Calpain-5 Expression in the Retina Localizes to Photoreceptor Synapses

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Purpose. We characterize calpain-5 (*CAPN5*) expression in retinal and neuronal subcellular compartments.

Methods. *CAPN5* gene variants were classified using the exome variant server, and RNA-sequencing was used to compare expression of *CAPN5* mRNA in the mouse and human retina and in retinoblastoma cells. Expression of CAPN5 protein was ascertained in humans and mice in silico, in mouse retina by immunohistochemistry, and in neuronal cancer cell lines and fractionated central nervous system tissue extracts by Western analysis with eight antibodies targeting different CAPN5 regions.

Results. Most *CAPN5* genetic variation occurs outside its protease core; and searches of cancer and epilepsy/autism genetic databases found no variants similar to hyperactivating retinal disease alleles. The mouse retina expressed one transcript for *CAPN5* plus those of nine other calpains, similar to the human retina. In Y79 retinoblastoma cells, the level of *CAPN5* transcript was very low. Immunohistochemistry detected CAPN5 expression in the inner and outer nuclear layers and at synapses in the outer plexiform layer. Western analysis of fractionated retinal extracts confirmed CAPN5 synapse localization. Western blots of fractionated brain neuronal extracts revealed distinct subcellular patterns and the potential presence of autoproteolytic CAPN5 domains.

Conclusions. *CAPN5* is moderately expressed in the retina and, despite higher expression in other tissues, hyperactive disease mutants of *CAPN5* only manifest as eye disease. At the cellular level, CAPN5 is expressed in several different functional compartments. CAPN5 localization at the photoreceptor synapse and with mitochondria explains the neural circuitry phenotype in human *CAPN5* disease alleles.

Keywords: CAPN5, calpain, autosomal dominant neovascular inflammatory vitreoretinopathy, ADNIV

Mutation of the calcium-activated protease calpain-5 (CAPN5) can cause a severe blinding disease, autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV, OMIM #193235).[1–5] In their twenties, ADNIV patients begin to display a synaptic signaling defect and intraocular inflammation (uveitis). Over the ensuing five decades, they experience retinal degeneration, retinal neovascularization, and intraocular fibrosis, culminating in phthisis and blindness.[1–3] Although *CAPN5* is expressed in many tissues, ADNIV patients only manifest disease in the eye.[6] Autosomal dominant neovascular inflammatory vitreoretinopathy CAPN5 is hyperactive, since the disease allele reduces the calcium level required for protease activity.[7] Thus, the eye-restricted phenotype likely reflects the extraordinarily high calcium concentrations in the retina, where such a hyperactive calcium-dependent protease could be particularly damaging.[3,5]

Increased calpain activity is a feature of many eye-related pathologies, including retinal degeneration,[8,9] retinal hypoxia,[10–13] retinitis pigmentosa,[14–16] retinal detachment,[17] and glaucoma.[18,19] Retinal damage from these pathologies can be lessened by administering the calpain inhibitor SJA6017.[8,20–22] However, since the human retina expresses several calpains, it

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is not known which isoform(s) SJA6017 inhibits. Both *CAPN1* and *CAPN2* are expressed in the retina and show increased activity in other neurodegenerative conditions and hypoxic cell death.[8,20] *CAPN10* and calpastatin also are expressed in the retina[23,24] and *CAPN3* expresses a retina-specific splice variant in rats.[8,25] Although CAPN3 is linked to limb-girdle muscular dystrophy type 2A,[26] it is not associated with any known retinal disease. CAPN5, the most distant calpain family ortholog,[7] is the only retinal calpain known directly to trigger retinal disease in humans. Inhibition of CAPN5 might be therapeutic, but a specific inhibitor has never been isolated; and sequence analysis shows CAPN5 does not bind calpastatin, the endogenous calpain inhibitor.[7,27]

To increase our understanding of CAPN5 in the healthy retina and during ADNIV, we characterized CAPN5 mRNA and protein expression in the normal retina. We also drew from rich compilations of genetic-variance expression databases and performed antibody epitope-structure analysis, immunohistochemistry, and subcellular fractionation.

Methods

Human ADNIV Electroretinogram (ERG)

The collection of data used in this study was approved by the Institutional Review Board for Human Subjects Research at the University of Iowa, was compliant with the Health Insurance Portability and Accountability Act, and adhered to the tenets of the Declaration of Helsinki. A full-field ERG was performed according to international standards. Briefly, the eyes were dilated and dark adapted for 30 minutes. Electroretinograms were recorded simultaneously from both eyes using Burian-Allen bipolar contact lens electrodes as described previously.[28] Evoked waveforms, a 100 μV calibration pulse, and a stimulus artifact were recorded on Polaroid film.

RNA Preparation and Next-Generation Sequencing

The Institutional Animal Care and Use Committee (IACUC) approved all experiments. Rodents were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the Policy for the Use of Animals in Neuroscience Research of the Society for Neuroscience. Total RNA was extracted from mouse retinas and cell lines using RNAeasy (Qiagen, Hilden, Germany), and submitted to Otogenetics Corporation (Norcross, GA, USA) for RNA-Seq assays. Libraries were sequenced via Illumina HiSeq2000. Paired-end 90 or 100-nucleotide reads were generated and checked for data quality using FASTQC (Babraham Institute, Cambridge, UK), and analyzed using DNAnexus (DNAnexus, Inc., Mountain View, CA, USA). Gene expression levels for human retina were collected from GEO Omnibus (accession number, GSE40524).

Variant Annotation and Filtering

Variants in the 1000 Genomes, Epi4k, and Autism datasets were annotated with minor allele frequencies (MAFs) from EVS and database of single nucleotide polymorphisms (dbSNP) using GATK's VariantAnnotator[29] and SNPSift/SNPEff.[30] Non-coding variants, those not passing quality filtering and those with a MAF >2% were removed. *CAPN5* variants were downloaded from the Exome Variant Server (EVS) website (available in the public domain at http://evs.gs.washington.edu/EVS/). Only nonsynonmous variants with an MAF <2% in either the EA or AA populations were analyzed.

We obtained vcf files from the database of genotypes and phenotypes (dbGAP) entry for the ARRA Autism Sequencing Collaboration (phs000298). Only those consented for autism research only (AO) were downloaded. The data set(s) were deposited by the ARRA Autism Sequencing Collaborative, an ARRA funded research initiative. Support for the Autism Sequencing Collaborative was provided by Grants R01-MH089208 awarded to Mark Daly, R01-MH089175 awarded to Richard Gibbs, R01-MH089025 awarded to Joseph Buxbaum, R01-MH089004 awarded to Gerard Schellenberg, and R01-MH089482 awarded to James Sutcliffe.

Exome vcf files from the Epi4k Epilepsy Phenome/Genome Project (EPGP) were requested and downloaded from dbGAP (dbGAP Study Accession, phs000653.v2.p1). Our appreciation goes to the Epilepsy Epi4k consortium: Discovery in Epilepsy study (NINDS U01-NS077303) and the Epilepsy Genome/Phenome Project (EPGP-NINDS U01-NS053998).[31,32]

Cell Culture, Reagents, and Antibodies

Cell lines HEK293T, SH-SY5Y, and Y79 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Antibodies used were anti-GAPDH antibody (sc-32233), goat anti-rabbit, and goat anti-mouse secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit anti-CAPN5 polyclonal antibodies (GTX103264; GeneTex, Irvine, CA, USA; 12373-1-AP; Proteintech Group, Inc., Chicago, IL, USA; sc-50500 and sc-271271; Santa Cruz Biotechnology, Inc.) rabbit anti-myc tag antibody (ab9106) and other anti-CAPN5 antibodies (ab28280, ab38943, and ab97534; Abcam, Cambridge, MA, USA).

HEK293T adherent cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine system (FBS). SH-SY5Y adherent cells were cultured in a 1:1 mix of RPMI 1640 and F-12 media containing 10% FBS. Human retinoblastoma Y79 cells were grown in RPMI-1640 medium containing 20% FBS. All cultures contained penicillin (100 U/mL) and streptomycin (100 μg/mL).

Western Blot

Levels of CAPN5 were detected by immunoblot. Cells were lysed 24 hours after transfection, and total cellular protein measured using NanoDrop 2000c (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Samples were electrophoretically resolved by 4% to 12% Bis-Tris PAGE (Life Technologies; Grand Island, NY, USA) and transferred to nitrocellulose (iBlot; Life Technologies). Membranes were blocked with 5.0% nonfat dry milk in 1 × TBS 0.1% Tween-20. Primary antibodies were diluted 1:1000; secondary antibodies 1:10,000. Immunoreactive bands were visualized by Supersignal West Dura Extended Duration Substrate and a MYECL Imager (Thermo Fisher Scientific, Inc.).

