Supplemental Information

Calpains as mechanistic drivers and therapeutic targets for ocular diseases

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Supplemental Figures

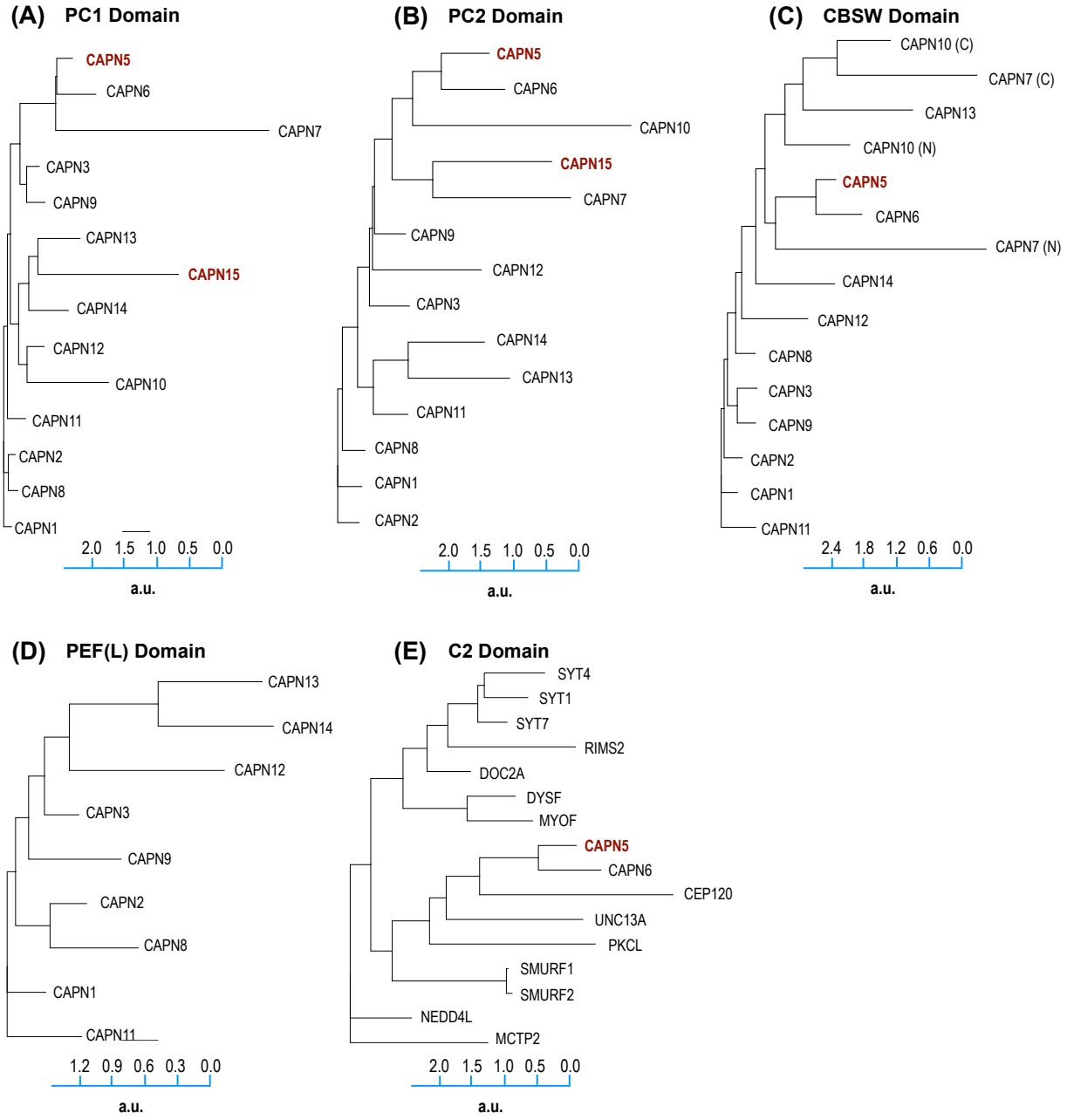

Figure S1. Sequence-based phylogenetic tree of human calpain domains (related to Figure 2).
Sequences representing individual human calpain domains (i.e., PC1, PC2, CBSW, PEF, and C2) underwent multiple sequence alignment (MSA) using MAFFT v7 (alignment strategy: FFT-NS-1). The MSA subsequently underwent phylogenetic reconstruction in IQ-TREE-1.6.2. The reconstructed human calpain domain (and C2-domain containing proteins, panel E) trees are shown: **(A)** PC1, **(B)** PC2, **(C)** CBSW, **(D)** PEF(L), **(E)** C2 demonstrating differences in clustering between one- and three-dimensional comparisons. Calpains in red text indicate that they are involved in ocular calpainopathies.

