Photoreceptor Cell–Derived CAPN5 Regulates Retinal Pigment Epithelium Cell Proliferation Through Direct Regulation of SLIT2 Cleavage

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PURPOSE. To identify the causative gene and investigate the corresponding mechanisms for an autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV) family.

METHODS. Clinical examination and genetic analysis were performed in a Chinese ADNIV family. To dissect the molecular consequence, we used gene targeting to knock-in a patient’s specific mutation in the mouse genome. Immunostaining and immunoprecipitation were harnessed to analyze the colocalization and interaction of CAPN5 with SLIT2 in photoreceptors. The purified SLIT2-N, SLIT2-C fragments, and the conditioned medium from 661W cells with the overexpression of CAPN5 were treated on ARPE-19 cells. The viability of ARPE-19 cells was determined by MTT assays. The activation of protein kinase A (PKA) was analyzed by immunofluorescence and Western blotting in 661W and ARPE-19 cells as well as in frozen retina tissue from wildtype (WT) and knock-in mice.

RESULTS. We identified a novel CAPN5 mutation (p.R289W) in a Chinese family and generated the knock-in CAPN5R289W mouse. This mutation caused abnormal proliferative RPE in both humans and mice. CAPN5 directly cleaved WT SLIT2 in vitro, but not the mutant SLIT2 (p.R1113I). CAPN5 interacted with the SLIT2 in mouse retinal photoreceptors (661W cells) and increased cleavage and secretion of the SLIT2 fragments (SLIT2-N and SLIT2-C). Conditioned medium induced higher levels of secreted SLIT2 fragments, which promoted PKA activation and promoted proliferation of ARPE-19 cells.

CONCLUSIONS. The novel CAPN5 mutation (p.R289W) is responsible for the present ADNIV family. The mutant CAPN5 stimulated secretion and cleavage of SLIT2 fragments that may act as a bystander to regulate abnormal RPE cell proliferation for ADNIV.

Keywords: ADNIV, CAPN5, SLIT2, PKA, cleavage, retinal pigment epithelium, cell proliferation
manner. The molecular mechanisms underlying several pathologies, such as photoreceptor degeneration and eye inflammation, are not clear in ADNIV. It has been shown that secreted proteins regulated the development, differentiation, and proliferation of the RPE layer as well as the vitreous space in a paracrine manner. Whether factors secreted by photoreceptor cells can also influence the function of RPE cells or other tissues in the eye has not been well studied. Therefore, here we identified a novel mutation in CAPN5 in an ADNIV family and investigated the molecular consequence of CAPN5 mutation. We found that it is physically associated with the secreted neural repulsion molecule SLIT2 in retinal photoreceptors.

**Materials and Methods**

**Genetic Analysis**

The family was recruited and ocular and hearing examinations were performed in the Eye Hospital of Wenzhou Medical University. Whole exome sequencing was utilized, and candidate disease-causing mutation in CAPN5 was evaluated in the ADNIV family using Sanger sequencing.

**Generation of CAPN5 Knock-In Mice**

Knock-in mice were created on a B6 (C57BL/6j/N) background. Before we generated B6 knock-in (CAPN5R289W) mice, we confirmed the strain background is wildtype (WT) B6, which ruled out C57BL/6j/N with Crb1RD8/RD8 linked to retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA). CAPN5R289W mice were maintained in the Wenzhou Medical University Animal Care Services Facility. All experimental procedures were approved by the Institutional Committee of Wenzhou Medical University in accordance with the Declaration of Helsinki and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were conducted in compliance with the Animal Research: Reporting of In Vivo Experiments guidelines.

The gene-targeting vector was sequenced to confirm that it had only the desired point mutation. Gene targeting was performed in mouse embryonic stem cells, and the knock-in mice were identified by analyzing genomic DNA isolated from tail tips. The CAPN5 knock-in mice contained FRT-neo-FRT (intron 5) and p.R289W point mutation (exon 6); CAPN5 knock-in mice were mated with FLP mice to remove the antibiotics gene. The final CAPN5 knock-in mice without the antibiotics gene were labeled with CAPN5R289W.

