve cupping, and by severe retinal nerve fiber layer (NFL) loss in mutant retinas (Fig. 1B) was detected with optical coherence tomography. Histological study of retinas from 12-month-old mice confirmed the clinical assessment, and revealed enlarged optic disc cup with thinning of the inner retinal layer in mutant mice (Fig. 1C). Whole-exome sequences (WES) revealed a G to T base-pair transversion in Pitx2 (Fig. 1D) in 12-month-old mutant mice. The presence of Pitx2<sup>egl1</sup> mutation in a segregating cross. Thus, a novel homozygous missense mutation, c. G344T (p.R115L) (NM_001042502.2), in the Pitx2 gene was identified and deemed the most likely candidate for the glaucoma-like symptom in egl1/ egl1 mice. Notably, the affected amino acid residue is located in the HD region of PITX2 protein (Fig. 2D, lower panel) and is highly conserved across species (Fig. 2E).

Generation of the Pitx2<sup>egl1</sup> mouse model

In order to confirm the causative nature of the egl1 mutation and explore the Pitx2 mutation further, a Pitx2<sup>egl1</sup> knock-in mouse model (named Ki) was generated using CRISPR/Cas9 technology (Fig. S3). Homozygous Pitx2<sup>Ki</sup> mice were born at the expected Mendelian ratio (49 out of 220 in Pitx2<sup>egl1</sup>/ Ki to Pitx2<sup>egl1</sup>–/– crosses) and was not significantly different from the expected, 55 out of 220 mice. No significant changes in the protein content and localization pattern of Pitx2 were observed in mutant retina.
photographs and optical coherence tomography images of determine whether the elevated IOP in
with aberrant ocular development. Mice were examined at E15.5, elevation of IOP measurements, 13.87 ± 0.89 mmHg (16.38 ± 1.13 mmHg (14.62 ± 2.34 mmHg (associated with glaucoma (Fig. 3C). Interestingly, nearly 53% of age, 47% of mutants presented with high IOP levels of >18 mmHg, IOP (gray bar, egli1 mice = 23.1 ± 1.4 and black bar, WT mice = 18.4 ± 1.7) at 12 months of age. The green lines indicate the plane in which the B-scan to the left of the fundus images were taken P, a marker for TM cells. This
50% of ARS patients develop elevated IOP and glaucomatous indicating incomplete penetrance of the disease. Similarly, only 50% of ARS patients develop elevated IOP and glaucomatous pathologies [33–35].

Previous studies have demonstrated that PITX2 is required for normal ocular development [21, 36]. We, therefore, sought to determine whether the elevated IOP in Pitx2wt mice was associated with aberrant ocular development. Mice were examined at E15.5, P9 and 6 weeks for morphological alterations by histology. The anterior chamber of mutant mice at E15.5 appeared slightly collapsed and showed delayed separation of the cornea from the lens surface compared to Pitx2wt mice, but otherwise no other suggestion of abnormal development was apparent (Fig. 3D, upper panel). By contrast at P9, multiple malformations of the anterior chamber were observed in mutant eyes. A distinct disruption of the iridocorneal angle with adhesion of the iris to cornea, creating a fully angle closure was observed in mutant eyes (Fig. 3D, middle panel). The iris-cornea adhesion was more pronounced in adults, at 7 weeks of age, and was accompanied by iris hypoplasia and ciliary body atrophy in all mutant eyes (Fig. 3D, lower panel).

Moreover, histological analysis in 7-week-old Pitx2 mutant mice revealed a thinner cornea compared to that of control (Fig. S3C, D). Immunofluorescent staining of sections from WT mice indicated that PITX2 was strongly expressed in the mouse GCL, where it colocalized with the RGC marker, Brn3a (Fig. S3D, upper panel), suggesting that Pitx2 may play a role in RGC function.