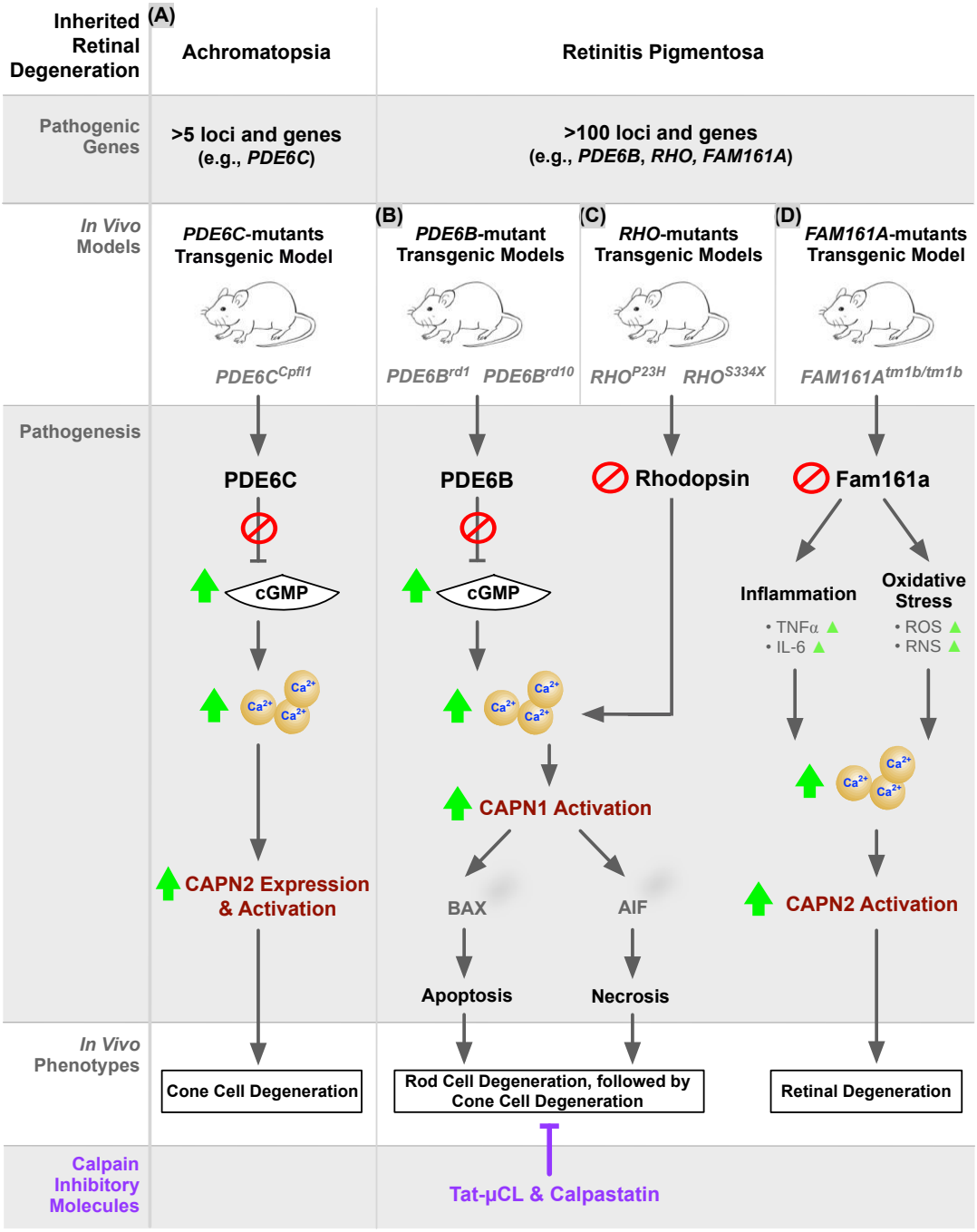

Figure S2. Pathology of calpain-mediated inherited retinal degenerations in in vivo models. Pathological mutations in **(A)** achromatopsia and **(B-D)** retinitis pigmentosa (RP) lead to calpain activation [1-3]. *PDE6C* mutations lead to cone cell death through increased CAPN2 expression and activation [1,4,5] while mutations in **(B)** *PDE6B* lead to rod cell death via increased CAPN1 activity [6,7]. **(C)** Rhodopsin (encoded by *RHO*) is a G-protein coupled receptor expressed on rod cells that is instrumental in conducting visual phototransduction. **(D)** Fam161a is a structural protein found in microtubule-organizing centers at the base of photoreceptor cells that is an integral part of the photoreceptor sensory cilium. Mutations that disrupt *RHO* or *FAM161A* cause retinal degeneration by activating CAPN1 and CAPN2, respectively [1,7-9].