**Electroretinography and Optical Coherence Tomography**

ERG was performed with a standard protocol. Differences between WT (n = 15) and CAPN5R289W (n = 12) mice were assessed for six ERG parameters in three age-matched groups by means of t-tests corrected for multiple comparisons (α = 0.05). In a subset of the mice (n = 7 for CAPN5R289W, n = 6 for WT), S- and M-opsin-mediated cone function was compared. Both stimuli were presented in a ganzfeld lined with aluminum foil. Retinal cross-sections of WT and CAPN5R289W mice were acquired with a 3.2-μm resolution spectral-domain optical coherence tomography (SD-OCT) system (Bioptigen, Inc., Durham, NC, USA). Corneas were lubricated with ophthalmic lubricant frequently during the imaging session (Systane Ultra; Alcon Ltd., Fort Worth, TX, USA). Using the fast fundus mode (200 raster scans of 200 longitudinal reflectivity profiles each), we first centered the location of the optic nerve head within a 1.6 × 1.6-mm field of view by rotating the cassette that holds the animal.

**Antibodies and Reagents**

Antibodies to the following antigens were used: SLIT2 (sc-16619, E-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CAPN5 (sc-271271, A-5; Santa Cruz Biotechnology), phosphor-PAKα/β/γ (Thr198) (sc-136461; Santa Cruz Biotechnology), mouse PAKα/β/γ (sc-98951, H-56; Santa Cruz Biotechnology), Ki67 (Ruying Biotechnology, Hangzhou, China), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Bioworld, Wuhan, China). Secondary antibodies coupled to horseradish peroxidase or fluorescein were rabbit anti-mouse IgG (Boshide, Wuhan, China), goat anti-mouse IgG (Boshide), goat anti-rabbit IgG (Boshide), Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 555-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

**Cell Culture**

Four different cell lines (HEK-293T cells, human neuroblastoma SHSY5Y cells or mouse photoreceptor-like 661W cells, human RPE ARPE-19 cells) were cultured in the present study. Transfection of SHSY5Y and 661W cells was performed using a reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After 72 hours of transfections of CAPN5 vectors in 661W cells, the fresh medium (400 μl DMEM with 0.1% FBS) was added in a 24-well plate, which is used as conditioned medium for the culture of human RPE ARPE-19 cells.

**Immunostaining**

Cells (5 × 10^5) were transfected with the plasmids on coverslips 60 hours post transfection, and cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at 25°C. Cells were incubated with 10% bovine serum albumin (BSA) diluted in PBS, then incubated with anti-Flag antibody and anti-SLIT2 antibody for 18 hours at 4°C. After three washes in PBS for 5 minutes each, slips were incubated with secondary antibodies, Alexa 555 goat anti-rabbit and Alexa 488 goat anti-mouse (1:700 dilution each, prepared in blocking solution). The coverslips were attached on glass sides with glycerol diluted in PBS (1:1). Images were captured in a digital format using a Zeiss microscope (Carl Zeiss, Chester, VA, USA). For retina analysis, mouse eyes were enucleated and embedded in optimal cutting temperature compound at –80°C. Sections (8 μm) were cut on a cryostat, placed on glass slides, and allowed to dry for 2 hours. Sections were fixed in cold acetone on ice for 10 minutes, followed by three washes with PBS, 5 minutes each. Sections were incubated in blocker (1% BSA, 0.2% skim milk, and 0.3% Triton X-100 in PBS) for 15 minutes at room temperature. Sections were then incubated with rabbit anti-CAPN5 (1:500; Santa Cruz Biotechnology) and anti-SLIT2 (1:300; Santa Cruz Biotechnology) overnight at 4°C in humid environment. After three 5-minute washes in PBS, sections were incubated with secondary antibodies.

