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Tubulin hyperglutamylation induces retinal degeneration by disrupting ciliary architecture and protein trafficking

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ABSTRACT

Tubulin post-translational modifications confer diverse functions to microtubules, with polyglutamylation representing a dynamic modification governed by coordinated glutamylation and deglutamylation. *AGBL5* functions as a deglutamylase that removes glutamate residues at branch points within polyglutamate chains. While pathogenic variants in human *AGBL5* are associated with retinitis pigmentosa, the underlying mechanism remains poorly defined. In the present study, an *Agb15* knockout mouse model was established and exhibited pronounced tubulin hyperglutamylation in photoreceptors, followed by progressive retinal degeneration. Transcriptomic profiling identified widespread disruption of ciliary function in *Agb15* knockout mice. Ultrastructural analysis by transmission electron microscopy revealed an impaired inner scaffold within the connecting cilium. Consistent with this defect, key phototransduction proteins were mislocalized or down-regulated in both mutant rod and cone photoreceptors, accompanied by severe disorganization of outer segment disk membranes. Immunofluorescence further demonstrated impaired recruitment of IFT88, kinesin-II, and dynein-2 to the connecting cilium, suggesting defective intraflagellar transport. Collectively, these findings indicate that *AGBL5*-dependent tubulin glutamylation homeostasis is essential for photoreceptor survival through preservation of connecting cilium architecture and normal protein

trafficking mediated by intraflagellar transport.

Keywords: *AGBL5*; Tubulin glutamylation; Connecting cilium; Intraflagellar transport; Photoreceptor; Retinal degeneration

INTRODUCTION

Inherited retinal disease represents a major cause of irreversible vision loss worldwide. Among these disorders, retinitis pigmentosa (RP) constitutes the most prevalent subtype, with an estimated global incidence of 1 in 3 000–1 in 7 000 individuals (Ferrari et al., 2011). Early stages of RP typically involve rod photoreceptor dysfunction, leading to night blindness and progressive constriction of peripheral visual fields (Hartong et al., 2006). Disease progression subsequently involves cone photoreceptors, culminating in tunnel vision and complete blindness in advanced stages. From a genetic perspective, RP exhibits marked heterogeneity, with more than 90 genes currently implicated in both nonsyndromic and syndromic forms of the disease (<https://sph.uth.edu/retnet/>).

Syndromic RP largely arises from mutations in genes essential for ciliary structure and function (Tatour & Ben-Yosef, 2020). Photoreceptor outer segments constitute highly specialized sensory cilia that retain core architectural features of primary cilia but have evolved distinct trafficking mechanisms that differ from those of primary cilia. Disruption

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of genes essential for ciliary structure and function may give

rise to syndromic ciliopathies characterized by photoreceptor degeneration together with abnormalities in ciliated cells across multiple organs and tissues. Representative syndromic ciliopathies include Joubert Syndrome (Parisi, 2019), Bardet-Biedl Syndrome (Chandra et al., 2022), and Senior-Løken Syndrome (Ronquillo et al., 2012). In contrast, selected variants in connecting cilium-associated genes selectively compromise photoreceptor cells and induce nonsyndromic retinal dystrophies. The photoreceptor cilium serves as the central organelle for light sensing and phototransduction, and is organized into three principal domains: basal body, connecting cilium (CC), and outer segment. An axonemal core extends from the basal body through the CC into the outer segment, providing the structural framework for the photoreceptor cilium. The axoneme consists primarily of microtubules and associated regulatory proteins. In primary cilia, axonemal microtubules assemble into doublets (MTDs) arranged in a canonical “9+0” configuration. Within the photoreceptor CC, the axonemal structure is further reinforced by an inner scaffold that stabilizes MTD cohesion (Mercey et al., 2022), together with Y-link structures that bridge MTDs to the ciliary membrane (Gilula & Satir, 1972).

Microtubules assemble from $\alpha\beta$ -tubulin heterodimers, and axonemal tubulins undergo multiple conserved post-translational modifications, including acetylation, glycylation, glutamylation, and detyrosination (Wloga et al., 2017). These modifications collectively constitute the proposed “tubulin code”, which modulates diverse microtubule-associated activities (Janke & Magiera, 2020). Acetylation of Lys40 of α -tubulin is enriched in long-lived microtubules (Wloga et al., 2017), including those within basal bodies and axonemes, and confers enhanced resistance to mechanical challenges (Xu et al., 2017). In parallel, glutamylation and polyglutamylation, together with glycylation and polyglycylation, generate side chains on the C-terminal tails of both α - and β -tubulins. While glycylation has been demonstrated to control axonemal dynein-2 activity and flagellar motility (Gadadhar et al., 2021), tubulin glutamylation participates in control of ciliary signal transduction and intraflagellar transport (IFT)-mediated protein trafficking (O'Hagan et al., 2011). Conversely, deglutamylation of tubulins alters kinesin-II-mediated anterograde IFT and Hedgehog signaling within cilia (Hong et al., 2018).

Intracellular tubulin glutamylation is governed by the combined action of glutamylases from the tubulin-tyrosine ligase-like (TTLL) family and deglutamylases from the cytosolic carboxypeptidase (CCP) family. In addition to catalyzing glutamylation, selected TTLL enzymes also mediate tubulin glycylation (Janke & Magiera, 2020). TTLL1, TTLL4, and TTLL7 initiate glutamylation (Janke et al., 2005; Mukai et al., 2009; Wloga et al., 2008), whereas TTLL3 and TTLL8 initiate glycylation (Rogowski et al., 2009; Wloga et al., 2009). Elongation of polyglutamate side chains is driven by TTLL1, TTLL6, and TTLL11 (Suryavanshi et al., 2010; van Dijk et al., 2007; Wloga et al., 2008), while TTLL10 mediates polyglycine chain extension. Maintenance of an appropriate balance between polyglycylation and polyglutamylation is essential for normal ciliary function. Mice lacking *Ttll3*, and therefore deficient in glycylation, display shortened connecting cilia and retinal degeneration. Levels of tubulin polyglycylation and polyglutamylation are further controlled by deglutamylation, as no deglycylating enzymes have been identified (Audebert et al., 1993). CCP1–6 function as tubulin deglutamylases (Janke & Magiera, 2020; Tort et al., 2014). Proper regulation of tubulin polyglutamylation is required for

the maintenance of ciliated cells and neurons (LaVail et al., 1982). A mouse strain harboring an enzymatically inactive form of *Agtbbp1*, which encodes CCP1, displays excessive polyglutamylation accompanied by photoreceptor degeneration and early-onset neurodegeneration (LaVail et al., 1982; Magiera et al., 2018). Pathogenic variants in AGLB carboxypeptidase 5 (*AGBL5*, also named *CCP5*) have been linked to RP (Astuti et al., 2016; Branham et al., 2016; Kastner et al., 2015; Paredes et al., 2024). Ablation of *Agbl5* in mice causes male infertility due to defective sperm flagella (Giordano et al., 2019; Wu et al., 2017). More recently, mice lacking AGLB5 were shown to develop cone-rod dystrophy through excessive tubulin glutamylation (Aljammal et al., 2024). Nevertheless, the mechanistic basis of retinal degeneration associated with AGLB5 dysfunction is yet to be elucidated.

In the present study, a global *Agbl5* knockout mouse model was generated. AGLB5 deficiency resulted in a substantial increase in tubulin glutamylation within the retina. Mutant mice exhibited impaired rod and cone responses followed by progressive retinal degeneration. Tubulin hyperglutamylation disrupted axonemal architecture by destabilizing Y-link structures within the connecting cilium, leading to reduced recruitment of IFT components and defective trafficking of cone opsins and rod rhodopsin. These findings establish that precise regulation of tubulin glutamylation by AGLB5 is critical for the preservation of axonemal structural integrity and efficient intraflagellar transport in photoreceptors.

MATERIALS AND METHODS

Animals

All mice were housed in the animal facility of Sichuan Provincial People's Hospital with *ad libitum* access to food and water. Animal care and experimental procedures followed the guidelines of the Association for Research in Vision and Ophthalmology for animal use in research. Ethical approval was granted by the Ethics Committee of the Sichuan Provincial People's Hospital (Approval No.: 2019-123). Mice of both sexes were randomly assigned to experimental groups.