Subcellular Fractionation

Frozen bovine retinas were thawed and gently vortexed in buffer A (50% sucrose, 10 mM HEPES, pH7.4, 1 mM EDTA, 5 mM MgCl2) supplemented with a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany). After 13,000*g* centrifugation (1 hour), rod outer segments (ROS) were collected and the pelleted "rest of retina" (ROR) resuspended in Buffer A without sucrose. A centrifugation step (750*g*, 10 minutes) isolated nuclei. The ROR was centrifuged (100,000*g*, 1 hour) to separate soluble proteins (ROR-S1) from membranes (ROR-P1). The ROR-P1 was loaded on a 1.2 M sucrose cushion and centrifuged (200,000*g*, 30 minutes). Synaptosomes (Syn-S2) were collected from the top of the sucrose cushion; pelleted material was the "rest of membranes" fraction (ROM-P2). Dilutions for antibodies to retinal markers were: anti-RDS (1:30, Per 2B6, gift from Robert Molday); mouse anti-NKA (1:1000, M7-PB-E9, Santa Cruz Biotechnology, Inc.); rabbit anti-

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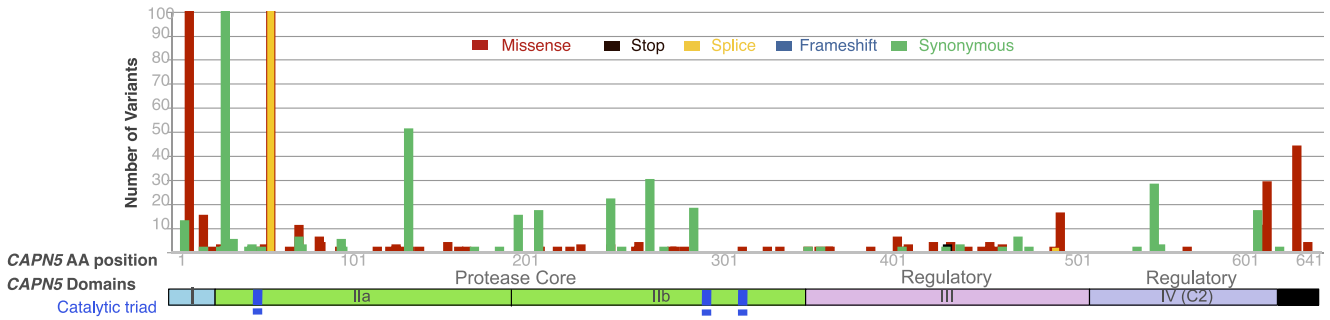

Figure 1. CAPN5 EVS Variants. Public exome databases show common variants in CAPN5 by amino acid position and type. The *CAPN5* gene is 59 kilobases, contains 13 exons, and generates a 2791 nucleotide transcript (encompassing all purported isoforms) that encodes a 640 amino acid protein. Shown here, this data set serves as a normative database for *CAPN5* variants, and depicts the relationship of these variants to functional residues and domains within CAPN5. Variants are rare within the proteolytic core, where retinal disease alleles are located. The EVS sequenced over 6476 individuals, with high *CAPN5* coverage, and found no frameshift variants, two premature stop-codon variants, 843 missense variants (of which 237 were splice-site mutations), and 681 synonymous variants. Of these variants, the majority was scored as either "unknown" or "benign" by PolyPhen. PolyPhen predicted that 1% and 0.83% of the population has "Possibly Damaging" and "Probably Damaging" alleles, respectively.

HCN1 (1:2500)[33]; mouse anti-actin (1:1000, AC-74, Sigma-Aldrich Corp., St. Louis, MO, USA); rabbit a-GAPDH (1:250, Abcam); rabbit anti-RAB3 (1:500, Thermo Fisher Scientific, Inc.); rabbit anti-VAMP2 (Synaptic Systems, Göttingen, Germany), mouse anti-PSD-95 (1:500, Millipore, Billerica, MA, USA). Rat brains were collected and fractionated according to the previously described protocol.[34]

Immunohistochemistry

Sections (7 μm) of fixed (4% paraformaldehyde) retina underwent immunohistochemistry (IHC) as described.[3] Images were captured using a Zeiss LSM 710 and Zen2009 software (Zeiss, New York, NY, USA).

Structural Modeling

The MODELLER server, ModWeb,[35,36] generated a CAPN5 structural model based on the crystal structure of rat calpain-2 (3BOW). Compared to the template, the RMSD was < 0.189 over 349 C_α atoms. PyMOL generated all structure figures.[37]

Results

CAPN5 Gene Variants Are Only Linked to Retinal Disease

Autosomal dominant neovascular inflammatory vitreoretinopathy is an extraordinarily rare disease. We reported only three independent ADNIV pedigrees[1]; all harbor missense mutations in *CAPN5* exon 6, which encodes the protease core domain. All ADNIV alleles change a single amino acid in a gating loop[1,4] and seem to generate a hyperactive protease.[7] We searched for ADNIV-like mutations in normal and disease-associated genomic databases to test whether other diseases that have been linked to increased calpain activity also are caused by hyperactive calpain mutants.

To determine the frequency of *CAPN5* variants, we mined the 1000 Genomes[38,39] and EVS databases,[40] which contain DNA sequences from a healthy human population. Using PolyPhen to estimate variant pathogenecity, our survey predicted that <2% of *CAPN5* gene variants are damaging alleles. This prediction was greater than the actual prevalence of intraocular inflammation (estimated between 17.5–111.1 per 100,000 population), consistent with the reported false-positive rate of PolyPhen predictions. Overall, mapping showed most *CAPN5* variants, especially missense and splice-site variants, exist outside the proteolytic core (Fig. 1). The absence of variants in the proteolytic core supports the idea that proteolytic core mutations are particularly damaging. This is consistent with the high penetrance, rarity, and severity of the ADNIV phenotype.

Increased calpain activity is associated with cancer,[41] and genetic studies link polycystic ovarian and rectal cancers to *CAPN5*.[25,42] We first searched for *CAPN5* variants in the Catalogue of Somatic Mutations in Cancer (COSMIC) Database, which holds sequences from 1.1 million sample genomes from 43 types of cancer.[43,44] Only 39 *CAPN5* missense variants occurred in the proteolytic core (and two stop codon variants), 27 of which could potentially alter CAPN5 activity (Supplementary Fig. S1).[1,5,8,23–25,45–49] No variants were found in the CAPN5 gating loop and none matched the previously identified ADNIV alleles. These results suggested *CAPN5* variants are rare in cancer, so higher calpain activity in cancer cells is not due to hyperactivating CAPN5 mutations.

CAPN5 is highly expressed in the brain,[50] and increased calpain activity has been associated with epilepsy.[51–57] To determine if rare *CAPN5* coding variants could be contributing to the incidence of neurodevelopmental disorders, we queried an epilepsy and autism dataset for mutations in *CAPN5* and compared the variant counts in each cohort to EVS and 1000 genomes. No significant enrichment was found in either the epilepsy or autism cohorts. Again, this suggests the increased calpain activity associated with epilepsy is not due to hyperactivating CAPN5 mutations.

CAPN5 mRNA Is Widely Expressed

Prior Northern analysis on adult mouse mRNA showed that *CAPN5* mRNA was widely expressed, particularly in the brain, eye, uterus, lung, submaxillary gland, prostate, and epididymis.[6] To further evaluate tissue-specific *CAPN5* expression, we used two recent online expression databases (Summarized in the Table). Biogps, which tracked mRNA levels in 79 human and 61 mouse tissues,[58,59] showed that CAPN5 expression is highest in human colon, heart, liver, prostate, and skeletal muscle. Human retinal tissue expressed *CAPN5* at levels slightly above average. Mice expressed CAPN5 highest in the uterus and digestive system, with only low to moderate expression in the eye. The EMBL-EBI gene expression database reported *CAPN5* expression in eight humans and 12 mice,[60] and confirmed high expression in the human digestive system. In contrast to the Biogps, however, these studies found significant expression of *CAPN5* in mouse brain tissue. This dataset did not track *CAPN5* expression in eyes.

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Table. Gene and Protein Expression of Calpain Family Members in the Retina

Gene	Calpain mRNA in Retina	Calpain Protein in Retina
CAPN1	Human,* mouse,* rat[49]	Rat,[23,45] pig,[23,45] human,[8] monkey,[8] rabbit[8]
CAPN2	Human,* mouse,* rat[49]	Rat,[23,45] pig,[23,45] human,[8] monkey,[8] rabbit,[8] cow[46]
CAPN3	Splice variants rat,[47] human,[25] monkey[25]	Splice variants rat[47]
CAPN5	Human[5], mouse*	Human,[1] mouse[5]
CAPN6	Human,* mouse*	N/A
CAPN7	Human,* mouse*	N/A
CAPN8	–	N/A
CAPN9	Human,* mouse*	N/A
CAPN10	Splice variants human,[48] mouse,[24] rat[49]	Splice variants rat,[24] mouse,[24] human[24]
CAPN11	Human,* mouse*	N/A
CAPN12	Human,* mouse*	N/A
CAPN13	N/A	N/A
CAPN14	N/A	N/A
CAPN15	N/A	N/A
CAPN16	N/A	N/A

N/A, not applicable.
* Current study.

The Retina Expresses Several But Not All Calpain Family Members

We applied RNA sequencing to compare expression of *CAPN5* mRNA to that of other calpains in the mouse retina (Fig. 2). Among the 10 calpain genes (*CAPN1*, *2*, *3*, *5*, *6*, *7*, *10*, *12*, *CAPNS1*, and *CAST*), *CAPN5*, *7*, *10*, *2*, and *CAPN1S1* were the most highly expressed (Fig. 2A). Interestingly, except for CAPN2, these variants do not contain an EF-hand domain (a calcium binding, helix-turn-helix motif) believed to mediate protein-protein interactions of the most abundant calpains.[61] The mouse retina expressed only the full-length *CAPN5* transcript, as we reported in humans.[1,3] Expression of *CAPN5* measured a mean of 0.003259 RKPM, approximately half that of the most abundant calpains, *CAPN2* and *CAPN7* (Fig. 2A). The relative expression level of *CAPN5* RNA was four orders of magnitude less than that of the highly expressed phototransduction genes (Fig. 2). Our transgenic mouse model of ADNIV shows a less severe inflammatory phenotype than humans,[5] but here we found that the calpain family mRNA expression pattern in mouse and human retina was similar (Fig. 2B). There were only minor differences between human and mouse. *CAPN1*, *CAPNS2*, and *CAST* were higher in human retina (Figs. 2B, 2C).