**Western Blotting Assay and Immunoprecipitation**

Western blotting analysis and immunoprecipitation was performed as described. In brief, cell lysates were incubated overnight at 4°C with anti-CAPN5 antibody or anti-SLIT2 antibody or with nonimmune rabbit or mouse IgG controls. Immunoprecipitations were then captured with protein G-Sepharose beads for 2 hours at 4°C and eluted with 10 mM glutathione according to manufacturer’s instructions (GE
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Healthcare, Shanghai, China). The cells were transfected with pSin CAPN5-Flag or pSin CAPN5 R289W vector for 60 hours, and cell lysates were incubated with anti-Flag M2 antibody conjugated with magnetic beads (Sigma-Aldrich Corp., St. Louis, MO, USA) for 2 hours at 25°C. Immunoprecipitates were then separated with a magnetic shell and eluted according to the manufacturer’s instructions (Sigma-Aldrich Corp.).

**Semiquantitative RT-PCR**

RT-PCR was performed using primers for mouse Capn5: sense 5'-GATCCCCGGCGCTTCCGTGATG-3', anti-sense 5'-GAGGATTA CGTCCACACCCACTG-3', for mouse SLIT2: sense 5'-GGCAGACA CTGTCCCCATCG-3', anti-sense 5'-ATCTATCTTGTTGAT CCTCGTGA-3'; for loading control mouse Gapdh: sense 5'-AG CCTGGGCAATATTTCCATCG-3', anti-sense 5'-CGTTTACCTCC CATGACAAAC-3'. Experiments were performed in triplicate and the density of bands was measured by ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and normalized to Gapdh.

**Plasmid Construct and Protein Purification**

The Flag-tagged full-length human CAPN5 isoform, CAPN5 R289W mutant, His-tagged single-chain fragment (scFv) against CAPN5 was inserted into the pSIN-EGFP vector. Site-directed mutagenesis of the SLIT2 was performed using high-fidelity enzyme Pfu (TIANGEN Biotech Co., Ltd., Beijing, China). The pET28a vector was used as backbone for the expression of CAPN5, SLIT2, SLIT2-N, and SLIT2-C. The plasmids were transformed into *Escherichia coli* BL21 cells, and His-tagged proteins were purified using Ni²⁺ affinity columns.

**Determination of Cell Viability**

ARPE19 (5 × 10⁵) cells were suspended in DMEM/F-12 medium with 0.1% FBS or conditioned medium from 661W transfected with CAPN5 vectors to a concentration of 5 × 10⁴ cells/mL, and 100 μL was added to each well of a 96-well plate. After treatment with recombinant SLIT2, SLIT2-N, and SLIT2-C proteins for 48 hours, cells were incubated separately with 10 μL MTT (500 μg/mL) for 4 hours. The culture medium was then removed, and 100 μL dimethylsulfoxide was added to each well, followed by a 30-minute incubation period at 25°C. Optical density was measured spectrophotometrically at 540 nm.

**Statistics**

Statistical analyses were performed with statistical software (SPSS 13.0; IBM Corporation, Armonk, NY, USA). All data are presented as mean ± SEM unless otherwise specified. Student’s t-test was used for comparison in experiments with only two groups. In experiments with more than two groups, ANOVA was performed, followed by Tukey’s post hoc test for pairwise comparisons among three and more than three groups. For analysis of more than two groups of nonparametric data, the Kruskal-Wallis test was used.

**RESULTS**

**Identification of a Novel CAPN5 Mutation (p. R289W) in an ADNIV Family**

The family included nine affected individuals in a four-generation family (Fig. 1A) originating from Anhui Province, China. The proband was a 9-year-old female (indicated with black arrow in Fig. 1A). She has cataracts (Fig. 1B), retinitis pigmentosa (RP, Fig. 1C), and mild dysaudia (Fig. 1D). Other affected individuals have a similar clinical manifestation, with the exception of the mild dysaudia, which is close to the phenotypes from reported ADNIV families. We identified a CAPN5 mutation (c. 865 C>T, p. Arg [R]289 Thr [W]) (Fig. 1E) in this family. The Arg289 residue located in exon 6 is highly conserved in different vertebrate species. Results from direct sequencing confirmed this mutation cosegregated with all affected individuals in the family and was not present in any of the unaffected family members or normal controls (from in-house exome databases with 1402 samples from a Chinese population). Thus, the p. R289W mutation in CAPN5 is a disease-causing mutation in this family.