Generation of *Agbl5* knockout mice

Agbl5 knockout mice on a C57BL/6J background were generated using CRISPR/Cas9-mediated genome editing. The murine *Agbl5* coding region comprises 16 exons. Two guide RNAs (gRNAs) were designed to target exons 2 and 12, respectively, resulting in deletion of exons 3–11 and partial loss of exons 2 and 12. Genomic DNA spanning the deletion junction was amplified by polymerase chain reaction (PCR) and validated by Sanger sequencing. Colony genotyping was performed by PCR using primers AGLB5-F (5'-TTGTTT CATTTTCTGCCCTTCCTC-3') and AGLB5-R1 (5'-AGTACT GCTCTGAAGTTATGCCG-3') to detect the mutant allele, and AGLB5-F and AGLB5-R2 (5'-CACTGAAGTAGAACCATGA CCTGA-3') to detect the wild-type (WT) allele.

Electroretinography (ERG)

Mice were anesthetized by intraperitoneal injection of ketamine (10 mg/kg) combined with xylazine (1 mg/kg) and dark-adapted overnight prior to recordings. Scotopic responses were elicited using single-flash stimuli ranging from 0.003–50 cd·s/m², with interstimulus intervals of 20 s to 2 min. For photopic recordings, mice were light-adapted under a steady background light of 10 cd·s/m², followed by single-flash stimuli ranging from 0.3 to 50 cd·s/m². All recording

procedures were performed on an ERG machine with a heating platform (IRC Medical Equipment, China) under a dim red illumination.

Immunoblotting

Mice were sacrificed, and retinas were rapidly isolated. Retinal tissue was lysed in 1×phosphate-buffered saline (PBS) by brief sonication. Proteins were resolved by 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were incubated with various primary antibodies, including anti-rhodopsin (1:5 000, custom-made (Avasthi et al., 2009)), anti-PDE6B (1:5 000, Proteintech, China), anti-GC2 (1:5 000, a gift from Dr. Wolfgang Baehr at the University of Utah), anti-GRK1 (1:3 000, Proteintech, China), anti-α-tubulin (1:30 000, Proteintech, China), anti-β-tubulin (1:30 000, Proteintech, China), anti-β-actin (1:10 000, Proteintech, China), anti-Ac-tubulin (1:5 000, Abcam, UK), and anti-polyglutamylation modification GT335 (1:5 000, Adipogen, USA). Horseradish peroxidase (HRP)-conjugated goat-anti-rabbit or goat-anti-mouse secondary antibodies (1:10 000, Proteintech, China) were applied for detection. Immunoreactive signals were visualized using an enhanced chemiluminescence (ECL) kit (4A Biotech, China).

Immunohistochemistry

Two types of retinal cryosections were used: sections derived from fixed eyeballs and sections prepared from fresh, unfixed eyeballs. Retinal sections from fixed tissue were generated as previously described (Li et al., 2020). For immunostaining, fixed sections were permeabilized and blocked using 1×PBS containing 5% normal donkey serum (NDS) (Jackson ImmunoResearch, USA) and 0.2% Triton X-100 (Sigma, USA) at room temperature for 30 min, followed by incubation with primary antibodies at 4°C overnight. Primary antibodies included anti-rhodopsin (1:900, custom-made) (Avasthi et al., 2009), anti-PDE6B (1:500, Proteintech, China), anti-GRK1 (1:500, Proteintech, China), anti-GC2 (1:500, a gift from Dr. Wolfgang Baehr at the University of Utah), anti-S-opsin (1:500, Millipore, USA), anti-ML-opsin (1:500, Millipore, USA), anti-Ac-tubulin (1:500, Abcam, UK), and anti-GT335 (1:500, Adipogen, USA). Alexa Fluor 488-conjugated goat-anti-rabbit and anti-mouse secondary antibodies (1:300, ThermoFisher, USA) were then applied for incubation at room temperature for 1 h. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1 000, Cell Signaling Technology, USA). For detection of axoneme-associated proteins, retinal sections were prepared from unfixed eyecups embedded in optimal cutting temperature (OCT) medium (Sakura, USA) as described previously (Li et al., 2020). Prior to permeabilization and blocking, the sections were fixed in 4% paraformaldehyde for 2 min. Subsequent staining procedures followed the same protocol used for fixed retinal sections. Fluorescence images were acquired using a Zeiss LSM900 confocal microscope (Zeiss, Germany).

Mouse retinal flat-mounts were prepared as described previously (Yang et al., 2023). Flat-mounted retinas were permeabilized and blocked in 1×PBS containing 5% NDS (Jackson ImmunoResearch, USA) and 0.2% Triton X-100 (Sigma, USA) at room temperature for 1 h, followed by incubation with anti-S-opsin (1:500, Millipore, USA) at 4°C overnight. Alexa Fluor 488-conjugated goat-anti-rabbit secondary antibody (1:300, ThermoFisher, USA), Alexa Fluor 594-conjugated PNA (1:200, Jackson ImmunoResearch, USA), and DAPI (1:1 000, Cell Signaling Technology, USA)

were applied for 1 h at room temperature. Fluorescence images were acquired using an LSM900 confocal microscope (Zeiss, Germany).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

TUNEL assay was performed using an *in situ* cell death detection kit (Roche, USA) following the manufacturer's instructions. Briefly, frozen retinal sections were permeabilized on ice for 5 min and incubated with TUNEL reaction solution at 37°C for 1 h. Nuclei were counterstained with DAPI (1:2 000, Cell Signaling Technology, USA). Fluorescence images were acquired using an LSM900 confocal microscope (Zeiss, Germany).

Histology

Eyeballs were fixed in 4% paraformaldehyde prepared in 1×PBS at room temperature for 24 h, followed by removal of the cornea and lens. Eyecups were dehydrated through increasing concentrations of ethanol solutions (30% to 100%), infiltrated with paraffin, and embedded. Paraffin blocks were sectioned at 5 μm thickness, then de-paraffinized by heating at 60°C for 1 h and washed in xylene. Subsequently, the sections were stained with hematoxylin and eosin (H&E) and rows of nuclei in the outer nuclear layer were quantified at multiple positions across each section.

Electron microscopy

Mouse eyeballs were briefly prefixed in 2.5% glutaraldehyde (Sigma, USA) at room temperature (approximately 25°C) for 10 min. A small corneal incision was made, followed by continued fixation at room temperature for 1 h. The cornea and lens were removed, and eyecups underwent further fixation at 4°C overnight. Samples were postfixed in 1% osmium tetroxide at 4°C for 2 h and dehydrated through a graded acetone series (30% to 100%), with small eyecup slices infiltrated and embedded in Epon 812 (Sigma, USA). Ultrathin sections (70 nm) were prepared using a diamond knife, stained with 2% uranyl acetate (SPI Supplies, USA) for 15 min and 1% lead citrate (Electron Microscopy Sciences, USA) for 3 min, and examined using a JEM-1400-FLASH transmission electron microscope (JEOL, Japan).

RNA sequencing (RNA-seq)

Retinas were dissected from mouse eyeballs, and total RNA was extracted using TRIzol reagent (ThermoFisher, USA). RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (ThermoFisher, USA), and RNA integrity was assessed using an RNA Nano 6000 Assay Kit (Agilent, USA). The transcriptome sequencing library was constructed using an NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA), following the manufacturer's recommendations, with index codes incorporated for sample identification. Library quality was examined on an Agilent Bioanalyzer 2100 system. Cluster generation of indexed libraries was performed on the cBot Cluster Generation System using a TruSeq PE Cluster Kit v4-cBot-HS (Illumina, USA) according to the manufacturer's procedures. Paired-end sequencing was conducted on an Illumina platform. Raw reads in fastq format were processed using in-house Perl scripts to remove adapter sequences, reads containing poly-N, and low-quality reads. Clean reads were then mapped to the reference genome, and differential expression analysis between mutant and WT samples was carried out using edgeR. RNA-seq was performed by Oebiotech (China).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism v.10.1. Unpaired Student's *t*-test was used for statistical analysis. A difference with statistical significance was defined as $P < 0.05$. Specifically, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, and ****: $P < 0.0001$. $P > 0.05$ indicated no statistically significant difference between groups, which was denoted as ns.