**Pitx2wt** mice develop elevated IOP subsequent to anterior segment dysgenesis

Similar to the Pitx2wt mutant mice, we observed that approximately 59% of mutants (20 out of 34) exhibited a bulging and distended appearance of the eye with a mild corneal opacity (Fig. 3A), as early as 2 months of age. Moreover that roughly 80% of these cases were bilaterally affected, as reported for ARS patients [19]. Rebound tonometry confirmed elevated IOPs in mutants. As expected, Pitx2wt mice exhibited a progressive elevation of IOP measurements, 13.87 ± 0.89 mmHg (n = 16), 16.38 ± 1.13 mmHg (n = 16), and 18.4 ± 1.7 mmHg (n = 18) at 3, 6 weeks, and 4 months of age, respectively. In contrast, the IOP measuring of their control littermates at corresponding ages were 11.16 ± 1.58 mmHg (n = 16), 13.17 ± 1.88 mmHg (n = 16), and 14.62 ± 2.34 mmHg (n = 18) (Fig. 3B), which is consistent with previous measurements for C57BL/6jstrains [32]. By 4-months of age, 47% of mutants presented with high IOP levels of >18 mmHg, and ~14% mutants with IOP > 21 mmHg, a level commonly associated with glaucoma (Fig. 3C). Interestingly, nearly 53% of mutants did not develop ocular hypertension at this stage indicating incomplete penetrance of the disease. Similarly, only 50% of ARS patients develop elevated IOP and glaucomatous pathologies [33–35].

Previous studies have demonstrated that PITX2 is required for normal ocular development [21, 36]. We, therefore, sought to determine whether the elevated IOP in Pitx2wt mice was associated with aberrant ocular development. Mice were examined at E15.5, P9 and 6 weeks for morphological alterations by histology. The anterior chamber of mutant mice at E15.5 appeared slightly collapsed and showed delayed separation of the cornea from the lens surface compared to Pitx2wt mice, but otherwise no other suggestion of abnormal development was apparent (Fig. 3D, upper panel). To further examine the TM malformation, ocular frozen sections were stained for α-SMA, a marker for TM cells. This analysis revealed that the majority of mutants exhibited an absence or smaller area of α-SMA staining compare with that of control, corroborating the TM hypoplasia in Pitx2wt mice (Fig. 3G, H). It should be noted that all heterozygotes (Pitx2wt+/−) tested at 3 months of age were not significantly different in ocular morphology or in IOP levels compared to their WT littermates (Fig. S4A, B), and all retinal structures, including the optic nerve head and anterior segment appeared normal (Fig. S4B, C).
Retinal ganglion cell loss and optic nerve degeneration in Pitx2KI mice

To determine whether high IOP leads to optic neuropathy in Pitx2KI eyes, we assessed retinal and optic nerve morphology in histological sections ON. Eyes of P16 mutant mice, an age prior to IOP elevation, exhibited a normal retinal layer morphology (Fig. S5A). At 6 weeks, a mild loss of cells in the ganglion cell layer (GCL) was observed in mutant mice (Fig. S5B), and this trend generally progressed with age.

At 3 months, mutant mice showed a mild thinning of outer and inner nuclear layers (Fig. S6A, B), accompanied by a 61.2% decrease in GCL cell number compared to that of controls (Fig. 2).
(Fig. S6A, C), indicating significant RGC loss. This result was precisely confirmed with anti-Brn3a, an RGC-specific marker, stained retinal section from 6-week-old (Fig. 4A) and 3-month-old (Fig. 4B) mice, which demonstrated a progressive RGC death in mutants (Fig. 4C). RGC density was further examined with anti-Brn3a stained retinal flatmounts from 3-month-old animals. Each anti-Brn3a stained retinal flatmount was segmented into nasal, temporal, ventral, and dorsal quadrants, and RGC density.
quantiﬁed respectively. In mutant retinas, a signiﬁcant decrease in Brn3a-positive cells in all four quadrants was observed (Fig. 4D). Quantitative analysis revealed a 43.4%; 32.8%; 24.3%, and 27.5% reduction of Brn3a-positive cells compare to that of WT in nasal, temporal, ventral, and dorsal area, respectively (Fig. 4E).