**Elevated Ki67 Staining in the RPE Layer in CAPN5R289W Mice**

To investigate molecular mechanism of CAPN5 mutation, we generated CAPN5R289W knock-in mice. We did not observe any obvious differences in RPE layer thickness between 3- and 8-month-old knock-in and WT mice by OCT (Fig. 2A). Also no functional differences of photoreceptors by ERG examination between 8-month-old CAPN5R289W mice and their WT counterparts have been observed (Figs. 2B, 2C). However, when we examined levels of the proliferative antigen Ki67 by immunofluorescence, we observed a significant increase in both 3- and 8-month-old CAPN5R289W mice (n = 4) when compared with WT mice (n = 6) in RPE layers (Fig. 2D, 2E). This suggests that abnormal proliferative activation exists in CAPN5R289W mouse RPE layers.

CAPN5 Interacts With SLIT2 in Mouse Photoreceptor-Like Cells and Neuro-Like Cells. It has been reported that CAPN5 is specifically expressed in the nucleus and cytoplasm of neural-like cells and photoreceptor cells and that CAPN5 mutations commonly affect the subcellular location of CAPN5. SLIT2 is expressed in the cytoplasm, at the plasma membrane, and can also be secreted by neurons or retinal neural cells. Previous studies using a yeast two-hybrid system have shown that the large subunit 1 associates with SLIT2/3. To identify the subcellular location and interaction between CAPN5 and SLIT2, we transfected CAPN5 and CAPN5 R289W mutant plasmids into 661W cells, and immunostaining was performed. It showed that WT CAPN5 was expressed mainly in the nucleus and partly colocalized with SLIT2. CAPN5 R289W was expressed and translocated mainly into the cytoplasm, and most of the signals colocalized with SLIT2 in the cytoplasm (Fig. 3A). These results suggested that the mutant increased translocation of CAPN5 from the nucleus to the cytoplasm and promoted CAPN5 colocalization with SLIT2 in the cytoplasm. To further investigate whether CAPN5 interacted with SLIT2 in 661W cells, we used anti-Flag beads to precipitate overexpressed CAPN5 and CAPN5 mutant protein in 661W and SHSY5Y cells, respectively. We found that CAPN5 coprecipitated with SLIT2 using a specific anti-SLIT2 primary antibody in both 661W and human neuroblastsoma SHSY5Y cells (Fig. 3B). Taken together, these results demonstrated that CAPN5 colocalized and interacted with SLIT2 at cytoplasm in photoreceptor-like 661W cells and neural-like SHSY5Y cells, and mutation leads to the mislocalization of the CAPN5.

CAPN5 Associates With SLIT2 and Affects its Proteolytic Cleavage in Mouse Retinal Photoreceptors. To confirm the localization of tissue expression of CAPN5 and SLIT2 in the retina, we performed immunofluorescence and Western blotting in adult WT mice (n = 10) and knock-in CAPN5R289W mice (n = 8) using specific primary antibodies against CAPN5 and SLIT2. It has been reported that CAPN5 is
expressed on the photoreceptor layer, inner layer, and retinal ganglion cells (RGCs) in mouse retina. SLIT2 expression has been observed in retina neuronal cells. Here, we detected CAPN5 expression mainly in the outer segment of the retina and partly in the inner layer and ganglion cell layer. SLIT2 was expressed in the outer segment of photoreceptor cells and the inner layer (Fig. 4A). We also found that CAPN5 and SLIT2 were mainly coexpressed in the outer segments of photoreceptors in WT mice and CAPN5R289W mice (Fig. 4B). Both CAPN5 and SLIT2 proteins were also detected in the mouse retina (n = 3), but we did not detect a specific 75-kDa CAPN5 band in HEK-293T cells (Fig. 4C). We also did not find the differently expressed levels of SLIT2 and CAPN5 in adult CAPN5R289W/+ heterozygous mice and CAPN5R289W/+ homozygous mice (Fig. 4E, not significant; P > 0.05). These results indicate that CAPN5 associated with SLIT2 and regulated proteolysis of SLIT2 in retina photoreceptors, as levels of the cleaved SLIT2-N and SLIT2-C fragments were increased in CAPN5R289W mice retinas in vivo.