RESULTS

Generation of *Agbl5* knockout mice

CRISPR/Cas9-mediated genome editing was employed for *Agbl5* knockout (KO) in mice, targeting exons 2 and 12 with two independent gRNAs (Figure 1A). This strategy fused the 5' segment of exon 2 to the 3' segment of exon 12, producing deletion of exons 3–11 together with partial loss of exons 2 and 12. Junction sequencing across exons 2–12 confirmed the expected genomic rearrangement (Figure 1A), which was independently verified by PCR-based genotyping (Figure 1B). In the absence of a suitable anti-AGBL5 antibody, real-time quantitative reverse transcription PCR (RT-qPCR) was used to assess transcript expression within the targeted region, demonstrating complete loss of *Agbl5* mRNA in retinas from homozygous KO mice (Figure 1C). These data validated the successful inactivation of *Agbl5*. KO mice were viable and exhibited grossly normal development; however, males displayed infertility, consistent with prior reports (Giordano et al., 2019; Wu et al., 2017).

Tubulin hyperglutamylation in retinas lacking AGBL5

AGBL5 functions as a deglutamylase that removes the γ -linked branching glutamate residue at tubulin C-terminal tails, thereby preventing initiation of polyglutamylation, and is the

only enzyme known to selectively cleave this modification (Kimura et al., 2010; Rogowski et al., 2010). Through this activity, AGBL5 enables complete reversal of tubulin glutamylation. Loss of AGBL5 has been shown to induce tubulin hyperglutamylation in sperm and to cause male infertility (Giordano et al., 2019; Wu et al., 2017). To determine whether AGBL5 similarly affects retinal tubulin glutamylation, glutamylated proteins were examined using GT335, an antibody that specifically recognizes post-translationally added branch-point glutamate residues (Wolff et al., 1992). Immunoblot analysis revealed marked accumulation of glutamylated tubulin species at approximately 52 kDa in postnatal day 30 (P30) mutant retinas (Figure 2A, B). A higher-molecular-weight GT335-reactive band near 250 kDa, likely corresponding to glutamylated RPGR (Sun et al., 2016), remained unchanged. In parallel, α -tubulin abundance increased by approximately two-fold, whereas β -tubulin levels showed a modest reduction. Immunofluorescence analysis demonstrated low basal glutamylation in WT photoreceptors at P30, with enrichment at the CC, including contribution from glutamylated RPGR (Figure 2C). In contrast, *Agbl5*-deficient retinas exhibited pronounced accumulation of glutamylated tubulins throughout the microtubule network of photoreceptor inner segments. Collectively, these findings suggest that AGBL5 deficiency drives excessive tubulin glutamylation in photoreceptors.

Knockout of AGBL5 impairs retinal function and drives progressive degeneration

Retinal function following *Agbl5* ablation in mice was evaluated by ERG. Scotopic recordings demonstrated a marked reduction in a-wave amplitudes in P30 KO mice, indicating compromised rod activity (Figure 3A). Consistently,

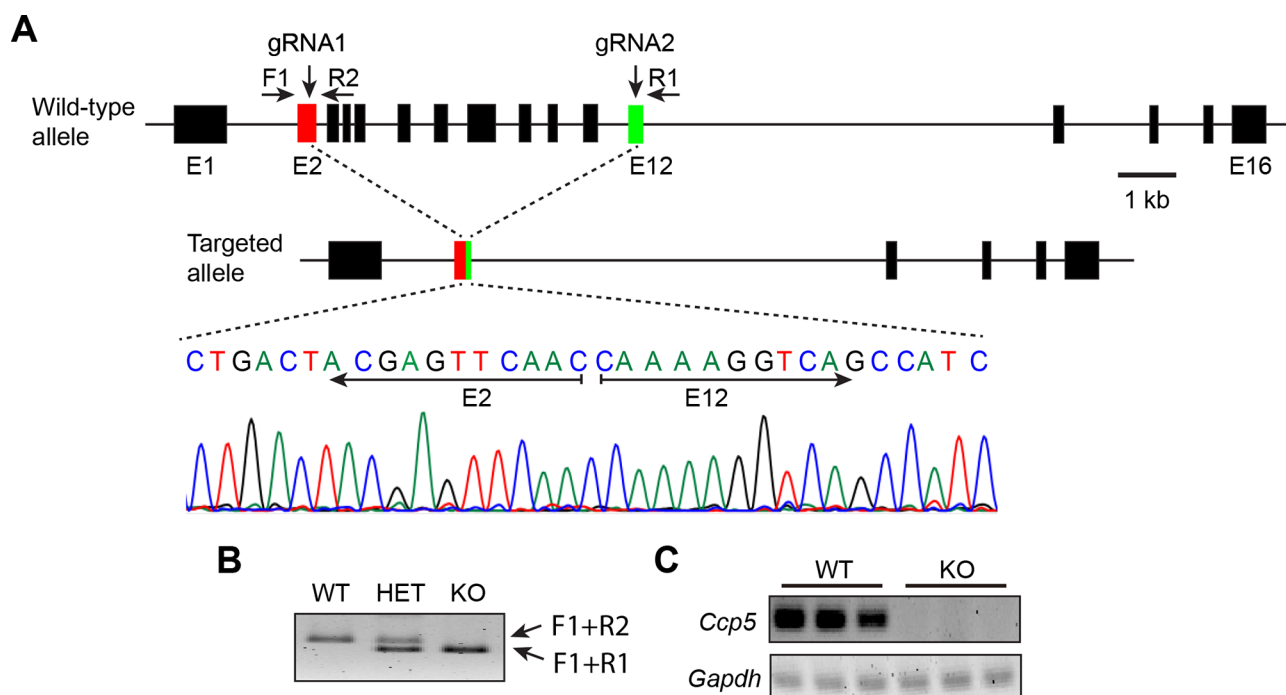


Figure 1 Generation and validation of *Agbl5* knockout mice

A: Schematic of CRISPR/Cas9-mediated disruption of *Agbl5*. gRNAs 1 and 2 were designed to target exon 2 (E2) and exon 12 (E12), respectively, resulting in deletion of E3–E11 together with partial deletion of E2 and E12. Junction sequencing confirmed the expected genomic rearrangement. Primers F1, R1, and R2 were designed for PCR-based genotyping. B: Representative PCR genotyping of wild-type (WT), heterozygous (HET), and homozygous knockout (KO) mice. Primers F1 and R2 were used to amplify the WT allele, whereas primers F1 and R1 were used to amplify the KO allele. C: RT-PCR analysis demonstrated absence of *Agbl5* mRNA in KO retinas, whereas robust expression was detected in WT samples. *Gapdh* served as an internal control.

