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

Yan Wang,1,2 Heming Li,1,2 Shihu Zang,3 Fanfan Li,4 Yingying Chen,4 Xiao Zhang,1,2 Zongming Song,1,2,5 Qing Peng,6 and Feng Gu1,2

1School of Ophthalmology and Optometry, Eye Hospital, Wenzhou Medical University, Wenzhou, Zhejiang Province, China
2State Key Laboratory and Key Laboratory of Vision Science, Ministry of Health and Zhejiang Provincial Key Laboratory of Ophthalmology and Optometry, Wenzhou, Zhejiang Province, China
3Fuyang City Suburban Middle School, Fuyang, Anhui, China
4The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China
5Henan Eye Institute, Henan Eye Hospital, Henan Provincial People's Hospital and People's Hospital of Zhengzhou University, Zhengzhou, Henan, China
6Shanghai Tenth People's Hospital, Tongji University, School of Medicine, Shanghai, China

Correspondence: Feng Gu, Eye Hospital, Wenzhou Medical University, 270 Xueyuan West Road, Wenzhou, 325027, Zhejiang, China; gufengwu@gmail.com.
Submitted: July 25, 2017
Accepted: March 7, 2018

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

Calpains are proteases that regulate many biological functions through their proteolytic activity.1 Active calpains have been linked to neurodegenerative diseases such as Huntington’s disease, Alzheimer’s disease, and several neurotraumas.1–5 CAPN1 and CAPN2 are well-known classical calpains and activated in neurodegenerative conditions and cell death. They also have been identified as important components of several eye pathologies, including photoreceptor degeneration, reactive retinal pigment epithelium (RPE) cells, retinal neovascularization, autosomal inflammation, and blindness.12,13 Unlike the classical calpains CAPN1 and CAPN2, CAPN5 is expressed mainly in the outer segments of the retina and also in synapses of photoreceptors, as well as in the inner plexiform layer and retinal ganglion cells.14 Although the CAPN5 protein has no calcium-binding domains unlike CAPN1/2, its activity is related to calcium ion levels in the retina in a fashion similar to CAPN1 and CAPN2.11

The catalytic domain of the calpain protease family is highly conserved in different isoforms and across species.15 Many substrates of classical calpains are known that are related to cell death, the cytoskeleton, and cell metabolism.6,17 A single amino acid mutant locating at the catalytic domain of CAPN5 increases its activation and is expressed in a very cell-specific
CAPN5 Regulates Cleavage of SLIT2

**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 (CAPN5<sup>R289W</sup>) mice, we confirmed the strain background is wildtype (WT) B6, which ruled out C57BL/6j/N with Crb1<sup>RD8/RD8</sup> linked to retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA). CAPN5<sup>R289W</sup> 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 mice were identified by analyzing genomic DNA isolated from tail tips. The CAPN5<sup>R289W</sup> knock-in mice were mated with FLP mice to remove the antibiotics gene. The final CAPN5<sup>R289W</sup> knock-in mice without the antibiotics gene were labeled with CAPN5<sup>R289W</sup>.

**Electroretinography and Optical Coherence Tomography**

ERG was performed with a standard protocol. Differences between WT (<i>n</i> = 15) and CAPN5<sup>R289W</sup> (<i>n</i> = 12) mice were assessed for six ERG parameters in three age-matched groups by means of <i>t</i>-tests corrected for multiple comparisons (<i>α</i> = 0.05). In a subset of the mice (<i>n</i> = 7 for CAPN5<sup>R289W</sup>; <i>n</i> = 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 CAPN5<sup>R289W</sup> mice were acquired with a 3.2-μm resolution spectral-domain optical coherence tomography (SD-OCT) system (Biotigen, 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.

**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<sup>4</sup>) 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, sections were incubated with secondary antibodies, Alexa Fluor 488-conjugated donkey anti-mouse IgG (Boshide, Wuhan, China), goat anti-mouse IgG (Boshide), goat anti-rabbit IgG (Boshide), Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA).

**Antibodies and Reagents**

Antibodies to the following antigens were used: SLIT2 (sc-16019, E-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CAPN5 (sc-271271, A-5; Santa Cruz Biotechnology), phosphor-PKAα/β/γ (Thr198) (sc-164161; Santa Cruz Biotechnology), mouse PKAα/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).