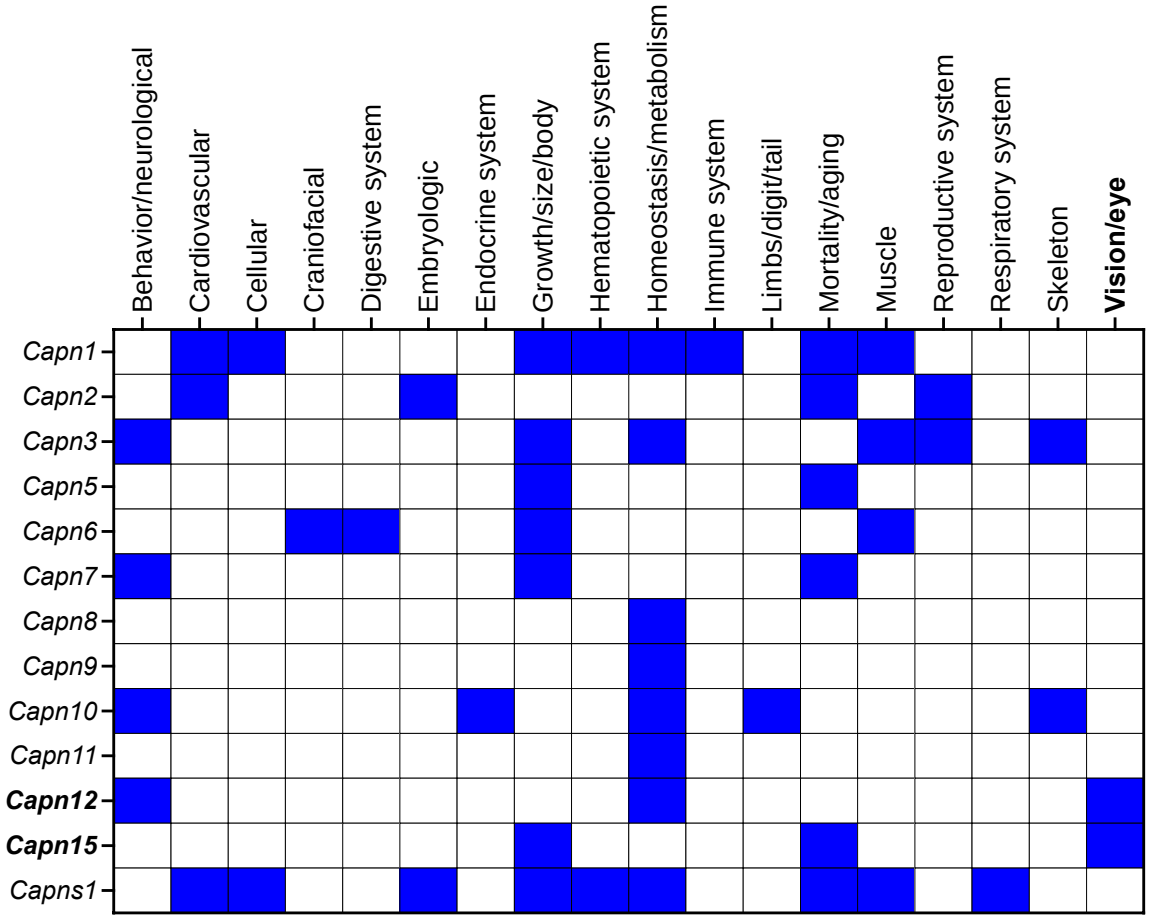

Figure S3. Selective calpain inhibition in murine knockout models. Interrogation of the International Mouse Phenotyping Consortium (IMPC) and the Mouse Genome Informatics (MGI) databases for calpain-related genes. Phenotypic summary data are represented as a heatmap where mouse orthologs are shown in rows and organ system phenotypes shown in columns. *Blue* indicates that deletion of the mouse ortholog is associated with a phenotype in that organ system (e.g., vision/eye). Selective deletion of two calpain orthologs (*Capn12* and *Capn15*) are associated with ocular phenotypes.