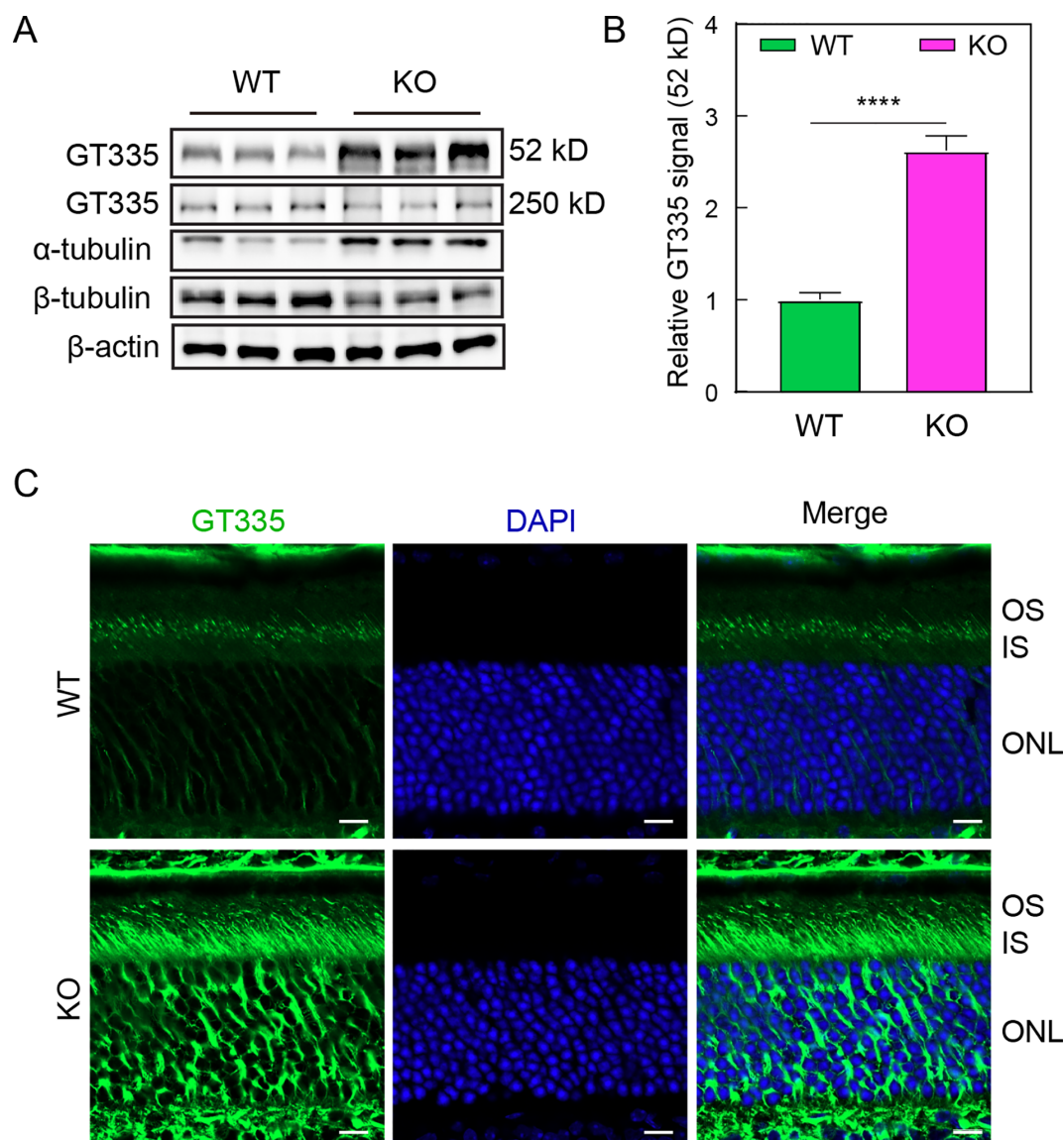


Figure 2 Tubulin hyperglutamylation in *Agbl5*^{-/-} retinas

A: Immunoblot analysis of postnatal day 30 (P30) retinal lysates probed with the polyglutamylation-specific monoclonal antibody GT335, which recognizes the first branch-point glutamate residues on tubulins and other substrates. B: Quantification of GT335-reactive signals shown in A. ****: $P < 0.0001$, unpaired t -test. C: Immunostaining of retinal sections with GT335 (green); nuclei were counterstained with DAPI (blue). Scale bar: 10 μ m. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer.

photopic recordings revealed decreased b-wave amplitudes, reflecting early cone dysfunction at the same developmental stage (Figure 3B). With increasing age, both rod- and cone-mediated responses deteriorated further (Figure 3C, D), demonstrating progressive retinal dysfunction. TUNEL analysis identified apoptotic cells within the outer nuclear layer (ONL) of one-month-old *Agbl5*^{-/-} retinas (Figure 3E, F), providing direct evidence of ongoing photoreceptor degeneration. H&E staining showed largely preserved retinal architecture at one month of age (Figure 3G); however, by 6 months, the ONL was significantly thinned across the entire retina relative to WT controls (Figure 3H). At 17 months, photoreceptors were completely degenerated (Supplementary Figure S1; note that KO mice in this experiment were generated previously using a different approach (Giordano et al., 2019)). Together, these data demonstrate that AGBL5 deficiency results in cone-rod dystrophy accompanied by progressive retinal degeneration.

Transcriptomic profiling reveals disruption of ciliary and

microtubule-associated gene programs in retinas lacking AGBL5

To determine the impact of *Agbl5* ablation on retinal function, retinal transcriptomes from KO and control mice were analyzed by RNA-seq, with controls comprising WT and heterozygous retinas, neither of which exhibited retinal dysfunction. Differential expression analysis identified more than 1 900 genes with significant expression changes in KO retinas (Figure 4A, B). Gene Ontology (GO) enrichment analysis of cellular components revealed predominant perturbation of non-motile cilia- and microtubule-associated categories (Figure 4C), consistent with extensive tubulin hyperglutamylation and suggesting altered composition of photoreceptor axonemes. This pattern likely reflects a complex feedback response to structural disruption of the axonemes. Accordingly, tubulin hyperglutamylation induced by *Agbl5* loss profoundly affected ciliary and microtubule architecture within CCs. GO enrichment analysis of biological processes further indicated significant alteration of phototransduction-related pathways in *Agbl5*^{-/-} retinas

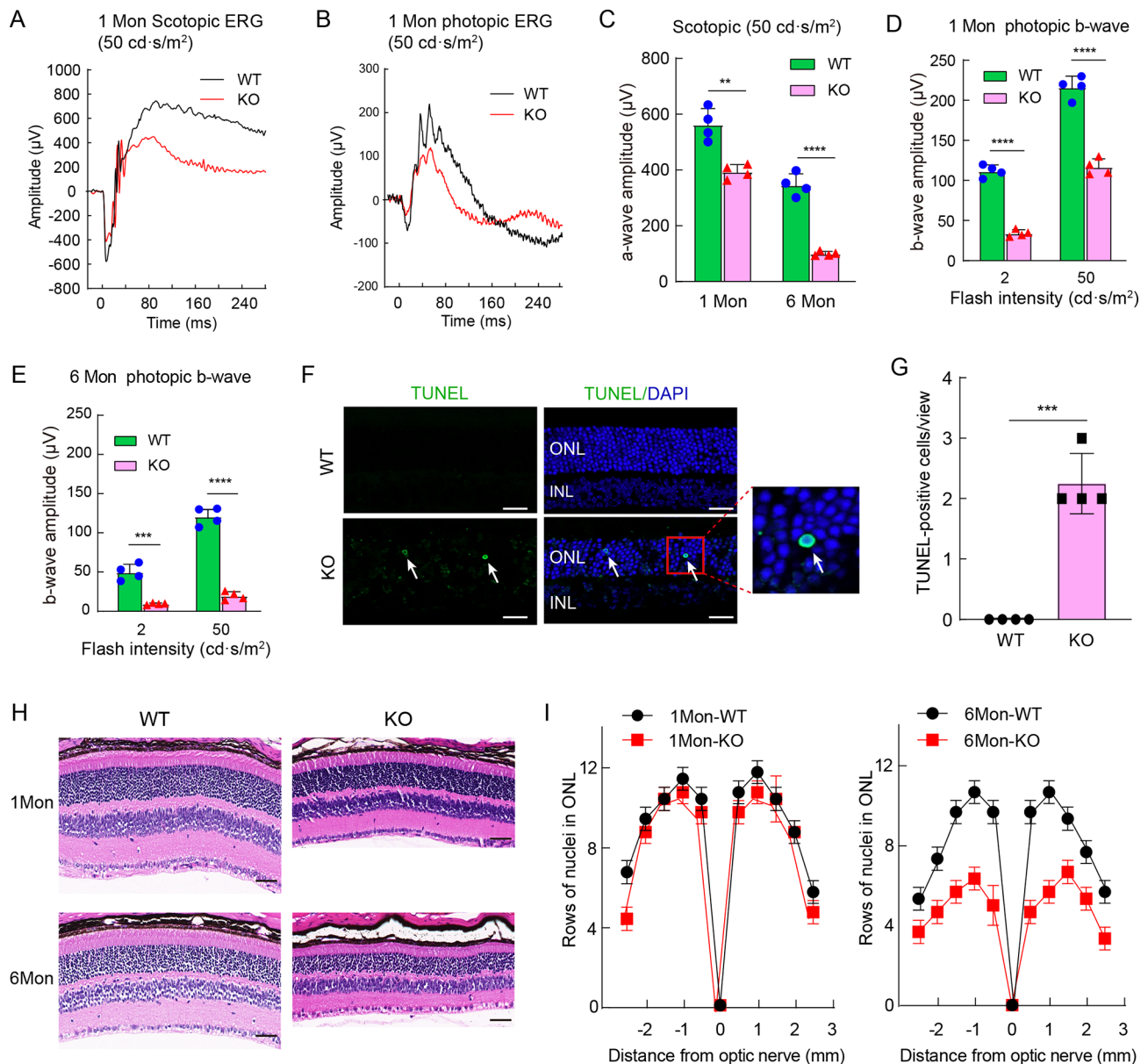


Figure 3 Progressive retinal dysfunction and degeneration in *Agbl5*^{-/-} mice

A, B: Representative scotopic (A) and photopic (B) single-flash ERG traces recorded at 50 cd·s/m² from WT (*n*=3) and KO (*n*=3) mice at one month of age. C: Quantification of scotopic a-wave amplitudes at 50 cd·s/m² at one and six months of age. *n*=3 for each genotype per age. D: Quantification of photopic b-wave amplitudes at 50 cd·s/m² in WT and KO mice at one and six months of age. *n*=3 for each genotype per age. E: TUNEL assay for WT and KO retinal sections from mice at the age of one month. Arrows indicate TUNEL-positive nuclei (green); all nuclei were counterstained with DAPI (blue). Scale bar: 25 μ m. ONL, outer nuclear layer; INL, inner nuclear layer. F: Quantification of TUNEL-positive cells (*n*=4). G: Retina morphology assessed by hematoxylin and eosin (H&E) staining. Scale bar: 50 μ m. H: Spider plots showing total number of rows of nuclei measured at 10 locations across the ONL in H&E-stained retinal sections through the optic nerve from WT and KO mice at one (left panel) and six months (right panel). Data in (C), (D), and (F) are expressed as mean $\pm$ standard deviation (SD); ***: *P*<0.001; **: *P*<0.01; unpaired *t*-test.