CAPN5 Promotes Proteolytic Cleavage of SLIT2 in Neuronal-Like Cell Lines. To demonstrate that CAPN5 regulates the cleavage of SLIT2 in vitro, we transfected CAPN5

and CAPN5 R289W mutant vectors into 661W cells and the human neuroblastoma SHSY5Y cells. After 72 hours post transfection, the levels of SLIT2 isoforms were measured by Western blotting. We found that an increase in both 140-kDa SLIT2-N and 55-kDa SLIT-C fragments was observed in 661W cells after CAPN5 transfection (Fig. 5A). The levels of cleaved SLIT2-N plus SLIT2-C fragments were increased with the overexpression of CAPN5 R289W compared to WT CAPN5 (Fig. 5B, $P < 0.05$). We also detected higher levels of SLIT2-C fragment compared with SLIT2-N fragment after CAPN5 R289W transfection (Fig. 5C, $P < 0.05$). These increased levels of secretion were also detected in SHSY5Y cells with the transfection of CAPN5 R289W. The levels of SLIT2-C versus SLIT2-N were unchanged after WT CAPN5 transfections (Fig. 5D, 5E, $P < 0.01$; Fig. 5F, $P < 0.05$). These data suggested that CAPN5 R289W overexpression induced higher levels of cleaved SLIT2-C than SLIT2-N in cells.

It has been shown that native secreted mouse SLIT2 is usually proteolytically cleaved at R1113 to form the long SLIT2-N and short SLIT2-C fragments. This cleavage site is highly conserved in different species.26 To further examine whether CAPN5 directly cleaves SLIT2, we incubated purified His-tagged recombinant human CAPN5 with purified recombinant mouse SLIT2 protein. Also, recombinant CAPN5 was incubated with His-tagged mouse SLIT2 protein and the non cleavable R1113I SLIT2 mutant protein in physiological buffers. After 1-hour incubation, proteins were analyzed by Western blotting using an anti-SLIT2 specific antibody. We found that WT recombinant SLIT2 was proteolytically cleaved into SLIT2-N and SLIT2-C fragments by human CAPN5. However, SLIT2 R1113I mutant was resistant to cleavage as expected (Fig. 5G). These results suggest that recombinant CAPN5 protein proteolytically cleaves SLIT2 into SLIT2-N and SLIT2-C fragments but did not proteolytically cleave the mutant SLIT2 R1113I protein.

To further explain how CAPN5 activity drives the cleavage of SLIT2, we used an intracellularly expressed antibody fragment plasmid, pSinscFv, to target and inhibit CAPN5. We cotransfected CAPN5 R289W and pSinscFv vectors into 661W cells and found that the total levels of SLIT2-N and SLIT2-C were significantly decreased 60 hours post transfection compared with CAPN5 R289W and the vector control group (Fig. 5H). Taken together, these results suggested that overexpression of CAPN5 induced increased proteolytic cleavage of SLIT2 in 661W and SHSY5Y neuronal-like cell lines. CAPN5 R289W showed a much stronger proteolytic activity on SLIT2 as a substrate compared to WT CAPN5. Also, recombinant WT SLIT2 protein was degraded by CAPN5, while...
CAPN5 did not proteolytically cleave the mutant SLIT2 (p.R1113I) protein in vitro. Moreover, a specific inhibitory intracellular antibody blocked the activity of CAPN5 and inhibited cleavage of SLIT2, which may have therapeutic effects for this disease.