The human retinoblastoma Y79 cell line offers an in vitro model of photoreceptors. In these cells, *CAPN5* was expressed at significantly lower levels (5.580e-005 ± 8.391e-005); otherwise, the pattern matched the human and mouse retinas where *CAPN2* and *CAPN7* were the most expressed calpains (Fig. 2C).

Calpains mediate inflammatory necrotic cell death and frequently are compared to caspases, which mediate apoptosis.[62,63] Eight members of the caspase family were expressed in the retina at levels similar to calpains, which suggests the retina is not biased towards a specific cell-death pathway.

CAPN5 Protein Is Widely Expressed

The Human Protein Atlas describes protein expression across 80 major tissues and organs using antibody-based methods and transcriptomics (summarized in the Table).[64,65] CAPN5 protein was detected in 22 distinct normal tissues, mirroring data from the mRNA expression databases. Notably, the Human Protein Atlas data also described equivalently strong expression of CAPN5 in the kidney, bladder, testes, prostate, skin, lungs, and endocrine organs, and moderate expression throughout the digestive tract. CAPN5 also was detected in several brain regions (i.e., cerebral cortex, hippocampus, lateral ventricle, and cerebellum). CAPN5 protein was detected in the cytoplasm, nucleolus, and cell membrane. Limitations of this dataset were the lack of eye tissues and use of only one CAPN5 antibody.

CAPN5 Detection in Neuronal Cells Varies by Antibody

Seven commercial CAPN5 antibodies were tested in Western blots of HEK-293T cells expressing recombinant *CAPN5* cDNA. Antibodies had been generated against different CAPN5 regions (Fig. 3B). Antibodies Ab-2, -6, and -7 showed distinct CAPN5 immunoreactive bands. The strongest signal with the least background was detected by Ab-7, which was generated against CAPN5 domains I, II, and the first 38 amino acids of domain III (Fig. 3C). This antibody did not crossreact with other calpain family members (data not shown). The remaining antibodies detected CAPN5, but gave high background and nonspecific bands (data not shown). Interestingly, two neural cancer lines, SHSY5Y neuroblastoma and Y79 retinoblastoma cells, express endogenous CAPN5 and showed lower molecular weight species not present in transfected HEK-293T cells (Fig. 3D). Since self-cleavage is a known feature of other calpains,[61] and calpain autoproteolysis releases a catalytic protease core, the approximately 50 kDa species detected in SHSY5Y and Y79 cells could represent the protease core (domain II) of autoproteolyzed CAPN5. This suggests CAPN5 may undergo a basal level of cell-line specific autoproteolysis, which could impact expression studies.

In Western blots of extracts from mouse and human retina, Ab-7 detected endogenous CAPN5 species that appeared to be the full-length protein[1,5] (Fig. 3E); the retinal extracts did not contain low molecular weight species like those found in cancer cells, suggesting calpain autoproteolytic activity is not a feature of the normal healthy retina. Interestingly, Ab-7 also recognized Tra-3, the distant *Caenorhabditis elegans* paralog of CAPN5 (Fig. 3E) despite that an alignment of Tra-3 with human CAPN5 showed less than 40% identity (Fig. 4A). Together, these findings suggest that for optimal Western analysis of CAPN5, the best antibodies were polyclonal and likely targeted conformational epitopes in all four domains.[66] To test this, a custom antibody was generated using a polypeptide comprised of sequences from all four human CAPN5 domains that were homologous to Tra-3, distinct from other retinal calpain family members, and predicted to be on the CAPN5 surface (according to our published structural model: Figs. 4B, 4C and Supplementary Fig. S2).[4,5] This recombinant antigen was expressed in *Escherichia coli*, purified, and injected into rabbits to yield polyclonal Ab-8 (Supplementary Fig. S2). Antibody Ab-8 detected CAPN5 via Western blot at levels comparable to Ab-7 (Fig. 4D). Overall, these results suggest CAPN5 antibodies raised against different regions also might show differences in vivo, an important consideration when interpreting CAPN5 expression.

CAPN5 Is Expressed in Several Regions of the Mouse Retina

The retina contains several cell types in layers of highly organized circuits. Previously, we reported CAPN5 was expressed in the inner and outer segments (IS and OS,

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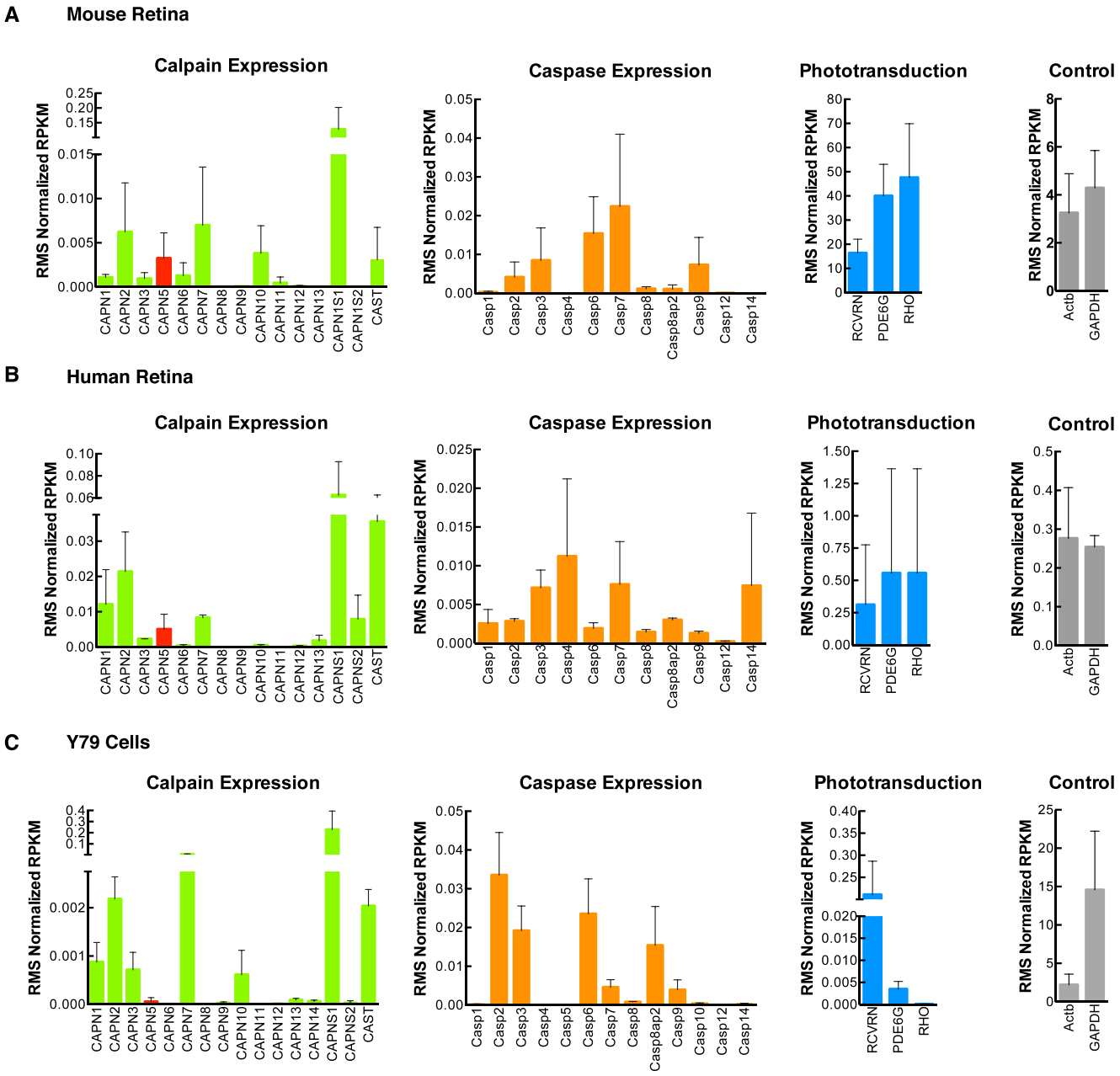
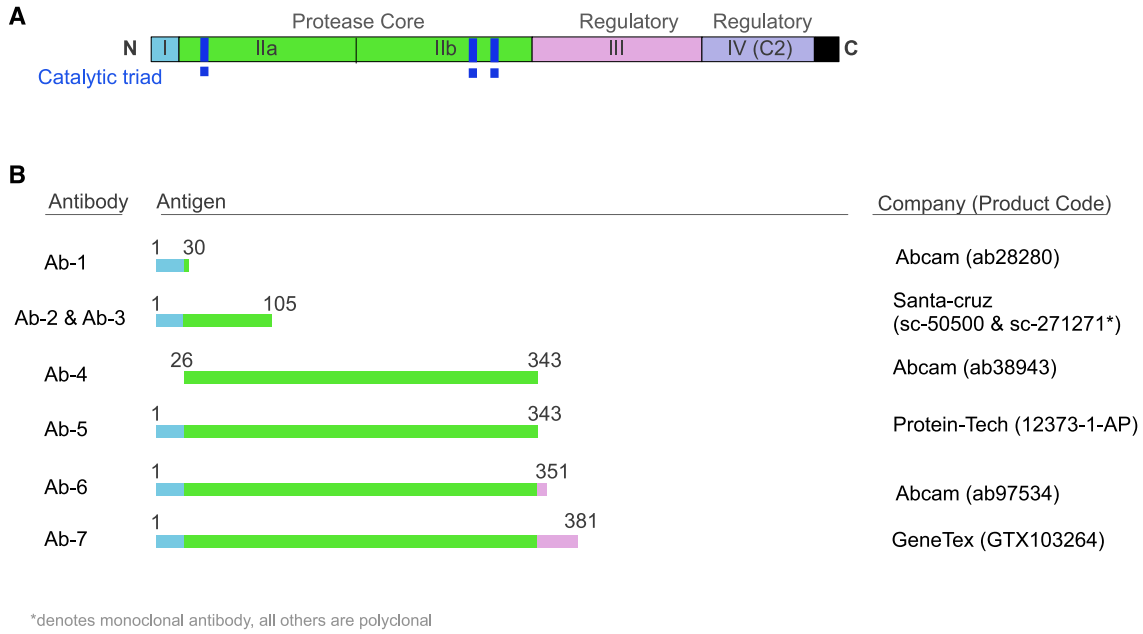