(Figure 4D). Although most components of the phototransduction cascade remained unchanged, several rod- and cone-expressed genes displayed differential regulation, including rhodopsin (*Rho*), cone arrestins (*Sag* and *Arr3*), and GC2 (*Gucy2f*) (Figure 4E). In contrast, transcripts encoding enzymes involved in the retinoid cycle responsible for regeneration of 11-*cis*-retinal showed no detectable changes. These transcriptomic alterations provide a mechanistic basis for the attenuated scotopic and photopic ERG responses observed in *Agbl5*-deficient mice.

AGBL5 deficiency disrupts axonemal architecture and reduces tubulin acetylation

Tubulin glutamylation is highly enriched within the CC

axoneme of photoreceptors, and transcriptomic analysis indicated perturbation of ciliary and microtubule-associated components in *Agbl5* mutant retinas. Ultrastructural examination by transmission electron microscopy (TEM) demonstrated that WT CC axonemes exhibited a normal “9+0” MTD arrangement with sharply defined boundaries (Figure 5A). This overall “9+0” pattern was preserved in mutant photoreceptors (Figure 5A). Y-link structures connecting MTDs to the CC membrane were present in both WT and mutant CCs. In contrast, the inner scaffold within the mutant CC appeared perturbed relative to controls, suggesting that excessive tubulin glutamylation impaired inner scaffold integrity but had little effect on Y-link stability. Immunostaining

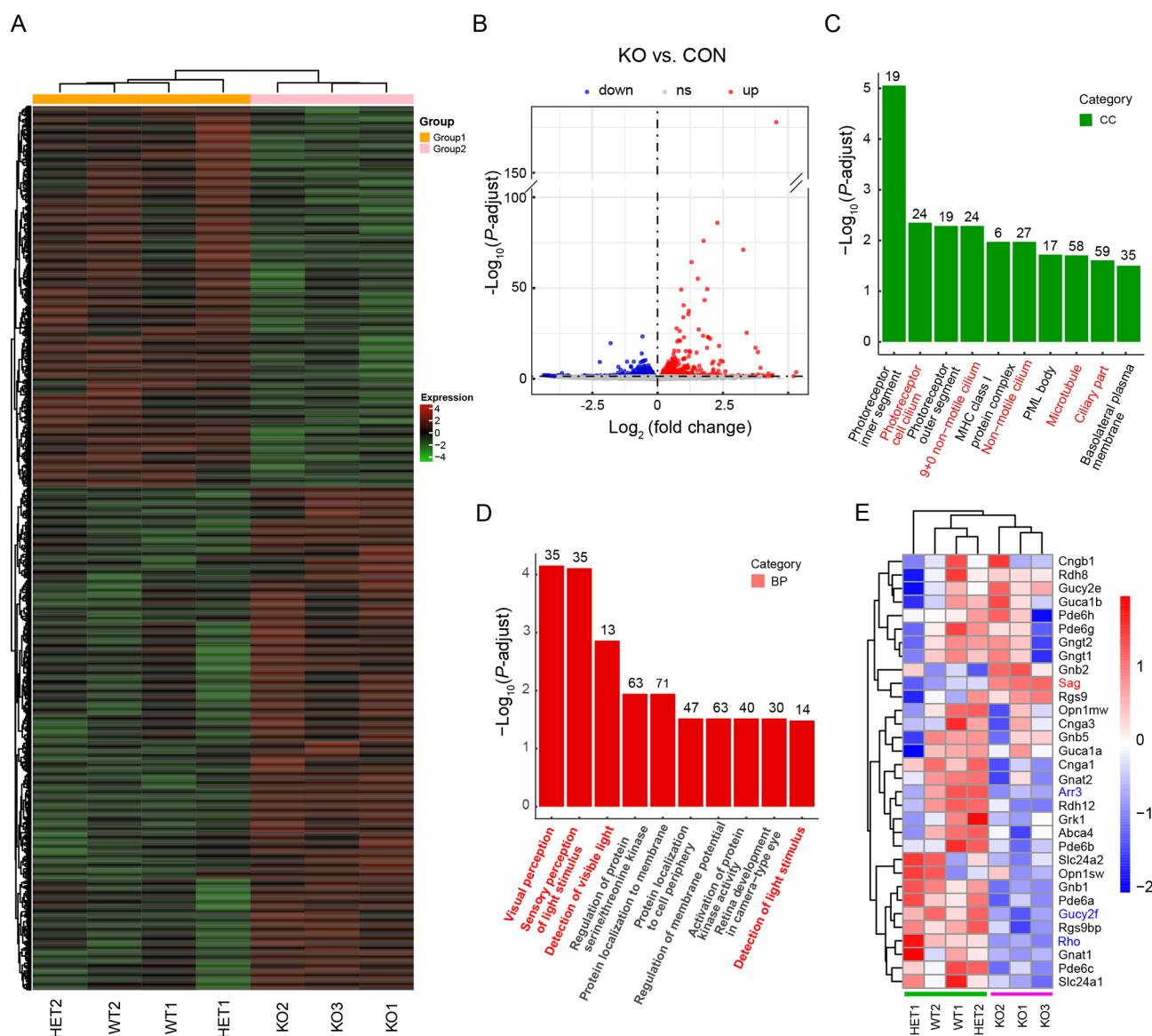


Figure 4 Transcriptomic analysis of P30 mouse retinas

A: Heatmap of differentially expressed genes (DEGs) identified by RNA-seq in *Agbl5*^{-/-} retinas relative to controls (WT and HET). B: Volcano plot showing differential gene expression in *Agbl5*^{-/-} and control retinas. C, D: Gene Ontology (GO) enrichment analysis of DEGs. CC: cellular component. BP: biological process. E: Heatmap of DEGs involved in rod and cone phototransduction cascades and photoreceptor-expressed retinoid cycle components, including *Abca4*, *Rdh8*, and *Rdh12*. Up-regulated genes (red); down-regulated genes (blue).