**CAPN5 Drives Secretion of SLIT2 Fragments and Promotes Proliferation of Human RPE Cells via Activation of Protein Kinase A (PKA).** We prepared SLIT2 recombinant protein (Fig. 6A). We found overexpression of CAPN5 leads to the increased expression of SLIT2-N and SLIT2-

![Figure 3. CAPN5 interacts with SLIT2 in vitro.](image-url)

(A) CAPN5 colocalizes with SLIT2 in 661W cells. Scale bar: 20 μm. (B) Sixty hours posttransfection with CAPN5 vectors, 661W cells and SHSY5Y cells were lysed and immunoprecipitated with M2 anti-Flag beads. The protein complex was then separated by SDS-PAGE and detected using an anti-SLIT2 antibody. CAPN5 was associated with SLIT2 in 661W and SHSY5Y cells.
C fragments compared to controls (Fig. 6B, 6C; \( P < 0.05 \)). The level of the SLIT2-C fragment was higher than the level of SLIT2-N (Fig. 6D; \( P < 0.05 \)). It has been reported that secreted SLIT2-C stimulates robust activation of PKA in adipocytes.27 We used the recombinant SLIT2, SLIT2-N, and SLIT2-C fragments to stimulate human RPE cells and found that by activating PKA, phosphorylation levels were increased. The phosphorylation of PKA was also enhanced by conditioned medium (from 661W cells after transfection with CAPN5 vectors; Fig. 6E). To investigate whether secretion of SLIT2-C from photoreceptors influences the viability of human ARPE-19 cells, we cultured ARPE-19 cells with conditioned medium or treated human RPE cells with recombinant SLIT2, SLIT2-N, and SLIT2-C fragments. The viability of retinal epithelium cells was higher in groups grown in conditioned medium or with SLIT2 and fragments treatment (Fig. 6F; \( P < 0.001 \), \( P < 0.01 \)). These results revealed that photoreceptor-like 661W cells secreted cleaved SLIT2-N and SLIT2-C fragments that both promoted ARPE-19 cell proliferation by activation of PKA.

CAPN5 \( R289W \) Mutant Decreased PKA Phosphorylation in Retinal Photoreceptor Cells. It has been shown that the inhibition of PKA may be of benefit in several neurodegenerative diseases.28 We therefore examined the level of phosphorylation and activation of PKA in retinas from WT and \( CAPN5^{R289W} \) mice by immunostaining. The level of phospho-PKA was decreased in the outer segments of photoreceptors in transgenic \( CAPN5^{R289W} \) mice compared to WT (Fig. 7A). These results were consistent with the levels of phospho-PKA in 661W photoreceptor cells after CAPN5 R289W overexpression (Fig. 7B). We found that the levels of phospho-PKA were reduced in \( CAPN5^{R289W} \) mouse retinas (Fig. 7C, 7D). The levels of phospho-PKA were decreased in 661W cells with overexpression of \( CAPN5^{R289W} \) (Fig. 7E, 7F). Taken together, these results demonstrate that R289W mutation inhibits phosphorylation of PKA in photoreceptor cells both in vivo and in vitro.

CAPN5 \( R289W \) Mice Show Abnormalities in the Proliferative RPE Layer. To investigate whether \( CAPN5 \) R289W promoted proliferation in the RPE layer in vivo, we performed

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**Figure 4.** CAPN5 associates with SLIT2 and cleaves SLIT2 in vivo. Retina sections from 3-month-old WT and \( CAPN5^{R289W} \) mice. (A) Low-magnification images of CAPN5 (red) and SLIT2 (green) in retina sections. ONL, outer nuclear layer; INL, inner nuclear layer; OS/IS, outer segment/inner segment of photoreceptor cells. Scale bar: 50 \( \mu \)m. (B) Higher-magnification images of OS/IS. Scale bar: 20 \( \mu \)m. (C) SLIT2 and CAPN5 were expressed in mouse retina by Western blotting. The cell lysate from HEK-293T cells was set as a positive control for SLIT2 expression. The loading control was anti-mouse IgG heavy chain, and positive control is shown as detected CAPN5. The protein and mRNA levels of CAPN5 and SLIT2 in WT mice (\( n = 5 \)) and \( CAPN5^{R289W} \) mice (\( n = 3 \)) were measured by Western blotting. (D) The expression levels of CAPN5 and SLIT2 in the mouse retina. The density of SLIT2 bands was measured and calculated compared to the values of GAPDH loading control; the mean ± SEM values from three independent experiments (WT, \( n = 5 \); \( CAPN5^{R289W} \)) are shown compared with WT mice. (E) The mRNA levels of CAPN5 and SLIT2 in the mouse retina. The density of SLIT2 bands was measured and calculated compared to the values of a GAPDH loading control; the mean ± SEM values from the \( CAPN5^{R289W} \) heterozygous mice (\( n = 4 \)) and \( CAPN5^{R289W} \) homozygous (\( n = 4 \)) mice in three independent experiments were compared to WT mice and are shown on the lower bands. ***\( P < 0.001 \), Student’s t-test. n.s., no significance.
hematoxylin and eosin (H&E) staining and immunofluorescence analysis. We found an abnormal proliferative RPE layer in CAPN5R289W mice, while no notable degeneration of the photoreceptor layer or other layers were observed (Fig. 8A).