Figure 2. RNA sequence analysis of human and mouse retina. (**A**) Calpain expression in the mouse retina, as compared to levels of caspase expression, phototransduction genes, and housekeeping genes as controls ($n = 3$). Results show *CAPN5* expression is approximately half that of the most abundant calpains, *CAPN2* and *CAPN7*. Calpain expression is comparable to caspase expression in the mouse retina. Expression of *CAPN5* is several orders of magnitude less than phototransduction genes and control genes. (**B**) Slightly different patterns of expression are seen in the human retina ($n = 3$). (**C**) Calpain expression observed in Y79 cells as compared to levels of caspase expression, phototransduction genes, and controls ($n = 3$). Similar to human and mouse retina, *CAPN2* and *CAPN7* are the most abundant calpains in Y79 cells, but *CAPN5* expression is lower.

respectively) of human and mouse photoreceptors using Ab-2.[1,5] Probing the mouse retina with Ab-4, however, revealed that CAPN5 also was modestly expressed in the outer plexiform layer (OPL).[5] With these disparities, we used the newly characterized Ab-7. This labeled the photoreceptor inner segments as well as the OPL, where photoreceptors form synaptic connections with bipolar cells and others in the inner nuclear layer (INL; Fig. 5). CAPN5 colocalized with PSD-95, a protein expressed specifically in photoreceptor synapses, where a signaling defect could correlate with the electronegative ERG found in ADNIV patients (Fig. 5). Additionally CAPN5 was detected at a lower level in some, but not all retinal ganglion cells (RGC). Lower levels of CAPN5 were detected in the INL and inner plexiform layer (IPL; Fig. 5). Finding CAPN5 localized to these sites suggests a role in synaptic transmission.

CAPN5 Localizes to Photoreceptor Synapses

To explore CAPN5 subcellular localization, retinal extracts were fractionated and analyzed by Western blot (Fig. 6). Rod outer segments were first isolated by sucrose flotation. CAPN5 was detected in the crude ROS fraction. The remaining retinal proteins (ROR) were separated into soluble (ROR-S1) and membrane (ROR-P1) fractions. CAPN5 was detected largely in



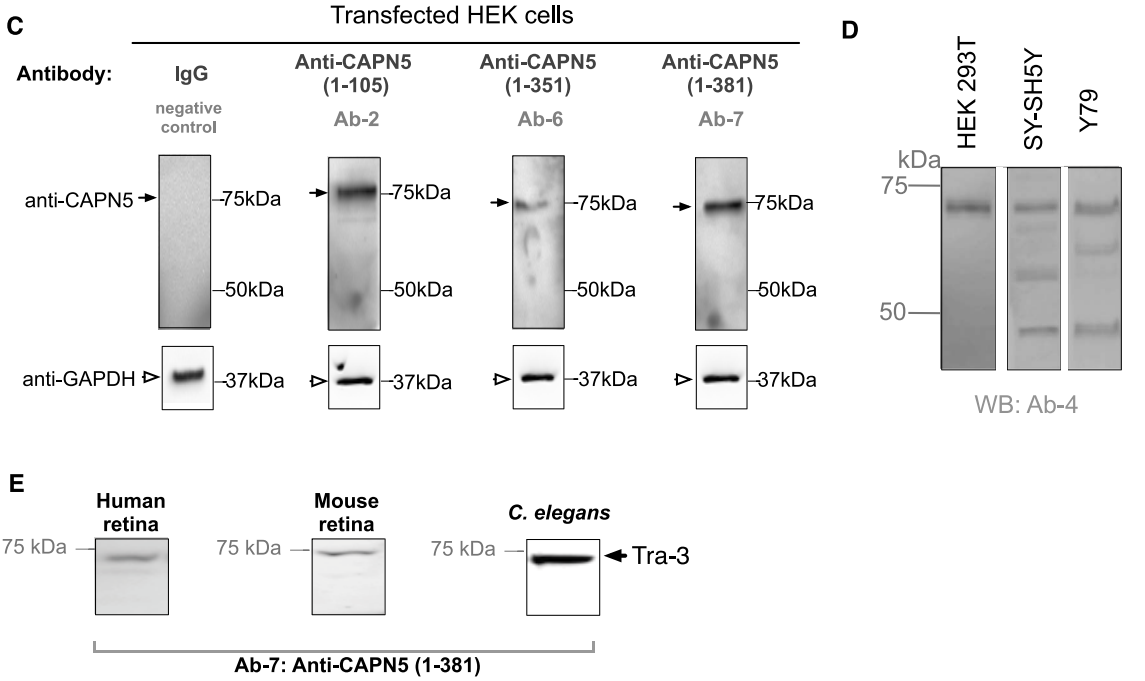


Figure 3. CAPN5 Western blot shows specificity of multiple CAPN5 antibodies. (**A**) Schematic diagram of human CAPN5 protein domain structure. CAPN5 is composed of 640 amino acids that comprise four domains (**A**). Domain-I spans amino acid residues 1 to 25. Domain-II is the protease core and spans amino acid residues 26 to 343; it consists of two subdomains that together contain the catalytic triad and calcium binding sites. Domain II also contains the three different ADNIV-causing mutations.[3] Domain-III spans amino acid residues 344 to 496 and contains a calcium-binding site leading to speculation that it is a regulatory region. Domain-IV (C2) also is believed to be regulatory; it spans amino acid residues 518 to 619, and contains three calcium-binding sites. (**B**) A diagram showing the epitopes of the seven different commercially available anti-CAPN5 antibodies tested, as well as the companies they were obtained from. Antibody Ab-1 (ab28280) mainly targeted domain I; Ab-2 (sc-50500) and Ab-3 (sc271271) targeted all of domain I, and part of domain II (catalytic domain); Ab-4 (ab38943) targeted the entire domain II and part of domain III; Ab-5 (12373-1-AP) and Ab-6 (ab97534) targeted domains I, II, and part of III. All antibodies were rabbit polyclonal antibodies, except for Ab-3, which was a mouse monoclonal antibody. (**C**) Immunoblots of HEK-293 cells transfected with full-length *CAPN5* (tagged with c-myc and flag tag) and probed separately with three unique anti-CAPN5 antibodies. Anti-GAPDH antibody was used as a protein loading control. Immunoglobulin G (IgG) was used as a negative control. The *closed arrows* indicate CAPN5 bands at approximately 75 kDa, while the *open arrowheads* indicate GAPDH bands at approximately 37 kDa. Immunoblot images were captured with an ECL imager. Antibody Ab-7 consistently gave the best bands with the least amount of background, indicating higher specificity for CAPN5. (**D**) Immunoblots of HEK-293, SY-SH5Y, and Y79 cells transfected with full length *CAPN5* and probed with Ab-4. (**E**) Immunoblots of retinas belonging to human and mice were probed separately with CAPN5 specific Ab-7. Immunoblot of protein obtained from wildtype (N2) *C. elegans*. 10 µg of protein were loaded.