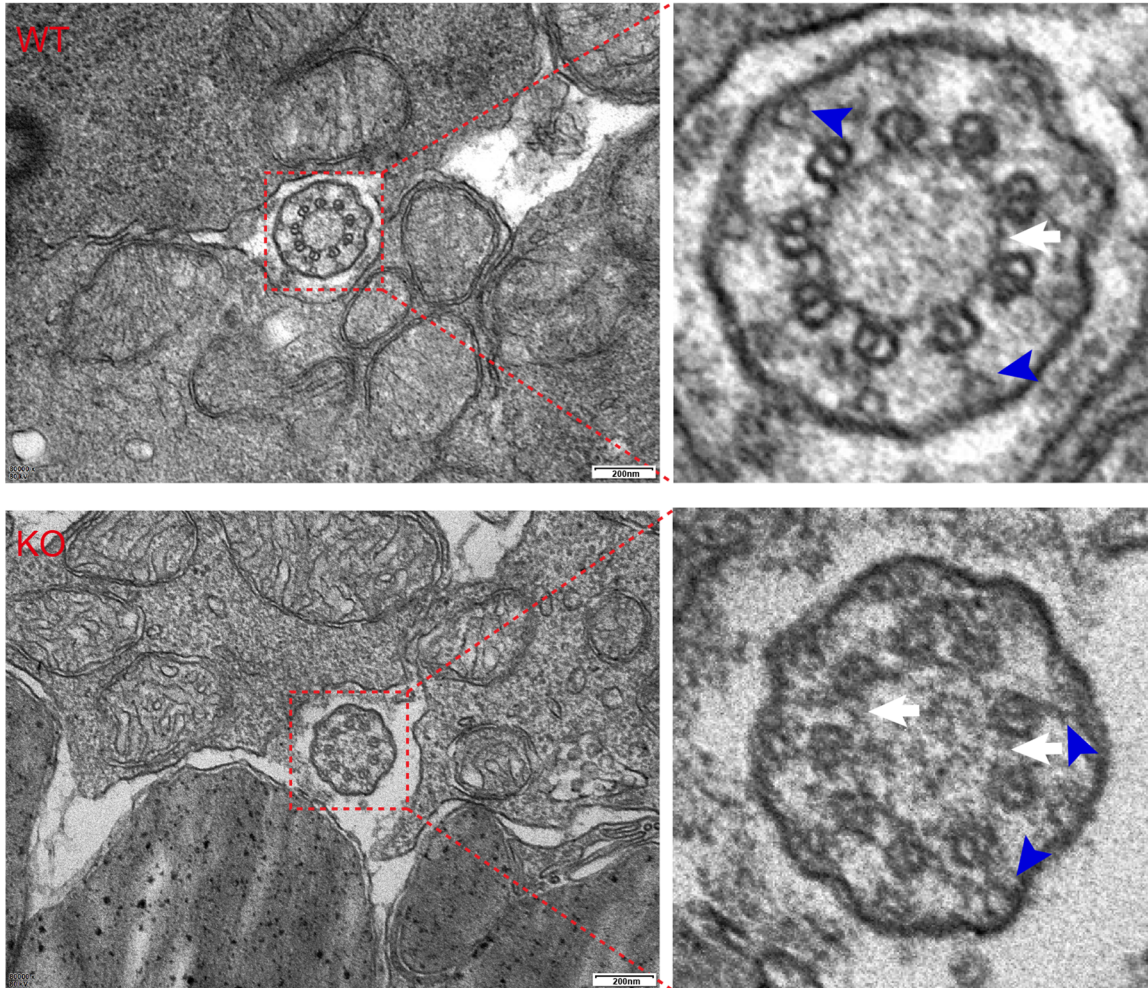
for CEP290, a protein proposed to participate in Y-link architecture (Craig et al., 2010; Potter et al., 2021), showed normal distribution within the CCs of KO mice (Supplementary Figure S2), consistent with intact Y-link structures observed by TEM. However, labeling of acetylated tubulin was markedly reduced in mutant photoreceptors (Figure 5B), as confirmed by immunoblotting (Figure 5C). These findings suggest that excessive tubulin glutamylation is accompanied by diminished microtubule acetylation and selective destabilization of the axonemal inner scaffold without overt disruption of Y-links.

AGBL5 deficiency impairs phototransduction protein expression and induces opsin mistrafficking in cone photoreceptors

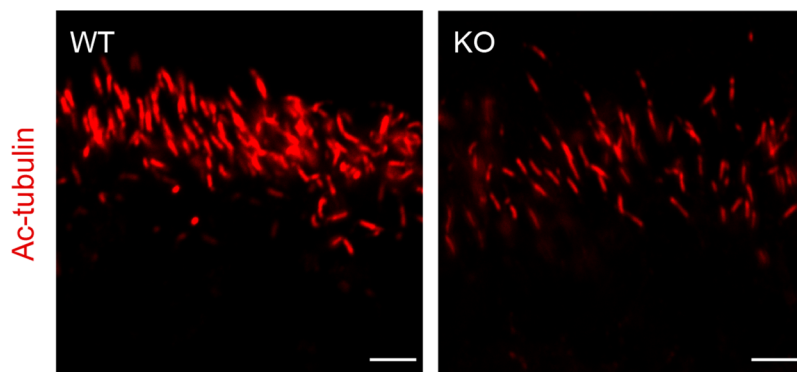
Axonemal microtubules within the CC provide the structural framework for directional trafficking between inner and outer segments. Structural perturbation of the CC in *Agbl5*-deficient photoreceptors was therefore predicted to compromise protein transport. Immunofluorescence analysis revealed that rod-enriched proteins, including RHO and PDE6B, were

transported to outer segments normally in *Agbl5* KO photoreceptors (Figure 6A). In contrast, GC2 and GRK1 exhibited partial mislocalization to inner segments, indicating impaired trafficking. All four proteins showed lower abundance in KO outer segments relative to WT outer segments. Immunoblotting confirmed reduced abundance of transmembrane proteins RHO and GC2, as well as GC1, whereas peripheral membrane proteins, such as PDE6B, remained largely unchanged (Figure 6B, C). Cone photoreceptors displayed pronounced mislocalization of ML-opsin and S-opsin to inner segments and synaptic terminals (Figure 6D, E). Retinal flat-mount staining with anti-S-opsin further revealed distorted cone outer segment morphology and disorganized orientation (Figure 6F, G). These observations demonstrate that axonemal hyperglutamylation disrupts efficient delivery of proteins to cone outer segments. Although RHO mistrafficking was not evident, its reduced abundance may stem from transcriptional down-regulation in combination with the degradation of mislocalized proteins.

A



B



C

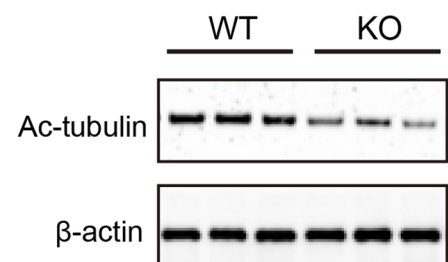


Figure 5 Structural abnormalities of photoreceptor axonemes in *Agbl5*^{-/-} retinas

A: Representative transmission electron microscopy images of transverse sections through connecting cilia of WT (P13) and *Agbl5*^{-/-} (P30) mice. Insets in left panels are enlarged and shown on the right. Blue arrowheads indicate Y-link structures bridging axonemal microtubule doublets and peripheral membrane. White arrows indicate axonemal inner scaffold. Scale bar: 200 nm. B: Immunostaining of frozen retinal sections using anti-acetylated tubulin (red). Scale bar: 2 μm. C: Immunoblotting analysis of proteins prepared from P30 retinas using anti-acetylated tubulin (red). β-actin was used as a loading control.

TEM showed substantial disorganization of outer segment disc membranes (Figure 6H, I), consistent with defective delivery of phototransduction proteins critical for outer segment morphogenesis. Occasional whorled membrane structures were detected in transverse sections of mutant outer segments (Figure 6J, K), consistent with recent findings

(Aljammal et al., 2024). Collectively, these data indicate that proper glutamylation of axonemal microtubules is critical for the normal function of IFT in cone photoreceptors.

Reduced recruitment of IFT88 and KIF3A to the basal body in photoreceptors lacking AGLB5