We observed that phosphorylation of PKA was increased in CAPN5R289W mouse RPE layers but reduced in the photoreceptor layer (Fig. 8B). These results indicated that the CAPN5 R289W mutant led to abnormal proliferation and increased

**FIGURE 5.** CAPN5 promotes proteolytic cleavage of SLIT2 in neuronal cells. Cells (661W and SHSY5Y) were transfected with vectors. Sixty hours post transfection, SLIT2 expression was measured. (A) Isoforms of SLIT2 expressed in 661W cells. (B) Relative levels of SLIT2-N plus (+) SLIT2-C fragments in 661W cells. (C) The normalized levels of SLIT2, SLIT2-N, SLIT2-C are shown in 661W cells. (D) Forms of SLIT2 expressed in SHSY5Y cells. (E) Levels of SLIT2-N plus SLIT2-C fragments in SHSY5Y cells. (F) The normalized levels of SLIT2, SLIT2-N, SLIT2-C are shown in SHSY5Y cells. GAPDH was used as the loading control. Expression levels of CAPN5 are shown in the lower panels as the positive control. The density of SLIT2-N plus (+) SLIT2-C fragment bands, SLIT2, SLIT2-N, SLIT2-C bands compared and normalized to GAPDH bands were measured and calculated using Image J software. The mean ± SEM values from three independent experiments were compared between CAPN5 WT and CAPN5 R289W groups. *P < 0.05, **P < 0.01; 1-way ANOVA, with Tukey’s test was used. Human recombinant CAPN5/His protein was incubated with mouse recombinant SLIT2/His and SLIT2 mutant R1113I protein at the indicated concentrations. After 1-hour incubation, proteins were detected by Western blotting with an anti-SLIT2 antibody. (G) The native recombinant CAPN5 cleaved SLIT2. –, untreated control. (H) Inhibitory intracellular antibody pSinscFv decreased CAPN5-mediated cleavage of SLIT2. *P < 0.05, **P < 0.01, Student’s t-test. –, cells transfected with pSin empty vector control; +, pSinscFv/CAPN5 R289W vector transfections.
phosphorylation of PKA in RPE cells (Fig. 8C), but reduced phosphorylation of PKA in the photoreceptor layer when compared with WT mice in vivo.

**DISCUSSION**

Mutations in *CAPN5* cause human ADNIV, the process of multiple pathologies including photoreceptor degeneration, autoinflammatory responses, and proliferative vitreoretinopathy.12,13 The molecular mechanisms underlying many of these pathologies have not been well clarified. We identified a novel mutation in *CAPN5* (p.R289W) in a Chinese ADNIV family. We speculated that this mutant stabilized catalytic core domain II and enhanced CAPN5 catalytic activation in the protein conformation. The proband shows features of cataracts, RP, and mild dysaudia. To dissect its molecular consequence, we generated a knock-in mouse. While, to our surprise, no obvious abnormal features (cataracts or retinitis pigmentosa) have been observed in mice, we did find that *CAPN5R289W* mice have an abnormally proliferative RPE layer. Thus, the different phenotypes between the ADNIV patients and *CAPN5R289W* mice could be caused by activated CAPN5-mediated multipathway proteolytic regulation of pathways in different species.