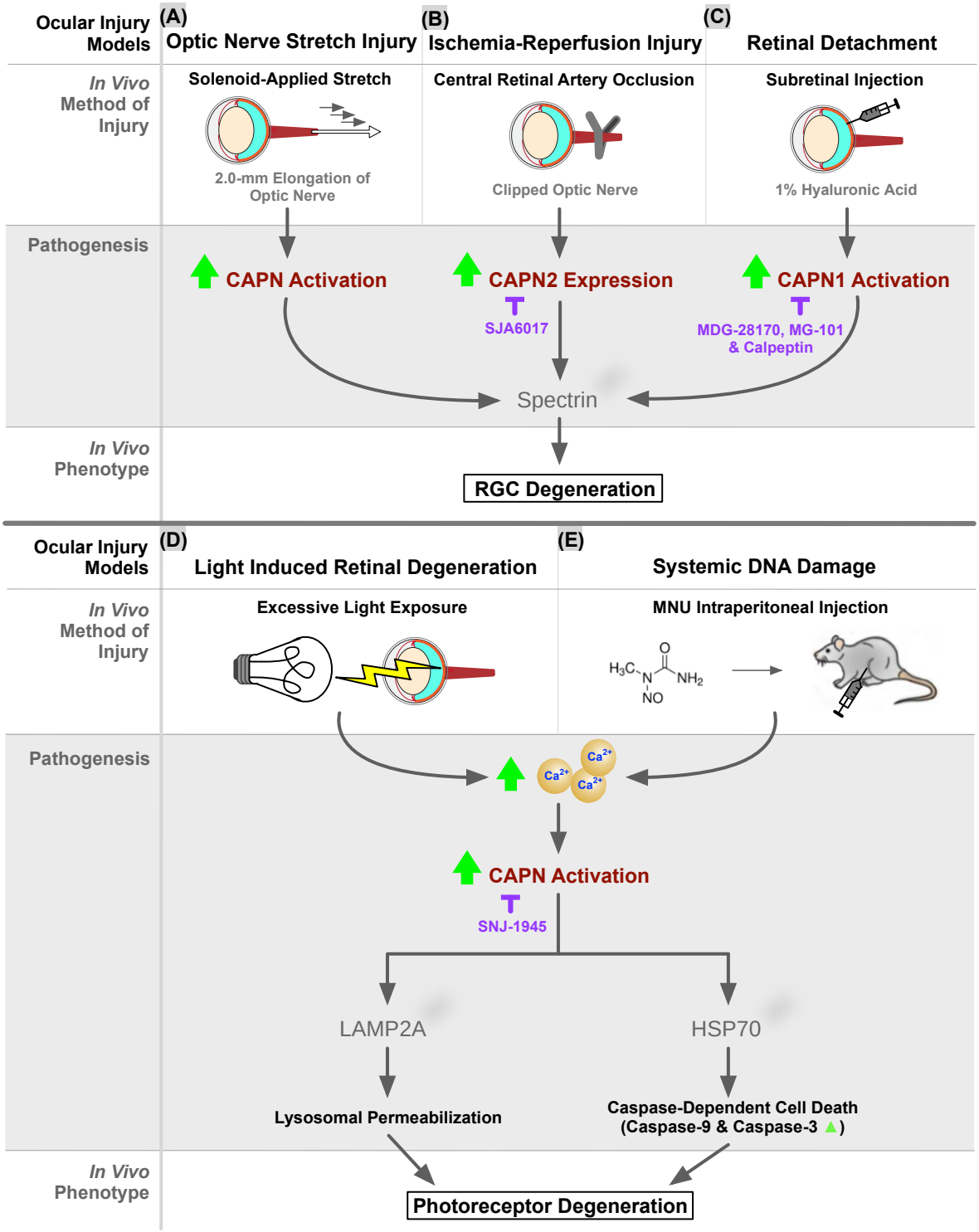

Figure S4. Ocular injuries and cellular stress induce calpain hyperactivity. Calpain activation is widely implicated in the damaging effects of retina injuries. In various *in vivo* models, calpain upregulation and calpain-caspase crosstalk appear to activate cell-death pathways. **(A)** In traumatic axonal injury (TAI), calpain activity was studied in mice subjected to a 2.0-mm optic nerve stretch injury using a solenoid-applied stretch [10]. In subsequent axonal degeneration, immunohistology detected calpain-mediated proteolysis of the cytoskeletal protein, spectrin, implicating calpains as early mediators of TAI that contribute to progressive intra-axonal structural damage. **(B)** Calpain activity is also implicated in retinal ischemia-reperfusion injury [11]. In a