We next evaluated optic disc cupping, a characteristic pathology of glaucoma. Clinical fundus examination revealed a glaucomatous fundus appearance with an asymmetric and severely excavated ON head and peripapillary chorioretinal atrophy in mutant eyes (Fig. 4F). Consistent with clinical fundus examinations, histological analysis revealed optic disc cupping and thinning of the NFL near the ON in mutant mice at 7 weeks of age, and these phenotypes became more severe at 3 months (Fig. 4G, H). Moreover, severe axon loss was also visible in the central retina of mutant mice, as evidenced by an extremely atrophied NFL, which was conﬁrmed by Neuroﬁlament-L staining (Fig. 4I). To determine details of axonopathy, toluidine blue-stained semithin sections of the ON from 3-month-old animals were prepared and the number of axons were quantiﬁed. Compared with WT mice, the axons density was clearly lower in mutant mice and was
accomppanied by axonal swelling and extensive myelin debris (Fig. 4), indicating severe axonal damage. Additionally, to assess if pathological changes in mutants with normal IOPs (≤18) occurred, we also examined these mutants. Interestingly, pathologic features of glaucomatous injuries, such as RGC loss (Fig. 5A, B) and ON damage (Fig. 5C–E) were observed but the phenotypes appeared milder compared to mutants with elevated IOPs.

Vision loss after ON degeneration is also a hallmark of glaucoma. We thus tested vision function of 3-month-old mice using ERG recordings. The scotopic b-wave amplitude was reduced at 0.3, 3, and 20 cd s/m² flash intensities (Fig. S8A–C, G), and the photopic b-wave (Fig. S8D, E, H) and flicker amplitude (Fig. S8F, I) was also reduced in mutant mice, while no difference was observed for the scotopic a-wave amplitude (Fig. S8A–C, G), suggesting impaired visual function in the inner retina. Additionally, the amplitudes of the oscillatory potentials (OPs), which originate from the functional inner retina in mice [37], were also significantly reduced in mutant mice (Fig. S9), reflecting inner retinal dysfunction.

Biochemical analysis of mutant PITX2 proteins

RT-PCR was performed on mRNA extracted from retina of adult WT mice. Among the three alternatively-spliced murine isoforms (Pitx2a, Pitx2b and Pitx2c) (Fig. S10A), RT-PCR analysis revealed that only Pitx2c isoform was expressed in the mouse retina (Fig. S10B). c. G344T variant was introduced into Pitx2c coding sequence by site-directed mutagenesis to examine the impact of this variant. F Fundus photograph at 5 months showed abnormal morphology of optic nerve head (arrowhead) in the eyes of Pitx2mutant mice. G Representative images of histologic sections taken through nerve heads from Pitx2fl/fl mice at 7 weeks and 3 months of age. The optic nerve head appeared abnormal in Pitx2mutant mice at both ages, with thinning of the nerve fiber layer (NFL) (red arrowheads) and optic nerve excavation is noted (asterisk). Black arrows indicate NFL entering the optic nerve (ON); V: Central blood vessel. Scale bars: 200 μm. H Quantitation and comparison of the NFL thickness near the optic nerve in 7-week and 3-month-old mice (n = 6 per cohort). G Protein localization of the H&E staining (upper panel) and the immunofluorescent staining (lower panel) of retinal cross sections are shown. Retinal nerve fibers were labeled with neurofilament-L (NF-L), and the nuclei were counter-stained with DAPI. Scale bars: 25 μm. J Optic nerve atrophy was assessed in cross semithin sections of resin-embedded optic nerves from 5-month-old WT and mutant mice stained with toluidine blue staining. Scale bars: 100 μm. K Quantification of axon number in the optic nerve of 5-month-old mice (n = 10 per cohort). The Pitx2mutant mice have significantly less axonal projections compared to Pitx2fl/fl mice. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The statistics were calculated based on data from all tested mutants. The representative images of Pitx2mutant were from mice with elevated IOPs. **P < 0.01; ***P < 0.001, #, no significant difference. The data are presented as mean ± SEM.