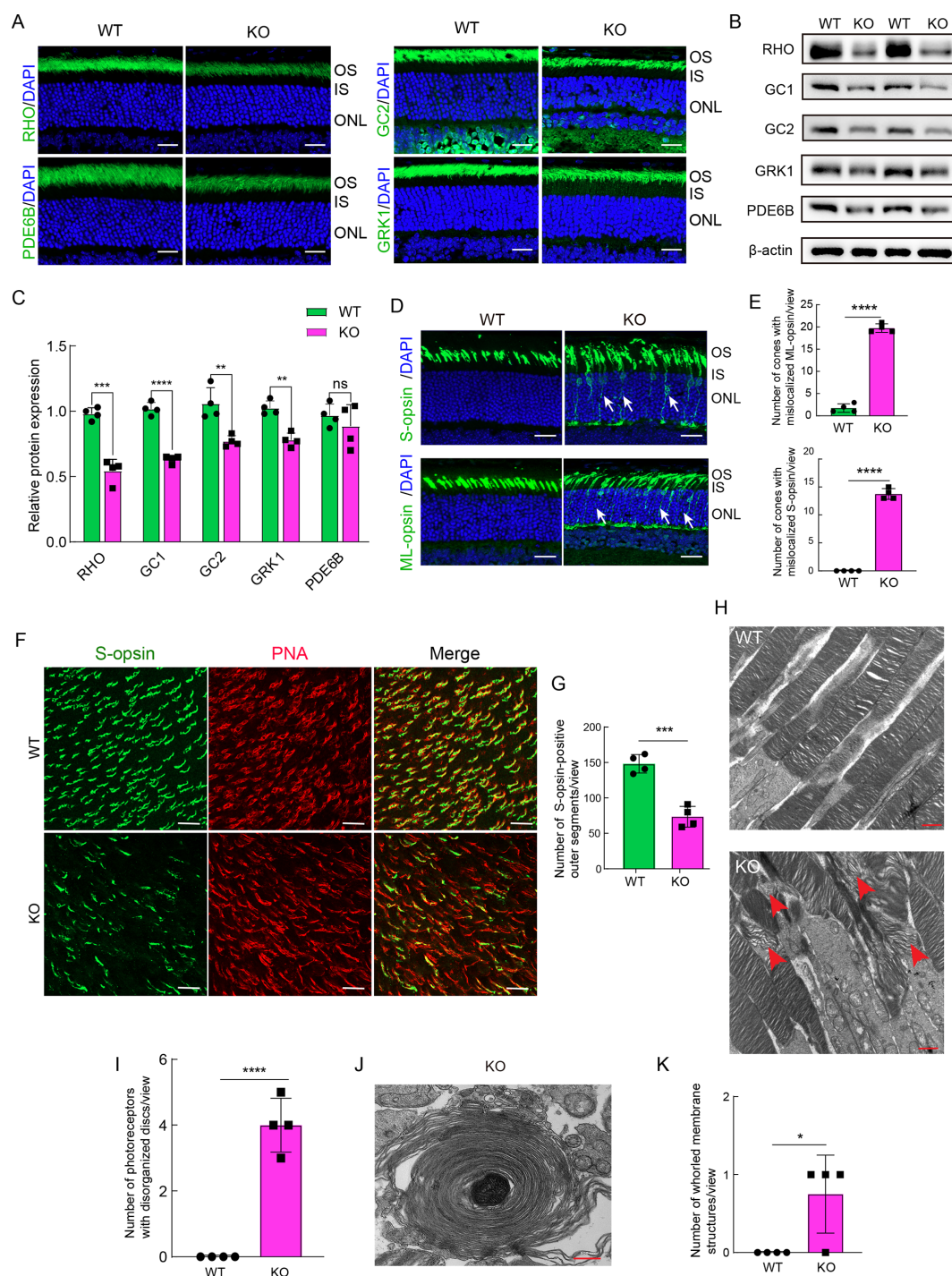


Figure 6 Attenuated phototransduction protein expression, cone opsin mistrafficking, and outer segment membrane disorganization in *Agbl5*^{-/-} mice

A: Immunostaining of P30 retinal sections showing localization of phototransduction proteins (green), including rhodopsin (RHO), PDE6B, GC2, and GRK1. Nuclei were counterstained with DAPI (blue). Scale bar: 20 μ m. B: Immunoblotting analysis of RHO, GC1, GC2, PDE6B, and GRK1 expression in P30 retinas. β -actin served as a loading control. C: Quantification of protein abundance in WT and KO retinas ($n=4$). D: Immunostaining of P30 retinal sections showing localization of cone opsins (green), including S-opsin and ML-opsin. Nuclei were counterstained with DAPI (blue). Arrows indicate mislocalized opsins in cone cells. Scale bar: 25 μ m. E: Quantification of cone photoreceptor cells with mislocalized S-opsin and ML-opsin in WT and KO retinas ($n=4$). F: Immunostaining of P30 whole-mount retinas using PNA (red) and anti-S-opsin (green). Scale bar: 20 μ m. G: Quantification of S-opsin-positive outer segments in WT and KO retinas ($n=4$). H: Transmission electron microscopy (TEM) images showing longitudinal sections of photoreceptor outer segments in P30 mice. Arrowheads indicate abnormal membrane disks in KO outer segments. Scale bar: 1 μ m. I: Quantification of photoreceptors with disorganized discs in WT and KO mice ($n=4$). J: TEM image of a transverse section through the outer segment of a KO photoreceptor from a P30 mouse, showing whorled membrane structures. Scale bar: 500 nm. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer, IPL, inner plexiform layer. K: Quantification of whorled membrane structures in WT and KO outer segments ($n=4$). Data in (C), (E), (G), (I), and (K) are expressed as mean $\pm$ SD; ****: $P < 0.0001$; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; ns: not significant; unpaired t -test.

Anterograde IFT is mediated by the heterotrimeric kinesin-II motor complex. Mistrafficking of cone opsins to inner segments and synaptic pedicles in AGBL5-deficient retinas is consistent with impaired kinesin-II-dependent transport, as cone-specific deletion of KIF3A, a kinesin-II subunit, produces a similar cone phenotype (Avasthi et al., 2009). In addition, AGBL5-deficient mice displayed shortened rod outer segments with preserved rhodopsin trafficking and CC formation, resembling early-stage phenotypes reported following rod-specific depletion of KIF3A and IFT88 during outer segment development (Jiang et al., 2015). Based on these parallels, localization of KIF3A and IFT88, key components of the IFT-B complex, was examined in *Agbl5*^{-/-} photoreceptors. In WT retinas, KIF3A immunoreactivity appeared as punctate bead-like structures distributed along the CC (Figure 7A), consistent with association of KIF3A with cargo undergoing axonemal transport. In contrast, these bead-like signals were largely absent in the mutant CC, indicating reduced cargo-associated KIF3A within the CC (Figure 7A, E). IFT88 was localized to both the proximal and distal regions of the WT CC (Figure 7B). However, in the mutant photoreceptors, IFT88 puncta were predominantly confined to the proximal end and markedly diminished distally, consistent with reduced recruitment to the CC (Figure 7B, E). In parallel, DynC2H1, a dynein-2 subunit required for retrograde IFT, was less abundant in the outer segment (Figure 7C, E), suggesting that retrograde transport was also compromised, in agreement with the established role of dynein-2 in axonemal trafficking (Dahl & Baehr, 2021). In contrast, localization of KIF5B, a kinesin-I component not implicated in IFT, remained unchanged (Figure 7D, E).

Together, these results suggest that tubulin hyperglutamylation induced by AGBL5 loss disrupts IFT through reduced recruitment of IFT88 and IFT-associated motor proteins to the CC, thereby impairing bidirectional trafficking in photoreceptors.

DISCUSSION

This study established an *Agbl5* KO mouse model that developed progressive visual dysfunction accompanied by degeneration of both rod and cone photoreceptors. Loss of AGBL5 led to pronounced accumulation of polyglutamylated tubulins, disruption of photoreceptor axonemal architecture, and defective trafficking of multiple phototransduction proteins, with particularly severe effects on cone opsin localization. These results indicate that tight regulation of tubulin glutamylation is critical for preservation of ciliary integrity and photoreceptor function.

Several deglutamylases participate in the removal of polyglutamylated chains from tubulins (Rogowski et al., 2010). However, AGBL5 represents the only enzyme in this family capable of excising the branch-point glutamate residue (Berezniuk et al., 2013). In the absence of AGBL5, complete reversal of glutamylation becomes impossible. Accordingly, in *Agbl5*^{-/-} photoreceptors, installation of the initial branching glutamate residue on tubulin cannot be reversed and therefore persists until tubulin turnover occurs. Within photoreceptors, axonemal tubulins constitute a primary target of glutamylation. Axonemal tubulins exhibit prolonged stability and slow replacement, creating conditions that favor progressive accumulation of polyglutamylated chains when deglutamylation is impaired. Consistent with this model, marked hyperglutamylation was observed in the CC of *Agbl5*^{-/-}

photoreceptors. Although previous research has reported that GT335 immunoreactivity in WT CC largely reflects glutamylated RPGR rather than tubulin (Sun et al., 2016), immunoblotting in the present study demonstrated a substantial increase in tubulin glutamylation without a corresponding change in RPGR modification. Accordingly, the elevated GT335 signals revealed by immunostaining in inner segments and CC of *Agbl5*^{-/-} photoreceptors can primarily be attributed to hyperglutamylated tubulins, identifying axonemal microtubules as the principal pathological target of AGBL5 loss.