relevant model, *CAPN1* mRNA expression, which was downregulated immediately after injury (i.e., central retinal artery occlusion [CRAO]-induced retinal ischemia for one hour), began to recover by day three of the subsequent seven-day reperfusion. In contrast, *CAPN2* mRNA expression was upregulated immediately after reperfusion and continued to rise after twenty-four hours. For both cases, spectrin proteolysis in the retina provided biochemical evidence that injury activated calpains. Further, treatment with a calpain inhibitor (SJA6017) following the ischemia-reperfusion event reduced the severity of cell density loss in the ganglion cell layer, indicative of the neuroprotective effect of calpain inhibition. **(C)** A rat model of retinal detachment (induced by subretinal injection with 1% hyaluronic acid [12]) demonstrated involvement of calpains in retinal injury. Mechanistically, CAPN1 activity (measured by spectrin proteolysis) peaked at seven days post-detachment and was associated with decreased autophagy, a proxy for photoreceptor death. *In vivo* stimulation of autophagy by multiple calpain inhibitors (i.e., MDG28170, MG-101, and calpeptin) led to attenuated cleavage of spectrin, upregulation of autophagic markers (Atg5 and Atg5-12) and reduced TUNEL-positive photoreceptors. Indeed, the fact that calpain inhibitors protected photoreceptors from apoptosis and increased cell survival suggests the neuroprotective power of calpain inhibition for patients with retinal injury. **(D)** Light-induced retinal degeneration (LIRD) caused by repetitive light exposure irreversibly damages retinal photoreceptors; furthermore, light exposure can exacerbate problems in a retina already made vulnerable by degenerative diseases such as RP, AMD, and DR. Hypothetically, light irradiation triggers apoptotic retinal photoreceptor cell death through increased intracellular calcium-mediated calpain activation [13]. This model is advantageous to use as the damage can be controlled by light intensity and exposure duration to render roughly all photoreceptors in an animal susceptible to cell death and more rapidly than transgenic models [14]. Several LIRD models demonstrate calpain upregulation during photoreceptor cell death. For example, *CAPN5* in Müller glia of zebrafish is upregulated in response to photoreceptor degeneration induced by acute light exposure [15,16]. In a LIRD rat model, the downstream targets of activated calpain in apoptotic pathways were identified: i calpain was postulated to cleave lysosomal associated membrane protein 2 (LAMP2A) leading to lysosomal permeabilization and subsequent cell death [17,18]. Importantly, SNJ-1945, a calpain inhibitor, protected eye function in a mouse model of LIRD (as measured by ERG and retina-histology) [19]. Indeed, LIRD models hold considerable value when probing the precise mechanisms of calpains in retinal degeneration. **(E)** *N*-methyl-*N*-nitrosourea (MNU) is an alkylating agent used in animal models to induce retinal degeneration. By methylating DNA, MNU kills only the photoreceptor cells in the RPE [20], a process that in turn activates the convergence of calpain/calcium and lipid peroxidation, and finally cell death. When MNU was intraperitoneally injected into rats, retinal intracellular calcium ion levels rose, as did calpain activity [21]. Moreover, the same study found that calpain inhibition with oral SNJ-1945 protected MNU-treated rats from photoreceptor cell loss, confirming calpain involvement in photoreceptor degeneration. Similar to rats, intraperitoneally injection of MNU into mice resulted in photoreceptor cell death which was ameliorated by treatment with SNJ-1945 [22]. Finally, another study corroborated this result: inhibiting calpain by MG-101 prevented MNU-induced photoreceptor cell death by suppressing the cleavage of heat-shock protein 70 (HSP70) [23].