The missense mutation disrupts the interaction of PITX2 with Nrf2/Yap1 and impairs the transcriptional activity of PITX2 for antioxidant genes

Previous studies suggest PITX2 expression and activity depends on Nrf2/Yap1-activated transcription and nuclear shuttling, where PITX2 binds to YAP1 and cooperatively activates transcription of antioxidant genes in mouse myocardium after injury [38]. We reasoned that the interactions of PITX2 with Nrf2/YAP1 could also play key roles in the transcription of antioxidant genes under injury condition in the retina. Thus, we analyzed the interactions between WT or mutant PITX2 and Nrf2/YAP1 by coimmunoprecipitation, in transiently transfected 293T cells. Upon co-expression of Flag-tagged WT PITX2 and HA-tagged NRF2, a distinct anti-HA signal (NRF2) was detected in cell lysates as well as Flag precipitates (Fig. 5D, left panel), confirming that PITX2 interacts with NRF2. We similarly confirmed interactions of PITX2 with YAP1 (Fig. 5D, right panel). By contrast, in the presence of mutant PITX2, the anti-HA signal (NRF2) was significantly weakened in Flag precipitates using the same procedure (Fig. 5E, left panel), and that of YAP1 was almost undetectable (Fig. 5E, right panel). Relative quantification (detail in methods) indicated that the binding capacity of mutant PITX2 to Nrf2 and YAP1 was reduced 53.78% and 89.12% of WT PITX2, respectively (Fig. 6C), indicating that the missense mutation disrupts the interaction of PITX2 with Nrf2 and YAP1, which might in turn affect its transcriptional activity. We then evaluated the transcriptional activity of the mutant PITX2 by assessing luciferase activity in transfected 293T cells using the LF-2700 promoter constructs [29]. Compared with the empty vector, WT PITX2 activated transcription from the LF-2700 promoter construct by approximately 12-fold (Fig. 5G). By contrast, the mutant PITX2 lost ~69.7% of its transcriptional activity compared to WT version (Fig. 5G). Thus, the missense mutation impairs the ability of PITX2 protein to recognize and bind to its target sequences and activate transcription of downstream genes. Additionally, to further determine whether the Nrf2/Yap1 axis participates in anterior chamber failure in Pitx2 animals, ocular cyrossections were immunofluorescently labeled using antibodies against Nrf2 and Yap1. No significant difference in staining was observed between WT and mutant mice (Fig. S11), implying that other mechanisms may underly the anterior chamber malformation.

We next performed transcriptomic analysis using of retinas from 2-month-old Pitx2 mutant mice and WT littermate controls to obtain insights into the molecular mechanisms. Analyses of the RNA-seq data revealed that expression of the antioxidative response-related genes (Cyp2a5, Upk3b1, Rtn2, Cyp26a1, Plet1, Slc25a24, Cyp2s1, Cyp2f2, Cyp2db1, and Cyp4a12b) were significantly downregulated in Pitx2 mutant retinas (Fig. 5H). These results were further confirmed by real-time quantitative PCR (qPCR) (Fig. 5I). Taken together, based on the transcriptome results, we propose that the rapid RGC loss and ON dystrophy in mutant retina results not only from the elevated IOP caused by anterior segment dysgenesis, but also to the compromised antioxidant capacity caused by impaired PITX2 transcriptional activity of antioxidant genes.

Pitx2fl/fl mutant retinas exhibit increased oxidative stress

Given the impaired PITX2-mediated transcription of antioxidant genes in mutant mice, we next examined the oxidative status of Pitx2fl/fl retinas of 6-week-old mice. Several indicators of oxidative stress were evaluated by ELISA using isolated retinal lysates. Compared to controls, decreased activities of the antioxidant...
enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and increased malondialdehyde (MDA) content were observed in mutant retinas (Fig. 6A–C), suggesting compromised antioxidant capacity and peroxidation in mutant retinas.

Retinal cryosections stained with dihydroethidium (DHE) (Fig. 6D) showed increased DHE reaction in n mutant retinas. Quantification of fluorescence intensity revealed a significant increase in superoxide production in mutant mice, compared to...
that of controls (Fig. 6E). In addition, both immunofluorescence and western blot demonstrated a significant increase in the NRF2 level in mutant retinas compared to controls (Fig. 6F–H). NRF2 regulates the expression of antioxidant proteins that attenuate cellular oxidative stress, thus its increased expression signifies activation of oxidative stress.