**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
CAPN5 Regulates Cleavage of SLIT2

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'-GATCCCGGTCTCCGTTGATG-3', anti-sense 5'-GAGCAATAGTGACACTCACTG-3'; for mouse SLIT2: sense, 5'-GGCACACACTG-3', anti-sense, 5'-ATCATCTCGTGATCTCGTGAG-3'; for loading control mouse Gapdh: sense, 5'-AGCTTGCATCTTCATCTG-3', anti-sense, 5'-GGTTCACCTCCCATGACAACA-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 plasmid CAPN5 construct was prepared by inserting the full-length human CAPN5 cDNA into the pET28a vector (Novagen, Madison, WI, USA) for 48 hours, cells were incubated separately with 100 ng/ml of recombinant SLIT2, SLIT2-N, and SLIT2-C for 5 hours, followed by a 30-minute incubation period at 25°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 x 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 x 10⁵ 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 ADNI 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 dysautria (Fig. 1D). Other affected individuals have a similar clinical manifestation, with the exception of the mild dysautria, which is close to the phenotypes from reported ADNI families. We identified a novel mutation (c. 865 C>T, p. Arg [R] 289 Trp [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 CAPN5 R289W Mice

To investigate molecular mechanism of CAPN5 mutation, we generated CAPN5 R289W 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 CAPN5 R289W 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 CAPN5 R289W mice (n = 4) when compared with WT mice (n = 6) in RPE layers (Fig. 2D, 2E). This suggests that abnormal proliferative activation exists in CAPN5 R289W mouse RPE layers.

CAPN5 Interacts With SLIT2 in Mouse Photoreceptor-Like Cells and Neural-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 neuroblastoma 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 Protozoic Lyeage 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 CAPN5 R289W mice (n = 8) using specific primary antibodies against CAPN5 and SLIT2. It has been reported that CAPN5 is...
CAPN5 expresses 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); it has been shown that SLIT2 is expressed by HEK-293T cells.

To further clarify the possible association between CAPN5 and SLIT2 in vivo, we used a CAPN5 antibody to immunoprecipitate CAPN5 protein complexes from whole retina lysates in adult WT mice (n = 4). Both full-length SLIT2 and 140-kDa SLIT-N forms were detected using a specific anti-SLIT2 antibody in the complex captured by CAPN5 antibody by Western blotting. SLIT2 was not present in mouse IgG control groups. IgG heavy-chain bands were present in input, IgG, and WT mouse retina lysates (Fig. 4D). The protein levels of CAPN5 were not altered in WT mice and CAPN5R289W mice. The full-length SLIT2-N and SLIT2-C fragments were all detected in CAPN5R289W mice (n = 4) retinas, while we could not detect the SLIT2-C fragment in WT mice retinas (n = 4) (Fig. 2E, P < 0.001). We also did not find the differently expressed levels of SLIT2 and CAPN5 in adult CAPN5R289W heterozygous mice and CAPN5R289W homozygous mice (Fig. 4E, 4F; 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 mouse 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. 5E, P < 0.05). These data suggested that CAPN5 R289W overexpression induced higher levels of cleaved SLIT2-C than SLIT2-N in cells.

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 fragments were significantly decreased 60 hours post transfection compared with WT CAPN5 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 SLIT2 protein. Also, recombinant CAPN5 was incubated with His-tagged mouse SLIT2 protein and the noncleavable 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 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 noncleavable 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 fragments were significantly decreased 60 hours post transfection compared with WT CAPN5 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-
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-C 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 phosphor-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 phosphor-PKA in 661W photoreceptor cells after CAPN5 R289W overexpression (Fig. 7B). We found that the levels of phosphor-PKA were reduced in CAPN5\(^{R289W}\) mouse retinas (Fig. 7C, 7D). The levels of phosphor-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
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 phosphorylation of PKA.

**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 pSinsFv decreased CAPN5-mediated cleavage of SLIT2. *P < 0.05, **P < 0.01, Student’s t-test. –, cells transfected with pSin empty vector control; +, pSinsFv/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 \textit{CAPN5} cause human ADNIV, the process of multiple pathologies including photoreceptor degeneration, autoinflammatory responses, and proliferative vitreoretinopathy.\textsuperscript{12,13} The molecular mechanisms underlying many of these pathologies have not been well clarified. We identified a novel mutation in \textit{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 \textit{CAPN5R289W} mice have an abnormally proliferative RPE layer. Thus, the different phenotypes between the ADNIV patients and \textit{CAPN5R289W} mice could be caused by activated CAPN5-mediated multipathway proteolytic regulation of pathways in different species.