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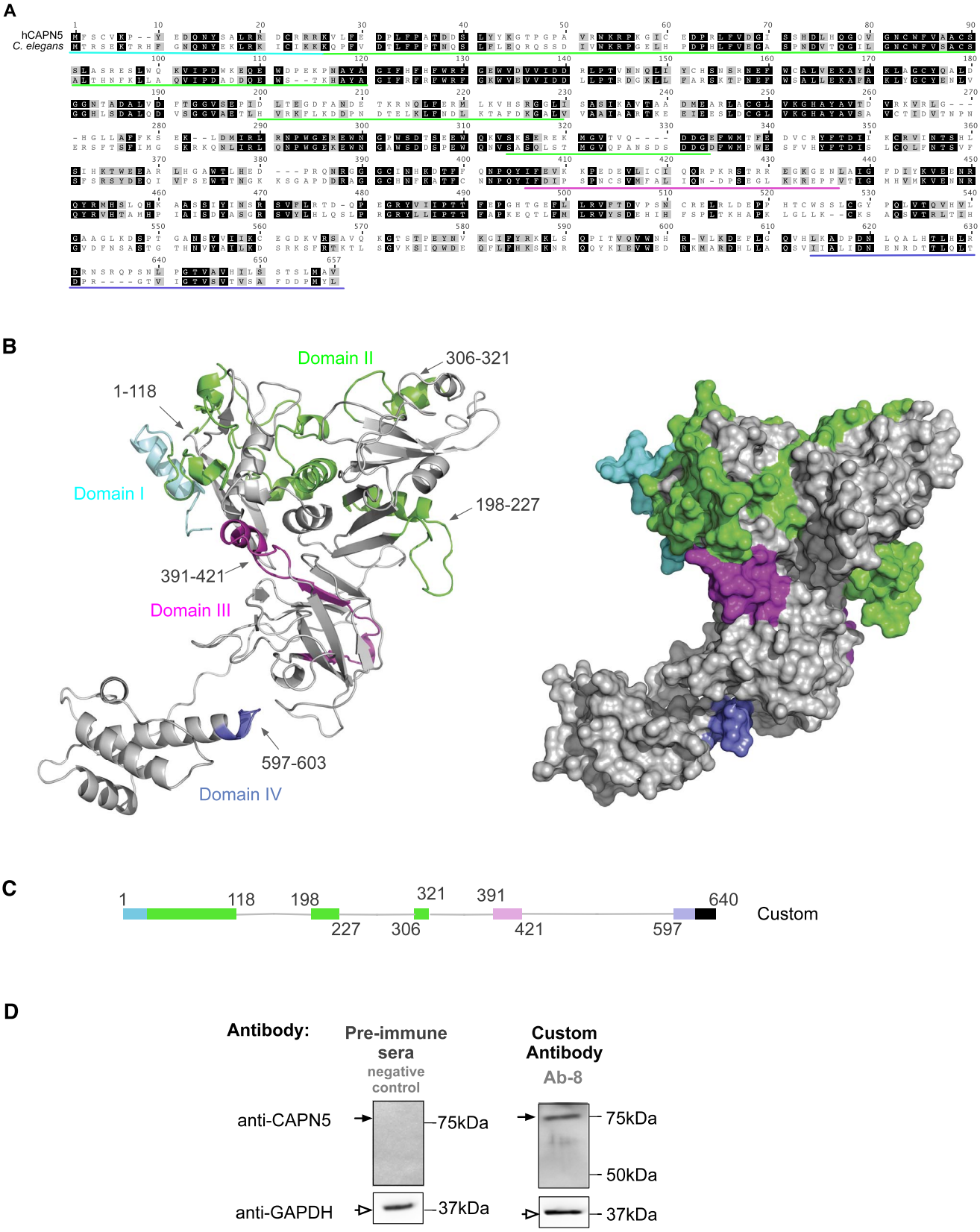

Figure 4. Customized antigen raises highly specific CAPN5 antibodies. (**A**) Alignment of *CAPN5* and *Tra-3* sequences. Regions of homology are shown in *black*. Regions corresponding to the customized CAPN5 antigen are underlined with colors corresponding to the different CAPN5 domains. (**B**) A structural model of CAPN5. The *highlighted regions* correspond to those recognized by our custom antibody. (**C**) A diagram showing the epitopes of our custom anti-CAPN5 antibody. (**D**) Immunoblots of HEK-293 cells transfected with full-length *CAPN5* and probed with our custom anti-CAPN5 antibody. We used GAPDH as a protein loading control. Preimmune-serum obtained from the same animal was used as a negative control. The *closed arrows* indicate CAPN5 bands at approximately 75 kDa, while the *open arrowheads* indicate GAPDH bands at approximately 37 kDa. Immunoblot images were captured with an ECL imager.

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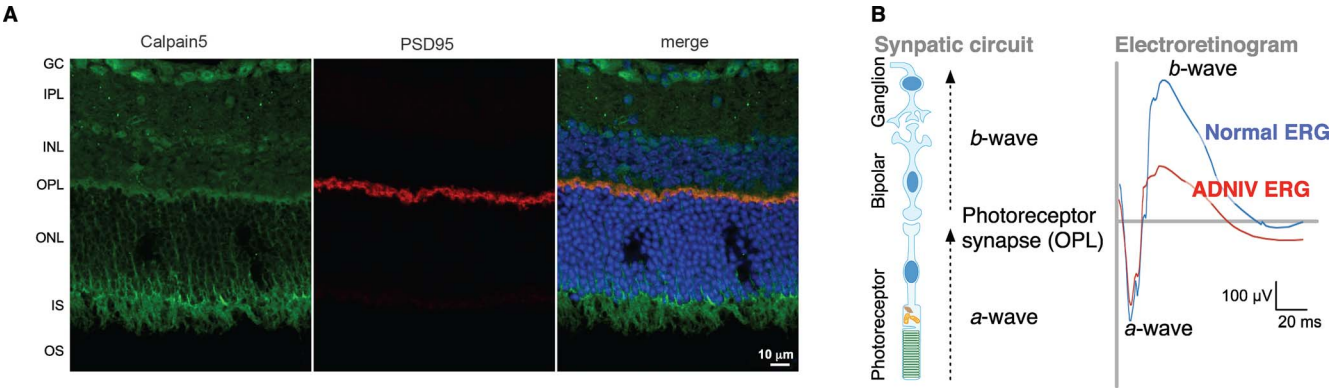

Figure 5. CAPN5 Immunohistochemistry in the retina synapse. (**A**) CAPN5 expression was present along a region corresponding to the OPL of the retina, where photoreceptors form synaptic connections with bipolar cells. PSD95 expression, a marker of neural postsynaptic densities, colocalized with CAPN5 expression along the synapses forming the outer plexiform layer. (**B**) The retinal synaptic circuit begins with light depolarization (a-wave) at the photoreceptor. Synaptic transmission from the photoreceptor to the bipolar cell and then to the RGC generates the hyperpolarized b-wave. This is shown in human ERG that trace the a- and b-waves in an ADNIV patient and a wildtype individual. Early in ADNIV patients and transgenic mouse models, the a-wave is intact but the b-wave is reduced (electronegative), indicating a defect in synaptic signaling between the photoreceptor and bipolar cell. The unusual phenotype is explained by CAPN5 localization to this synaptic junction. *Scale bar*: 10 μm. *Blue* = DAPI; *Red* = PSD95; *green* = CAPN5.

the soluble pool (Fig. 6). Interestingly, CAPN5 also was detected in the membrane fraction that is enriched for synaptosomes (Syn-Soluble, Fig. 6). This analysis was consistent with immunohistochemical localization of CAPN5 to photoreceptor synapses. Expression at these synapses explains a defining feature of ADNIV, loss of the electroretinogram b-wave (see Discussion).

Subcellular Localization of CAPN5 in the Rat Brain

The compartmentalization of CAPN5 in the retina suggests that CAPN5 function might be regulated by subcellular localization. To examine localization further, we used rodent brain cells as a source of material, since the brain expresses more CAPN5 than the retina, and brain tissue is far more abundant.[50] Brain extracts were fractionated and probed with CAPN5 Ab-1, -2, and -7. In parallel, fractions also were probed with a panel of antibodies that marked various subcellular compartments (Fig. 7).

As seen in cultured neural cells, the brain expressed multiple species of CAPN5 immunoreactivity, potentially reflecting autoproteolysis. Comparing expression patterns on Western blots, as detected by Ab-1, -2, and -7 (all polyclonal), we noticed each antibody gave a distinct pattern. Antibody Ab-1, which targets the 30 N-terminal amino acids, primarily recognized the smaller, more processed forms of CAPN5. Antibody Ab-2, which targets Domain I and part of Domain II, appeared to recognize both full-length and proteolytically processed forms, but the electrophoretic mobility of the high molecular weight forms were the same as those detected by Ab-1. Antibody Ab-7, which targets full-length CAPN5, recognized few approximately 50 kD species, preferring the full-length form. Thus, the presence of smaller species could indicate cellular compartments with greater CAPN5 proteolytic activity or the accumulation of the autoproteolyzed CAPN5 proteolytic core (~50 kD).

The patterns on Western blots suggest the different antibodies preferentially recognized different forms of CAPN5. The banding patterns indicates Ab-2 recognized the full-length and proteolytically processed forms of CAPN5, while Ab-1 and Ab-7 preferentially recognized the processed and full-length forms, respectively. The blots show the 50 kD form was enriched for in a crude synaptosomal/mitochondrial fraction (syn-mt).[67,68] The link between CAPN5 and the synapse and mitochondria has interesting implications, since mitochondria are highly enriched in the synapse due to energy needs associated with neurotransmitter loading and release.[69] Moreover, synapses and mitochondria are rich in calcium stores, which might activate CAPN5 and trigger autoproteolysis.

Discussion

The calpains are calcium-dependent, intracellular, cysteine proteases[70,71] that proteolytically process targets into new functional forms. Calpains are regulatory proteases and have been linked to a broad spectrum of cellular processes, including cell death, cell movement, sex determination, and intracellular signaling.[14,15] Excess, uncontrolled calpain activity is implicated in cancer pathogenesis,[72] muscular dystrophy,[73] diabetes,[74] and a number of neurologic diseases, such as multiple sclerosis,[75] Alzheimer's disease,[76,77] Huntington's disease,[78] and traumatic brain injury.[71,79] Compared to the rest of the calpain family, CAPN5 is especially interesting because it is the only known calpain with a human disease mutation that renders the protease intrinsically hyperactive.

Autosomal dominant neovascular inflammatory vitreoretinopathy phenotypically overlaps with several other eye diseases. For example, electronegative ERGs also are observed in retinoschisis, quinine toxicity, and congenital stationary night blindness (CSNB). Interestingly, CSNB and retinal degeneration in the *rd1* mouse are associated with excess calpain activity.[80] Additionally, the proliferative retinal neovascularization in ADNIV patients has been confused with diabetic retinopathy, but ADNIV patients are not diabetic and do not show retinal capillary nonperfusion or other signs of retinal or ocular ischemia. Instead, the proliferative changes are likely driven by uveitic inflammatory signals. It is interesting to speculate that calpains might be active in shared downstream pathways.