**Glial activation and RGC apoptosis in Pitx2^KI retinas**

We assessed the status of glial activation in mutant retinas from 2-month-old mice. In control retinas, CD68 immunoreactivity was weak and punctate, while in mutant retinas staining was intense and agminated in the inner retina (Fig. S12A), suggestive of an activated state. Moreover, the expression level of another
microglia marker Iba1 was found to be increased in mutant retinas by western blot analysis (Fig. S12B, C). Additionally, activated Müller glia were also distinctly recognizable in mutant retinas by immunostaining with an antibody against glial fibrillary acidic protein (GFAP) (Fig. S12A). These data confirmed astroglia in mutant retinas characterized by upregulation of GFAP level relative to controls (Fig. S12B, C). TUNEL assay revealed increased cell apoptosis in the GCL of mutant retinas, likely due to prolonged oxidative stress and excessive inflammatory reaction (Fig. S12D, E).

**Treatment with N-Acetyl-L-cysteine mitigates high intraocular pressure-induced retinal injury by inhibiting oxidative stress in Pitx2<sup>KI</sup> retinas**

The apparent oxidative stress and subsequent ocular injury prompted us to investigate whether an oxidative reductant would have protective efficacy in Pitx2 mutant mice. N-Acetyl-L-cysteine, an antioxidant, provides increased glutathione and exerts antioxidative protection. Thus, to rescue the RGC degeneration, N-Acetyl-L-cysteine (25 mg/kg/day) or PBS (serve as vehicle control), were intraperitoneally administered daily to Pitx2 WT or mutant mice after 5-weeks of age (Fig. 7A). After 5 weeks of treatment, mice were sacrificed for analysis.

N-Acetyl-L-cysteine-treated mutant mice (hereafter treated) showed no significant differences in IOP from vehicle-treated mutant mice (hereafter vehicle) (Fig. 7B). However, ROS activity was significantly affected in retinas of treated mutant mice. Imaging of vehicle mutant retinas demonstrated an increased DHE reaction in the GCL, INL, and ONL (Fig. 7C), while the DHE-superoxide reaction was largely prevented in the treated mutant retinas, which showed comparable staining intensity to that in WT retina (Fig. 7D). N-Acetyl-L-cysteine administration likely improved the oxidative stress response induced by elevated IOP in mutant retinas.

The RGC loss and ON damage was also evaluated by histological analysis. In vehicle mutant mice, the number of cells in GCL was drastically decreased compared to vehicle-treated WT controls. By contrast, the number of cells retained in the GCL were significantly greater in mutant mice administered N-Acetyl-L-cysteine, compared with corresponding vehicle-treated mice (Fig. 7E, F). The RGC density was further evaluated by immunofluorescent staining of Brn3a of retinal flatmounts. Consistent with H&E staining, N-Acetyl-L-cysteine treatment appeared to increase the number of Brn3a-positive cells in mutant retina compared to that of vehicle mutant mice in all four retinal quadrants (Fig. 7G), but still did not reach WT levels (Fig. 7H). To directly monitor the therapeutic effect on RGC death, the RGC number between vehicle- and treated-group of each genotype (indicated as Δ RGC number) were quantified. An increased number of Brn3a-positive cells after N-Acetyl-L-cysteine treatment was observed (Fig. 7I). Together, these data suggested that N-Acetyl-L-cysteine displays significant therapeutic effect on glaucomatous neuropathies through mitigation of oxidative stress and subsequent partial preservation of RGC in Pitx2<sup>KI</sup> retinas (Fig. 8).

**DISCUSSION**

The anatomical and physiological similarity between human and mouse eyes and the ability to genetically manipulate mice make them an excellent model to investigate disease mechanisms and potential intervention of diseases, such as glaucoma. The heritable DBA/2J glaucoma model has provided invaluable information on disease cell biology and intervention [39–41]. However, elevated IOP and glaucoma phenotypes do not manifest until 9–12 months, making mechanistic studies and testing of therapeutic strategies expensive and time-consuming. As a novel ENU induced Pitx2<sup>pR115L</sup> and the CRISPR/Cas9 mediated Pitx2<sup>pR115L</sup> glaucoma models exhibit early-onset glaucoma, including elevated IOP and RGC degenerative phenotypes, they provide valuable insight into degeneration of RGC and its related molecular pathways. At the molecular level, the Pitx2 p.R115L missense mutation disrupts binding of PITX2 to YAP1 and NRF2 and impairs its transcriptional activation of downstream genes involved in regulating oxidative stress (Figs. 5 and 6). Reduced activities of the antioxidant enzymes SOD and GSH-Px were observed in Pitx2 mutant retinas, indicating compromised antioxidant capacity and peroxidation (Fig. 5). In support of this, treatment with N-Acetyl-L-cysteine mitigates the RGC injury observed, presumably by inhibiting oxidative stress in mutant retinas (Fig. 7).