Disruption of AGBL5 produced a selective defect in the inner scaffold of the CC axoneme. This scaffold consists of POC5, CENTRIN, and FAM161A (Mercey et al., 2022) and spans adjacent MTDs to provide structural support for the CC. Pathogenic variants in FAM161A are associated with RP28 (Bandah-Rozenfeld et al., 2010; Langmann et al., 2010), underscoring the functional importance of this architectural element. In contrast to the compromised inner scaffold, Y-link structures connecting microtubule doublets to the ciliary membrane remained intact in *Agbl5*^{-/-} photoreceptors. These observations suggest that excessive tubulin glutamylation, likely through alteration of the electrostatic properties of the microtubule surface, selectively destabilizes the inner scaffold while sparing Y-link attachment to MTDs. Phototransduction proteins are synthesized in photoreceptor inner segments and transported to outer segments via IFT across the CC (Insinna & Besharse, 2008), a process that depends on axonemal microtubules as transport tracks. Impairment of this pathway results in mislocalization of rhodopsin and cone opsins to inner segments (Avasthi et al., 2009; Gupta et al., 2018). Structural abnormalities of the axoneme in *Agbl5*^{-/-} photoreceptors therefore provide a mechanistic basis for cone opsin mistrafficking, closely resembling phenotypes observed following cone-specific deletion of *Kif3a* (Avasthi et al., 2009). *Kif3a* encodes a kinesin-II subunit that drives ciliary anterograde trafficking of cargo along axonemal microtubules (Kozminski et al., 1995; Scholey, 2013). In *Agbl5*^{-/-} photoreceptors, hyperglutamylation of axonemal tubulins appears to prevent kinesin motor progression and is accompanied by reduced recruitment of IFT particles to the CC. Together, these defects impede delivery of cone opsins to outer segments, resulting in pronounced accumulation within inner segments. Rod photoreceptors exhibited milder transport defects. Only limited mislocalization of GC2 was detected in the mutant photoreceptors, and rhodopsin redistribution to the inner segments was not observed. Rapid degradation of mislocalized rhodopsin or diversion into extracellular vesicles may account for this difference (Hessa et al., 2011; Lewis et al., 2024; Spencer, 2023). Divergent behavior of rod and cone opsins has been reported in other models of impaired IFT, including cone-specific *Kif3a* ablation, which produces cone opsin mistargeting without overt rhodopsin mislocalization in rods (Avasthi et al., 2009). Accordingly, selective cone opsin mistrafficking in *Agbl5*^{-/-} retinas aligns with established consequences of disrupted IFT. Despite preserved rhodopsin localization, overall rhodopsin abundance declined in *Agbl5*^{-/-} photoreceptors and was accompanied by shortened outer segments, mirroring phenotypes reported following inducible retinal deletion of *Kif3a* (Jiang et al., 2015).

Tubulin glutamylation is dynamically maintained through coordinated activities of glutamylating and deglutamylating enzymes (He et al., 2020). In *Agbl5*^{-/-} photoreceptors, this

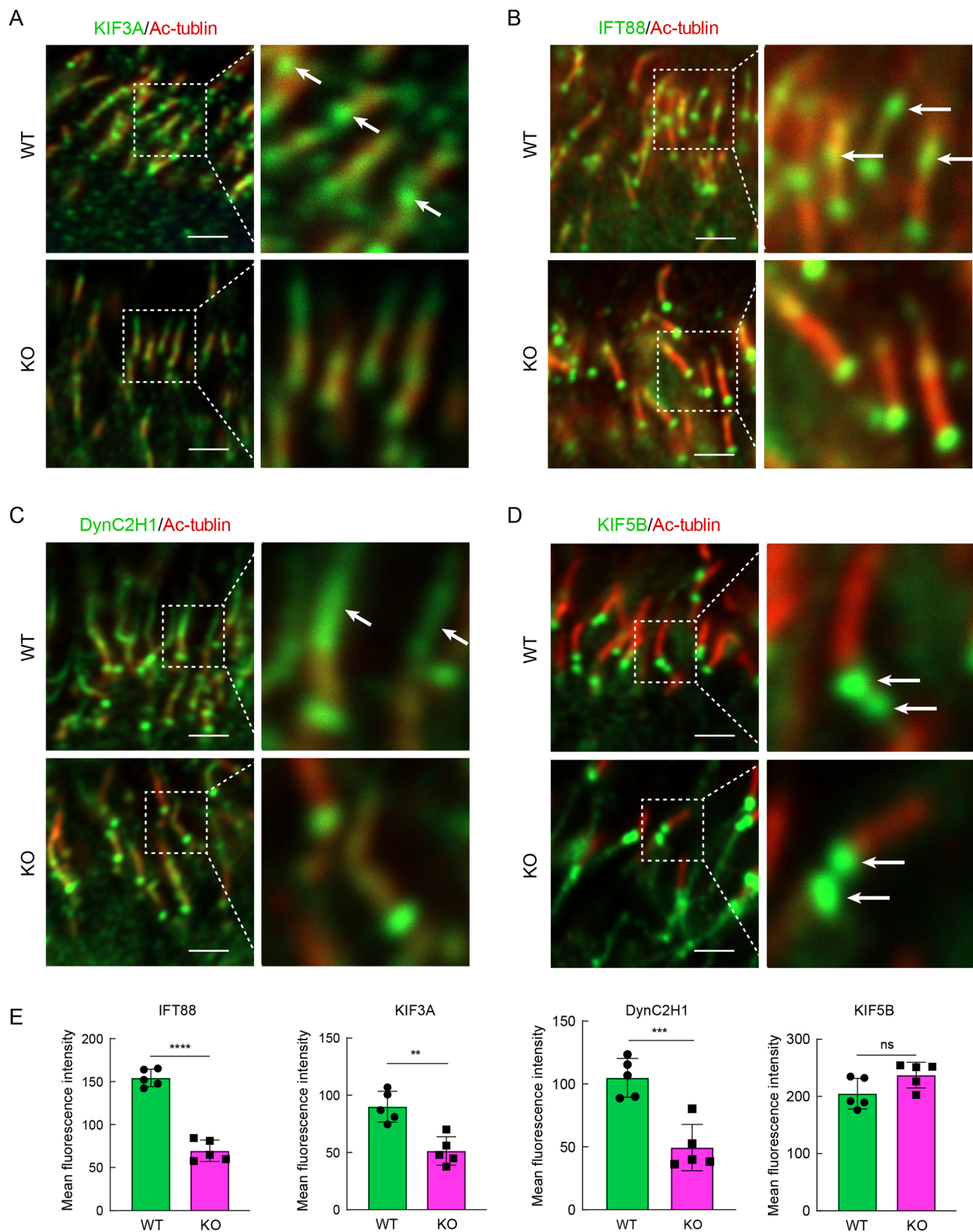


Figure 7 Abnormal distribution of kinesin-II, IFT, and dynein-2, but not kinesin-I, in the mutant mouse photoreceptor

A–D: Immunostaining of retinal sections prepared from unfixed P30 mouse eyecups using anti-KIF3A (A), anti-IFT88 (B), anti-DynC2H1 (C), or KIF5B (D) (green) with Ac-tubulin (red). Insets are enlarged and shown in the right panels. Arrows in (A, B) indicate KIF3A and IFT88 localization near distal regions of WT connecting cilium. Scale bar: 2 μ m. Arrows in (C) indicate DynC2H1 on the apical side of WT connecting cilium. Arrows in (D) indicate KIF5B in the basal body of both WT and KO photoreceptors. E: Quantification of fluorescence intensity of KIF3A, IFT88, DynC2H1, and KIF5B in connecting cilium of WT and KO mice ($n=4$). Data are expressed as mean $\pm$ SD; ****: $P<0.0001$; ***: $P<0.001$; **: $P<0.01$; ns: not significant; unpaired t -test.

equilibrium shifts toward excessive polyglutamylation, resulting in retinal degeneration. Therapeutic strategies could

therefore focus on restoring glutamylation homeostasis, either by attenuating glutamylase activity or by modulating expression of enzymes governing tubulin glutamylation dynamics. At present, no selective inhibitors targeting tubulin glutamylating enzymes have been identified, making pharmacological intervention unlikely at this stage. In contrast, gene-based approaches represent a more feasible alternative. Given that *AGBL5*-associated RP follows an autosomal recessive inheritance pattern, gene augmentation therapy constitutes a rational strategy for restoring *AGBL5* function in affected individuals.

DATA AVAILABILITY

Data are available upon reasonable request. The raw RNA-seq data and processed data generated in this study are available from the NCBI database (GEO, GSE305830), China National Center for Bioinformation Database of Genome Sequence Archive (GSA) (PRJCA049424), and Science Data Bank (<https://doi.org/10.57760/sciencedb.j00139.00305>).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

T.D.Z., H.L.W., T.W., and F.Y.Z. designed and performed the experiments and analyzed the data. J.X.G., Y.Q.L., Y.J.T., J.H.W., and M.M.M. performed the experiments and analyzed the data. J.J.W. and S.Q.D. analyzed the data. C.J. analyzed the data and edited the manuscript. H.B.Z. conceived the study, designed and supervised the study, performed the experiments, analyzed the data, and wrote the manuscript. H.L.W., H.B.Z., and T.D.Z. edited the paper. All authors read and approved the final version of the manuscript.

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