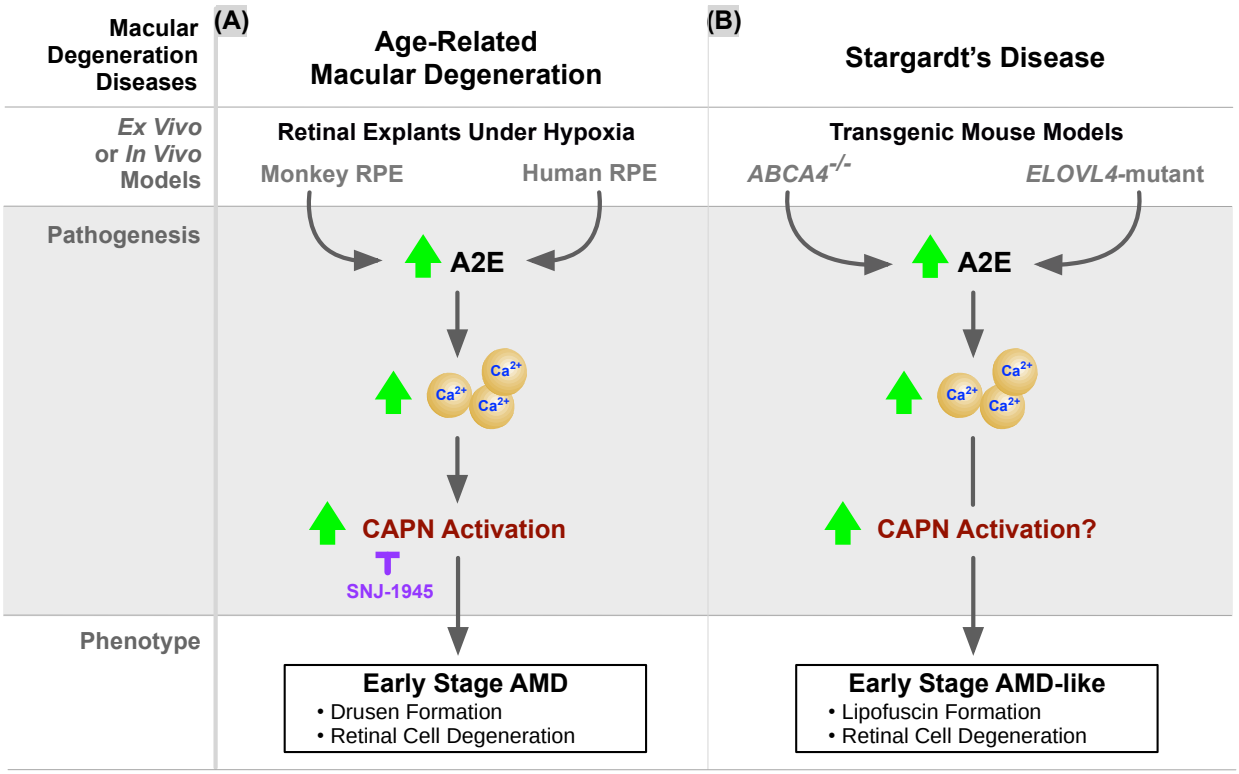

Figure S5. Age-related macular degeneration (AMD) and calpains. **(A)** Calpains were upregulated in age-related macular degeneration *ex vivo* and *in vivo* models. When primary retinal pigment epithelium cells (RPE) were subject to hypoxia, A2E—a principal component of drusen—increased, which was also found to increase calpain activation. A2E has been shown in a separate study to increase cytosolic calcium concentrations, which potentially further activates calpains. However, there are severe limitations to animal models of AMD. First, they do not fully mimic all stages of the human disease [24]. In addition, rodents lack a macula, therefore although they are cost effective and easy to genetically manipulate, they model only a limited range of AMD phenotypes. Further, non-human primates (NHP), which possess a macula, are expensive to maintain, difficult to genetically engineer, require a long-time course for disease progression and only develop early to intermediate stages of AMD (drusen, geographic atrophy) [25,26]. Therefore, advanced forms of AMD (neovascularization) must be induced by laser exposure or intravitreal injection with DL-alpha-aminodipic acid, for example. **(B)** Stargardt's disease is one of the most common forms of early-onset macular degeneration and is most often caused by mutations in genes *ABCA4* and *ELOVL4.* In photoreceptor cells, both ABCA4 and ELOVL4 play important roles in the visual cycle (visual phototransduction that converts light into electrical signals) by chemically regulating retinal/retinoid molecules. A key indicator of a dysfunctional RPE layer is the presence of lipofuscin, sub-retinal deposits of lipids and proteins. The principal component of lipofuscin is A2E as it is of drusen formation in AMD. Experimentally, *ABCA4* knock-out mice [27-29] and *ELOVL4* mutant knock-in mice (comparable to early-onset AMD) [30] develop a Stargardt's disease-like phenotype: A2E and lipofuscin accumulates in RPE, leading to eventual apoptosis of the RPE and overlying photoreceptors.

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