The expression of CAPN5 varies in abundance and in the appearance of proteolytically processed forms. Since our custom polyclonal CAPN5 antibody was raised to domains unique to CAPN5 that represented surface epitopes across the entire protein, it would be expected to recognize all forms of CAPN5 and all fragments that might be generated by autoproteolysis. Indeed, when interpreting immunohistochemical studies of calpains and their proteolytic targets, it is important to note that the various proteolyzed (or autoproteo-

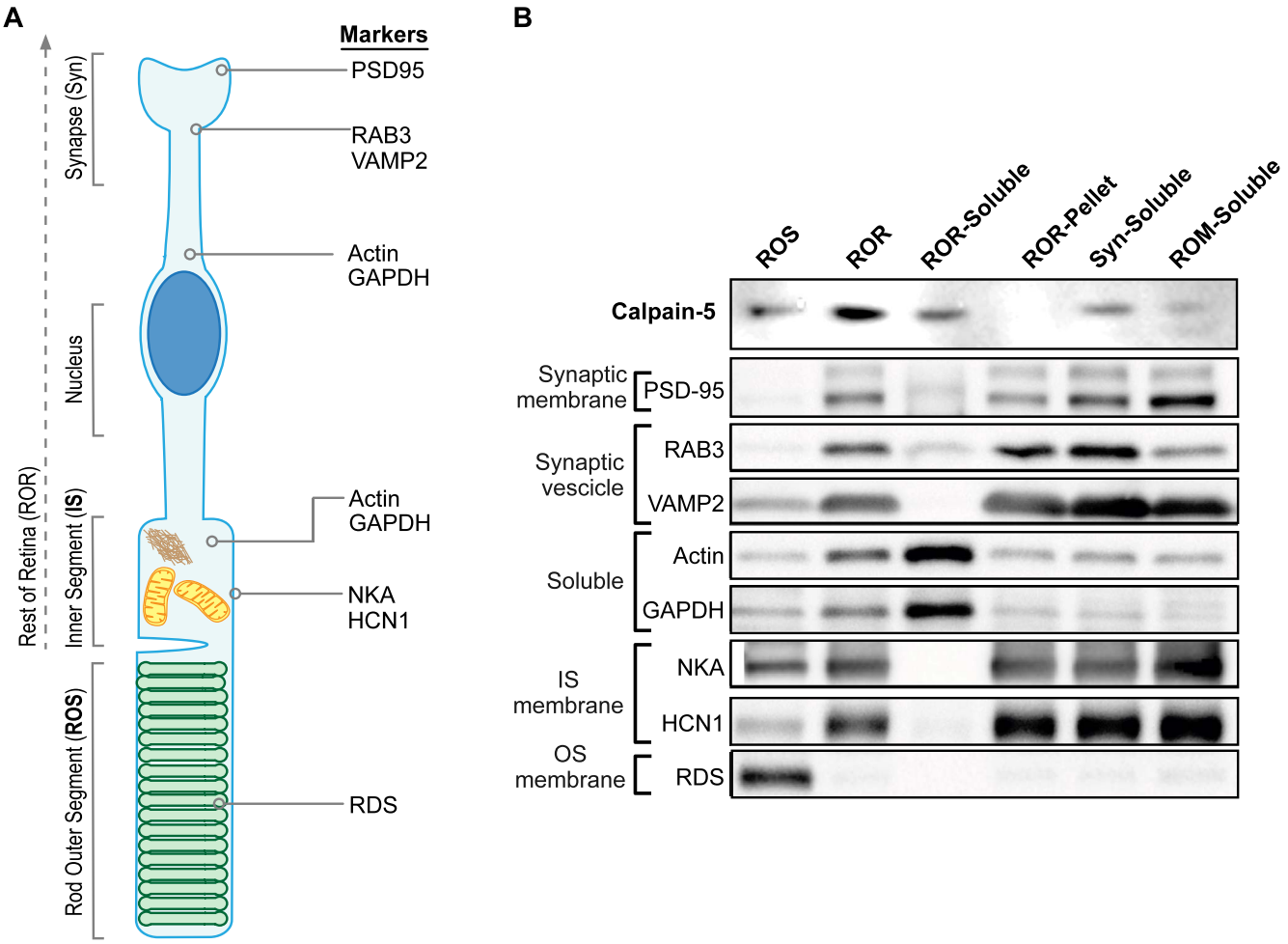

Figure 6. CAPN5 co-fractionates with both soluble and synaptic vesicle proteins. (**A**) A schematic illustration of a photoreceptor cell maps the location of subcellular marker proteins. (**B**) Antibodies against CAPN5 were used to probe retinal protein fractions alongside protein markers for specific retinal compartments. CAPN5 was detected in the ROS, but most of it remained in the ROR fraction. After the ROR was fractionated to enrich for soluble (ROR-S1), membrane (ROR-P1), and synaptic (Syn-S2) proteins, CAPN5 was found in the soluble and synaptic vesicle pools (along with Actin and GAPDH, or RAB3 and VAMP2, respectively). NKA, sodium potassium ATPase; HCN1, hyperpolarization-activated and cyclic nucleotide-gated channel, family member 1.

lyzed) forms would be expected to have different surface epitopes. The different patterns seen with different antibodies might reflect unique autoproteolytic domains of CAPN5, like that seen in the subcellular fractionation experiments.

In the retina, an examination by IHC using different antibodies revealed patterns of CAPN5 subcellular localization. In photoreceptors, for example, CAPN5 is in the outer segment,[1,5] where light is converted into cellular signals; the nucleus, where transcription takes place; and the synapse, which transmits signals to the inner retina. Newly available antibodies revealed expression in the inner nuclear layer and ganglion cell layer. In ADNIV patients, however, neither the RGCs nor the optic nerve degenerate. Instead, similar to patients with retinitis pigmentosa, ADNIV patients lose peripheral vision coincident with degeneration of their peripheral photoreceptors. Nevertheless, electrophysiologic testing of the optic nerve with pattern ERG (PERG), for example, may be worthwhile, since PERG can assess macular ganglion cell function. In addition, future immunohistochemistry studies using RGC markers might identify the subtypes of RGC cells expressing CAPN5.

Calpain proteolysis is linked with synaptic function.[81] Since the known calpain substrates include cytoskeletal proteins, membrane receptors, and postsynaptic-density proteins, calpain proteolysis of these substrates might be a feature of normal synaptic function.[81] Pharmacologic inhibition shows calpains have key roles in many neuronal processes including neurotransmitter release and signal transduction.[81] The high expression of CAPN5 we observed in the photoreceptor synaptic layer (OPL) and mitochondria supports a role for CAPN5 at synapses. This is interesting because it may explain the development of early ADNIV electronegative ERG where loss of the b-wave is a sign of defective inner retina neurotransmission.

The genomic survey was designed to help uncover why ADNIV *CAPN5* alleles cause retinal disease, and whether such alleles cause other diseases linked to high calpain activity. Our PolyPhen predictions of *CAPN5* variants in genome databases suggest damaging alleles arise at a very low frequency, implying they are highly detrimental. More specifically, we found very few protease-core variants, which are likely to be more damaging than variants in the N-terminal or C-terminal regions. Increased calpain activity has been associated with cancer and epilepsy, but we found few *CAPN5* mutations, suggesting that in those diseases calpain activity is elevated for reasons other than calpain mutations while the current *CAPN5* variants only display retinal disease.