**SLIT2** classically exists in one of three different forms: full-length, a long fragment (SLIT2-N), and a short fragment SLIT-C, which are generated by proteolytic cleavage. 22,23,29 However, the
The functional consequences of secreted SLIT2 fragments in the retina are still unknown. SLIT2 plays an important role as a secreted factor in neural guidance and migration and development of the retinal optical chiasma via the SLIT2-ROBO pathway.29,30 Recently, it has been shown that SLIT2-ROBO, especially SLIT2-N/ROBO signaling, enhances retinal neovascularization by regulating VEGF expression and increasing RPE cell proliferation via MAPK activation.31 We found, we believe for the first time, that CAPN5 interacts with and promotes proteolysis of SLIT2. This provides a novel molecular mechanism and therapeutic target for ADNIV pathologies caused by CAPN5 mutations.

Figure 7. Phosphorylation of PKA was decreased in photoreceptors in CAPN5R289W mice. (A) Immunofluorescence activity of phosphorylated PKA in the retina of mice. Double-stained CAPN5 (red) and phosphor-PKA (p-PKA, green) in mouse retina sections. The left panels show the lower magnification (20x), scale bar: 100 μm, and the right panels show higher magnification (40x), scale bar: 25 μm. Sections were 8 μm in thickness. WT (n = 5), mice were used as the control group. (B) Double-staining of p-PKA and CAPN5 in 661W cells. 661W cells transfected with WT CAPN5 vector and CAPN5 R289W vector. Sixty hours post transfection, cells were fixed and incubated with anti-CAPN5 (green) and anti-phosphor-PKA (red) followed by the appropriate secondary antibodies. Merged channels are shown. Scale bar: 5 μm. (C) Decreased levels of phosphor-PKA in CAPN5R289W mouse retinas. The retinal lysate from 3-month-old WT (n = 5) and CAPN5R289W mice (n = 7) were collected and separated by SDS-PAGE. (D) The levels of p-PKA, GAPDH, and PKA were detected by Western blotting. The bands were measured, calculated, and normalized to B6 WT group; the mean ± SEM values were from three independent experiments and are compared with WT control groups. **P < 0.01; Student's t-test was used. (E) Decreased levels of p-PKA in 661W cells, which were transfected with empty vector, CAPN5 WT vector, and CAPN5 R289W vector. Cells were lysed 60 hours post transfection, and Western blotting was performed to measure the levels of p-PKA, GAPDH, and PKA. Images are representative of experiments performed in triplicate. (F) The mean ± SEM values were from three independent experiments and are compared with vector control groups. *P < 0.05; 1-way ANOVA, with Tukey's test was used.
of RPE cells through activation of MAPK. Based on these results, CAPN5 promoted photoreceptor-derived SLIT2 expression that increased activation of PKA in RPE cells, but the CAPN5 R289W mutant decreased phosphorylation of PKA in photoreceptors in vitro and in vivo. This could be because PKA is a specific downstream pathway of activated CAPN5 in photoreceptors but not in RPE cells. Some studies proposed that inhibitors of cAMP upstream of PKA promoted proliferation of RPE cells in vitro. However, it is an oversimplification to say that proliferation of RPE cells is induced by activation or inactivation of PKA. It has also been reported that the photoreceptor cells release factors that affect neighboring cells; native secreted factors from other cell types could affect abnormal proliferation of the RPE layer. Here our results suggested that the secreted SLIT2-N and SLIT2-C fragments from photoreceptor cells may increase the activation of PKA in RPE cells. Therefore, we propose that photoreceptor cells released SLIT2-N and SLIT2-C and that this contributes to the abnormal proliferation of RPE cells in the CAPN5-induced ADNIV model.

**CONCLUSIONS**

In summary, we identified a novel CAPN5 mutation in the ADNIV family and generated the corresponding knock-in mice. We found that CAPN5 regulates cleavage and secretion of SLIT2 in retinal photoreceptors, which contributes the abnormal proliferation of RPE cells. Our study provides a novel molecular mechanism and therapeutic target for ADNIV pathologies caused by CAPN5 mutations.

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