\textbf{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. The full-length SLIT2 and SLIT2-N have been well studied as activators of roundabout axon guidance receptor (ROBO) signaling pathways in retinal axonal guidance.}\textsuperscript{22,23,29} However, the

\textbf{FIGURE 6.} Overexpression of CAPN5 promotes secretion of SLIT2 fragments from 661W cells as well as increasing cell proliferation and phosphorylation of PKA in RPE cells. (A) Purified SLIT2-N, SLIT2-C, and SLIT2 proteins were detected by anti-His tag antibody. Cells (661W) were transfected with vectors; 72 hours post transfection, the cultured media were collected, and detected by Western blotting with an anti-SLIT2 antibody. (B) Western blotting of SLIT2 isoforms in 100 \( \mu \)L 661W conditioned medium (CM). (C) Relative levels of SLIT2-N plus SLIT2-C fragments in CM. (D) Normalized levels of SLIT2, SLIT2-N, SLIT2-C in 100 \( \mu \)L 661W cell CM. The density of SLIT2-N plus (\( \pm \)) SLIT2-C, SLIT2-N, SLIT2-C bands were normalized to vector control groups. The mean \( \pm \) SEM values from three independent experiments were compared between CAPN5 WT and CAPN5 R289W groups. *\( P < 0.05 \), **\( P < 0.01 \). (E) SLIT2 and its fragments increased phosphorylation of PKA in human RPE cells. CM, the conditioned medium from CAPN5 vector-transfected 661W cells. Medium with 0.1\% FBS was used as a control. The images are representative of triplicate experiments. The density of p-PKA bands compared and normalized to GAPDH bands were measured and calculated using ImageJ software, respectively. The mean \( \pm \) SEM values from three independent experiments were compared to control medium or between CAPN5 WT and CAPN5 R289W groups. (F) ARPE-19 cells (\( 5 \times 10^3 \)) were treated with recombinant SLIT2-C fragment at 1 \( \mu \)g, the CM from CAPN5 vector-transfected 661W cells, and control medium with 0.1\% FBS. *\( P < 0.05 \), **\( P < 0.01 \).
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. 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. 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.

The SLIT2/ROBO pathway has been associated with retinal neovascularization, proliferative diabetic retinopathy, and RPE cells, as well as adipose-secreted SLIT2-C fragments that regulate thermogenesis and metabolic function of brown and beige fat. The secreted SLIT2 and SLIT2-N fragments promoted retinal neovascularization in the developing retina and abnormal pathologies in diabetic retinopathy. Indeed, these features of SLIT2-related diabetic retinopathy overlap with diabetic retinopathy-like features in CAPN5 mutation–caused ADNIV. Very few studies have been associated with the functional consequences of release of the SLIT2 C-terminal fragment through SLIT2-N/ROBO pathways because SLIT2-C is not associated with ROBO signals. The secreted full-length SLIT2 and N-terminal SLIT2 enhanced proliferation...
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 photoreceptor cells 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.

Acknowledgments

Supported by grants from the Natural Science Foundation of China (81201181 [FG], 81475295, 81670882 [ZS]), the Natural Science Foundation of Zhejiang Province, China (LQ17H120004 [YW]), Science Technology Project of Zhejiang Province (2017C37176

![Image](http://arvojournals.org/2018/April/Vol.59-No.5/1820/)

**Figure 8.** CAPN5R289W mice show an abnormal proliferative RPE layer and increased PKA phosphorylation in RPE cells. Retina sections from 3-month-old WT (n = 8) and CAPN5R289W mice (n = 9) were H&E stained. (A) Histopathology of CAPN5R289W mouse retinas. Magnification is 20×. Arrows denote the RPE layers. (B) Increased immunofluorescence of p-PKA in CAPN5R289W mice. The black arrows represent the RPE layers and white arrows p-PKA positive retinal epithelial cells. Section thickness was 8 μm. WT (n = 7) mice were the control group. Scale bar: 100 μm for upper panel, 25 μm for lower enlarged panel. (C) The relative intensity of p-PKA signal was measured and calculated; the mean ± SEM values were from CAPN5R289W (n = 8) mice and are compared with WT control groups (n = 7) in RPE layers. ***P < 0.001, Student’s t-test was used.
References