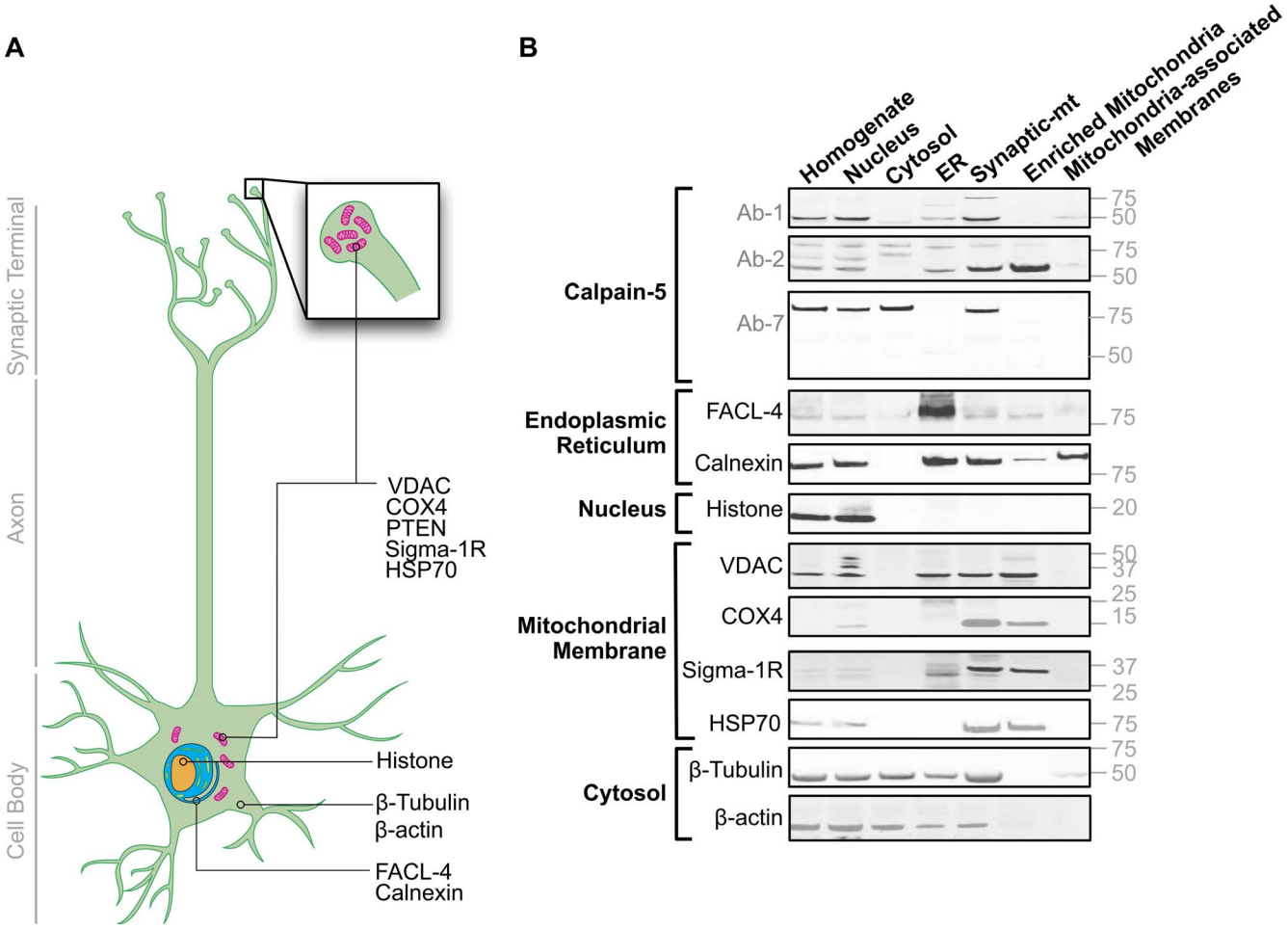

Figure 7. CAPN5 co-fractionates with specific cellular compartments. (**A**) A schematic illustration of a neuron maps the location of subcellular marker proteins. (**B**) Antibodies against CAPN5 were used to probe neuronal protein fractions alongside protein markers for specific cellular compartments. CAPN5 was detected in all compartments probed, depending on the antibody used. Most striking is the high levels of CAPN5 found in mitochondrial fractions, and the crude synaptosomal/mitochondrial fraction.

Even though we found calpain mRNA expression patterns were similar between mouse and humans, our previously reported ADNIV mice[3] had a less severe form of the disease; this may happen for several reasons. For example, in one mouse model CAPN5 was expressed from a retroviral vector, which gave a transient and patchy expression pattern in the host retina.[3] In a second, transgenic mouse model, the ADNIV phenotype might be ameliorated by the presence of two wild-type alleles.[5] In both models, expression is restricted to photoreceptors and not other retinal cells where CAPN5 is natively expressed. It also is possible that the short mouse lifespan ends before the disease can develop into full-blown ADNIV, since humans do not typically display severe disease until their fourth decade or later. To distinguish the possibilities from species differences, we will have to generate knock-in mice that replace the normal alleles with disease alleles under the native promoter.

Although ADNIV only affects the eye, *CAPN5* mRNA was expressed at only slightly above average levels in the retina and at far higher levels in the colon. Nevertheless, to date no ADNIV patients have gastrointestinal disorders. These data support the idea that CAPN5 is uniquely regulated and/or serving a highly specialized role in the retina. For example, ADNIV *CAPN5* mutations might specifically cause a retinal phenotype because photoreceptors use extremely high calcium levels for phototransduction. High calcium could not only activate CAPN5 transiently, but render it permanently hyperactive by autoproteolytically cleaving out an active fragment that is free of regulatory domains.[1] Alternatively, in the eye, the uniquely slow turnover rate of cells might allow retinal damage to accumulate and become more severe than in a tissue with a faster turnover rate (e.g., the digestive tract). Regardless of the mechanism that restricts the clinical disease to eye tissue, the subcelluar localization of CAPN5 indicates it likely targets several different proteins with nonoverlapping functions. This would account for the complex ocular phenotype. Thus, the next important step for understanding ADNIV and devising new therapies will be to identify the proteolytic targets of CAPN5.

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References

1. Mahajan VB, Skeie JM, Bassuk AG, et al. Calpain-5 mutations cause autoimmune uveitis, retinal neovascularization, and photoreceptor degeneration. *PLoS Genet*. 2012;8:e1003001.
2. Mahajan VB, Lin JH. Lymphocyte infiltration in CAPN5 autosomal dominant neovascular inflammatory vitreoretinopathy. *Clin Ophthalmol*. 2013;7:1339–1345.
3. Wert KJ, Skeie JM, Bassuk AG, Olivier AK, Tsang SH, Mahajan VB. Functional validation of a human CAPN5 exome variant by lentiviral transduction into mouse retina. *Hum Mol Genet*. 2014;23:2665–2677.
4. Bassuk AG, Yeh S, Wu S, et al. Structural modeling of a novel CAPN5 mutation that causes uveitis and neovascular retinal detachment. *PLoS One*. 2015;10:e0122352.
5. Wert KJ, Bassuk AG, Wu WH, et al. CAPN5 mutation in hereditary uveitis: the R243L mutation increases calpain catalytic activity and triggers intraocular inflammation in a mouse model. *Hum Mol Genet*. 2015;24:4584–4598.
6. Dear TN, Boehm T. Diverse mRNA expression patterns of the mouse calpain genes Capn5, Capn6 and Capn11 during development. *Mech Dev*. 1999;89:201–209.
7. Matena K, Boehm T, Dear N. Genomic organization of mouse Capn5 and Capn6 genes confirms that they are a distinct calpain subfamily. *Genomics*. 1998;48:117–120.
8. Azuma M, Shearer TR. The role of calcium-activated protease calpain in experimental retinal pathology. *Surv Ophthalmol*. 2008;53:150–163.
9. Imai S, Shimazawa M, Nakanishi T, Tsuruma K, Hara H. Calpain inhibitor protects cells against light-induced retinal degeneration. *J Pharmacol Exp Ther*. 2010;335:645–652.
10. Tamada Y, Nakajima E, Nakajima T, Shearer TR, Azuma M. Proteolysis of neuronal cytoskeletal proteins by calpain contributes to rat retinal cell death induced by hypoxia. *Brain Res*. 2005;1050:148–155.
11. Hoang MV, Smith LE, Senger DR. Calpain inhibitors reduce retinal hypoxia in ischemic retinopathy by improving neovascular architecture and functional perfusion. *Biochim Biophys Acta*. 2011;1812:549–557.
12. Nakajima E, Hammond KB, Rosales JL, Shearer TR, Azuma M. Calpain, not caspase, is the causative protease for hypoxic damage in cultured monkey retinal cells. *Invest Ophthalmol Vis Sci*. 2011;52:7059–7067.
13. Azuma M, Hammond KB, Nakajima E, Shearer TR. Calpain protease causes hypoxia-induced proteolysis in cultured human retina. *Curr Eye Res*. 2014;39:421–424.
14. Nakajima E, David LL, Bystrom C, Shearer TR, Azuma M. Calpain-specific proteolysis in primate retina: contribution of calpains in cell death. *Invest Ophthalmol Vis Sci*. 2006;47:5469–5475.
15. Kaur J, Mencl S, Sahaboglu A, et al. Calpain and PARP activation during photoreceptor cell death in P23H and S334ter rhodopsin mutant rats. *PLoS One*. 2011;6:e22181.
16. Shinde VM, Sizova OS, Lin JH, LaVail MM, Gorbatyuk MS. ER stress in retinal degeneration in S334ter Rho rats. *PLoS One*. 2012;7:e33266.
17. Chinskey ND, Zheng QD, Zacks DN. Control of photoreceptor autophagy after retinal detachment: the switch from survival to death. *Invest Ophthalmol Vis Sci*. 2014;55:688–695.
18. Huang W, Fileta J, Rawe I, Qu J, Grosskreutz CL. Calpain activation in experimental glaucoma. *Invest Ophthalmol Vis Sci*. 2010;51:3049–3054.
19. Ryu M, Yasuda M, Shi D, et al. Critical role of calpain in axonal damage-induced retinal ganglion cell death. *J Neurosci Res*. 2012;90:802–815.
20. Tamada Y, Fukiage C, Daibo S, Yoshida Y, Azuma M, Shearer TR. Involvement of calpain in hypoxia-induced damage in rat retina in vitro. *Comp Biochem Physiol B Biochem Mol Biol*. 2002;131:221–225.
21. Shirasaki Y, Nakamura M, Yamaguchi M, Miyashita H, Sakai O, Inoue J. Exploration of orally available calpain inhibitors 2: peptidyl hemiacetal derivatives. *J Med Chem*. 2006;49:3926–3932.
22. Sharma AK, Rohrer B. Sustained elevation of intracellular cGMP causes oxidative stress triggering calpain-mediated apoptosis in photoreceptor degeneration. *Curr Eye Res*. 2007;32:259–269.
23. Yoshimura N, Tsukahara I, Murachi T. Calpain and calpastatin in porcine retina. Identification and action on microtubule-associated proteins. *Biochem J*. 1984;223:47–51.
24. Ma H, Fukiage C, Kim YH, et al. Characterization and expression of calpain 10. A novel ubiquitous calpain with nuclear localization. *J Biol Chem*. 2001;276:28525–28531.
25. Nakajima T, Fukiage C, Azuma M, Ma H, Shearer TR. Different expression patterns for ubiquitous calpains and Capn3 splice variants in monkey ocular tissues. *Biochim Biophys Acta*. 2001;1519:55–64.
26. Zatz M, Starling A. Calpains and disease. *N Engl J Med*. 2005;352:2413–2423.
27. Dear N, Matena K, Vingron M, Boehm T. A new subfamily of vertebrate calpains lacking a calmodulin-like domain: implications for calpain regulation and evolution. *Genomics*. 1997;45:175–184.
28. Bennett SR, Folk JC, Kimura AE, Russell SR, Stone EM, Raphtis EM. Autosomal dominant neovascular inflammatory vitreoretinopathy. *Ophthalmology*. 1990;97:1125–1135, discussion 1135–1136.
29. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res*. 2010;20:1297–1303.
30. Cingolani P, Platts A, Wang le L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly (Austin)*. 2012;6:80–92.
31. EpiK Consortium, Epilepsy Phenome/Genome Project, Allen AS, et al. De novo mutations in epileptic encephalopathies. *Nature*. 2013;501:217–221.
32. EuroEPINOMICS-RES Consortium, Epilepsy Phenome/Genome Project, Epi4K Consortium. De novo mutations in synaptic transmission genes including DNM1 cause epileptic encephalopathies. *Am J Hum Genet*. 2014;95:360–370.
33. Pan Y, Bhattarai S, Modestou M, Drack AV, Chetkovich DM, Baker SA. TRIP8b is required for maximal expression of HCN1 in the mouse retina. *PLoS One*. 2014;9:e85850.
34. Bononi A, Pinton P. Study of PTEN subcellular localization. *Methods*. 2015;77-78:92–103.
35. Eswar N, John B, Mirkovic N, et al. Tools for comparative protein structure modeling and analysis. *Nucleic Acids Res*. 2003;31:3375–3380.
36. Eswar N, Webb B, Marti-Renom MA, et al. Comparative protein structure modeling using MODELLER. *Curr Protoc Protein Sci*. 2007;Chapter 2:Unit 2.9.