Alterations in the level of functional PITX2 protein (either increased or decreased) may contribute to the pathologies observed [42]. Previous studies reported that heterozygote Pitx2 null mice exhibited thinning of the ventral body, small body size, and ocular and tooth defects [21], whereas in other studies heterozygote alleles do not show obvious haploinsufficient phenotypes [43]. In the present study, heterozygous Pitx2<sup>pR115L</sup> mutants do not show any obvious glaucomatous phenotype (Fig. S3). This is probably due to the fact that the missense mutation studied here is likely to be a hypomorphic variant (Fig. 5). Additionally, incomplete penetrance was evident in our mouse models, with only 47% of mutants presenting with elevated IOP at 4 months of age (Fig. 3C), which is consistent with the observation that only 50% of patients with ARS develop elevated IOP and glaucoma. Moreover, while teeth hypodontia or microdontia is reported in some human ARS patients and not others, no apparent non-ocular abnormalities were observed in adult Pitx2<sup>pR115L</sup> mice. Thus, our mutants model recapitulates ocular phenotypes of ARS patients with PITX2 mutations [16, 44, 45].

We demonstrate two possible pathological mechanisms that underlie the glaucoma phenotypes induced by the missense mutation in Pitx2 (Fig. 8). The observed anterior segment dysplasia in Pitx2<sup>pR115L</sup> mice likely contributes to the dysfunction of the drainage system leading to an elevation in IOP, which serves as an initiator of the disease process. The mutation also exacerbates the high IOP-induced oxidative stress in the retina by disrupting the interaction of PITX2 with NRF2/YAP1, which consequently impairs the transcription of several antioxidant genes. The reduction of antioxidant capacity may accelerate RGC loss and optic neuropathy. The anterior segment dysplasia caused by Pitx2 variation has been well documented, but the precise mechanism through which subsequent pathogenesis occurs remains unclear. The present study...
mainly focuses on the latter, revealing a novel function of PITX2 in RGCs and in glaucomatous pathogenesis. We demonstrated that PITX2 moderates oxidative stress response through the NRF2-YAP1 axis in RGCs, whereas according to immunostaining data, this mechanism does not appear to be involved in the early post-natal phenotype (Fig. S11). The potential mechanisms triggering the anterior segment dysgenesis warrant further investigation.

Owing to the high demand of oxygen and energy necessary to generate action potentials, RGCs contain large number of mitochondria throughout the cell soma, axon and dendrites [46, 47]. Moreover, mitochondrial dysfunction has been recognized to be relevant to the development of glaucoma [48–52]. Transcriptional analysis of DBA/2J glaucoma model revealed mitochondrial dysfunction and metabolite depletion as a primary cause of neuronal damage in glaucoma [53]. These studies suggested that mitochondria dysfunction and oxidative stress play important roles in glaucoma. In our Pitx2KI early-onset glaucoma model, elevated expression levels of NRF2, a regulator of cellular...
resistance to oxidative stress, was observed (Fig. 6G–H). N-acetyl-L-cysteine treatment mitigated RGC loss by inhibiting oxidative stress in Pitx2 mutant retinas (Figs. 7 and 8). Cumulatively, our data identifies a potential target for development of therapeutic strategies for ARS induced glaucoma and also, a potential strategy for glaucoma therapy development.

**DATA AVAILABILITY**

All data generated or analyzed during this study are included in this published article and its supplementary information files. The data sets analyzed during the current study are available in Repository with Accession ID: CRA005112, Databank URL http://ngdc.cncb.ac.cn/gsa.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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