37. *PyMOL*. New York: Schrödinger; 2014 [cited 2015 Feb. 11]. Available from: http://www.pymol.org/.
38. The 1000 Genomes Project Consortium. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012; 491:56–65.
39. The 1000 Genomes Project Consortium. *1000 Genomes: A Deppt Catalog of Human Genetic Variation*. Bethesda, MD: National Human Genome Research Institute (NHGRI); 2008 [updated Sept 30, 2015]. Available from: http://www.1000genomes.org/.
40. NHLBI Exome Sequencing Project (ESP). *Exome Variant Server*. Seattle, WA: NHLBI GO Exome Sequencing Project (ESP) [updated May 14, 2015; cited 2015 Oct. 12]. Available from: http://evs.gs.washington.edu/EVS/.
41. Storr SJ, Thompson N, Pu X, Zhang Y, Martin SG. Calpain in breast cancer: role in disease progression and treatment response. *Pathobiology*. 2015;82:133–141.
42. Supiot S, Gouraud W, Campion L, et al. Early dynamic transcriptomic changes during preoperative radiotherapy in patients with rectal cancer: a feasibility study. *World J Gastroenterol*. 2013;19:3249–3254.
43. COSMIC: Catalogue of Somatic Mutations in Cancer. Hinxton, Cambridge, UK: Wellcome Trust Sanger Institute; 2004 [cited 2015 Oct 16]. Available from: http://cancer.sanger.ac.uk/cosmic.
44. Bamford S, Dawson E, Forbes S, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer*. 2004;91:355–358.
45. Tsung PK, Lombardini JB. Identification of low-Ca2+- and high-Ca2+-requiring neutral proteases in rat retina. *Exp Eye Res*. 1985;41:97–103.
46. Azarian SM, Schlamp CL, Williams DS. Characterization of calpain II in the retina and photoreceptor outer segments. *J Cell Sci*. 1993;105:787–798.
47. Azuma M, Fukiage C, Higashine M, Nakajima T, Ma H, Shearer TR. Identification and characterization of a retina-specific calpain (Rt88) from rat. *Curr Eye Res*. 2000;21:710–720.
48. Horikawa Y, Oda N, Cox NJ, et al. Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat Genet*. 2000;26:163–175.
49. Azuma M, Sakamoto-Mizutani K, Nakajima T, Kanaami-Daibo S, Tamada Y, Shearer TR. Involvement of calpain isoforms in retinal degeneration in WBN/Kob rats. *Comp Med*. 2004;54: 533–542.
50. Singh R, Brewer MK, Mashburn CB, et al. Calpain 5 is highly expressed in the central nervous system (CNS), carries dual nuclear localization signals, and is associated with nuclear promyelocytic leukemia protein bodies. *J Biol Chem*. 2014; 289:19383–19394.
51. Araujo IM, Gil JM, Carreira BP, et al. Calpain activation is involved in early caspase-independent neurodegeneration in the hippocampus following status epilepticus. *J Neurochem*. 2008;105:666–676.
52. Lopez-Meraz ML, Niquet J. Participation of mu-calpain in status epilepticus-induced hippocampal injury. *Brain Res Bull*. 2009; 78:131.
53. Tsao CY, Mendell JR. Partial epilepsy in an adolescent male with limb-girdle muscular dystrophy 1B. *J Child Neurol*. 2009; 24:346–348.
54. Xu J, Kurup P, Zhang Y, et al. Extrasynaptic NMDA receptors couple preferentially to excitotoxicity via calpain-mediated cleavage of STEP. *J Neurosci*. 2009;29:9330–9343.
55. Stankewich MC, Gwynn B, Ardito T, et al. Targeted deletion of betaIII spectrin impairs synaptogenesis and generates ataxic and seizure phenotypes. *Proc Natl Acad Sci U S A*. 2010;107: 6022–6027.
56. Feng ZH, Hao J, Ye L, et al. Overexpression of mu-calpain in the anterior temporal neocortex of patients with intractable epilepsy correlates with clinicopathological characteristics. *Seizure*. 2011;20:395–401.
57. Gao H, Geng Z. Calpain I activity and its relationship with hippocampal neuronal death in pilocarpine-induced status epilepticus rat model. *Cell Biochem Biophys*. 2013;66:371–377.
58. *BioGPS*. La Jolla, CA: The Scripps Research Institute; 2015 [cited 2015 Oct. 12]. Available from: http://biogps.org/.
59. Wu C, Orozco C, Boyer J, et al. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol*. 2009;10:R130.
60. The European Bioinformatics Institute. *EML-EBI*. Hinxton, Cambridgeshire, UK: European Molecular Biology Laboratory; 2015 [cited 2015 Oct. 2]. Available from: https://www.ebi.ac.uk/.
61. Campbell RL, Davies PL. Structure-function relationships in calpains. *Biochem J*. 2012;447:335–351.
62. Schultz DR, Harrington WJ Jr. Apoptosis: programmed cell death at a molecular level. *Semin Arthritis Rheum*. 2003;32: 345–369.
63. Fan TJ, Han LH, Cong RS, Liang J. Caspase family proteases and apoptosis. *Acta Biochim Biophys Sin (Shanghai)*. 2005;37: 719–727.
64. Uhlen M, Bjorling E, Agaton C, et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. *Mol Cell Proteomics*. 2005;4:1920–1932.
65. Uhlen M, Fagerberg L, Hallstrom BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. 2015;347: 1260419.
66. Huang J, Honda W. CED: a conformational epitope database. *BMC Immunol*. 2006;7:7.
67. Morgan IG. Synaptosomes and cell separation. *Neuroscience*. 1976;1:159–165.
68. Smith AD, Llinás R, Kostyuk PG. *Commentaries in the Neurosciences*. Oxford: Pergamon Press; 1980.
69. Picard M. Mitochondrial synapses: intracellular communication and signal integration. *Trends Neurosci*. 2015;38:468–474.
70. Hanna RA, Campbell RL, Davies PL. Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. *Nature*. 2008;456:409–412.
71. Yang J, Weimer RM, Kallop D, et al. Regulation of axon degeneration after injury and in development by the endogenous calpain inhibitor calpastatin. *Neuron*. 2013;80: 1175–1189.
72. Storr SJ, Carragher NO, Frame MC, Parr T, Martin SG. The calpain system and cancer. *Nat Rev Cancer*. 2011;11:364–374.
73. Richard I, Broux O, Allamand V, et al. Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell*. 1995;81:27–40.
74. Buraczynska M, Wacinski P, Stec A, Kuczmaszewska A. Calpain-10 gene polymorphisms in type 2 diabetes and its micro- and macrovascular complications. *J Diabetes Complications*. 2013;27:54–58.
75. Das A, Guyton MK, Butler JT, Ray SK, Banik NL. Activation of calpain and caspase pathways in demyelination and neurodegeneration in animal model of multiple sclerosis. *CNS Neurol Disord Drug Targets*. 2008;7:313–320.
76. Getz GS. Calpain inhibition as a potential treatment of Alzheimer's disease. *Am J Pathol*. 2012;181:388–391.

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77. Higuchi M, Iwata N, Matsuba Y, et al. Mechanistic involvement of the calpain-calpastatin system in Alzheimer neuropathology. *FASEB J*. 2012;26:1204–1217.
78. Landles C, Sathasivam K, Weiss A, et al. Proteolysis of mutant huntingtin produces an exon 1 fragment that accumulates as an aggregated protein in neuronal nuclei in Huntington disease. *J Biol Chem*. 2010;285:8808–8823.
79. Liu S, Yin F, Zhang J, Qian Y. The role of calpains in traumatic brain injury. *Brain Inj*. 2014;28:133–137.
80. Paquet-Durand F, Azadi S, Hauck SM, Ueffing M, van Veen T, Ekstrom P. Calpain is activated in degenerating photoreceptors in the rd1 mouse. *J Neurochem*. 2006;96:802–814.
81. Wu HY, Lynch DR. Calpain and synaptic function. *Mol Neurobiol*. 2006;33:215–236.

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