Supplementary Data:

Intra-vitreal αB crystallin fused to elastin-like polypeptide provides neuroprotection in a mouse model of age-related macular degeneration.

Authors: Parameswaran G. Sreekumar[1,*], Zhe Li[2,*], Wan Wang[2], Christine Spee[3], David R. Hinton[3,4,**], Ram Kannan[1,**], J. Andrew MacKay[2,3,5,**]

[1]Arnold and Mabel Beckman Macular Research Center, Doheny Eye Institute, Los Angeles, CA 90033, USA

[2]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy of the University of Southern California, Los Angeles, CA 90089, USA

[3]Department Ophthalmology, USC Roski Eye Institute, Keck School of Medicine of the University of Southern California, Los Angeles, CA 90033, USA

[4]Department of Pathology, Keck School of Medicine of the University of Southern California, Los Angeles, CA 90033, USA

[5]Department of Biomedical Engineering, Viterbi School of Engineering of the University of Southern California, Los Angeles, CA 90033, USA

*Authors contributed equally to this work

**Co-senior authors

Correspondence: J. Andrew MacKay (jamackay@usc.edu)

Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Ave, Los Angeles, CA 90089, USA

Supplemental Methods

Polarized RPE monolayer

Highly differentiated fetal human RPE cells were cultured on transwell filters as described [35]. In brief, 10^5 primary human fetal RPE cells / cm^2 were seeded on fibronectin-coated transwell filters (12-mm internal diameter, 0.4-μm pore size, Corning Inc, Corning, NY, USA) in medium containing 10% FBS for 1 day and 1% FBS for 4 weeks. Polarized RPE monolayers express tight junction markers, including ZO-1 [35]. Both apical and basal cellular compartments were treated with 1 or 2.5 mM $NaIO_3$ and co-treated with mini cry, SI or crySI for 24h (10 μM). Transepithelial electrical resistance (TER) of RPE monolayers was measured with an EVOM epithelial tissue voltohmmeter (World Precision Instruments, Sarasota, FL).

After the experiment, the morphologic features of polarization were characterized by ZO-1 staining. RPE monolayers were fixed in 4% PFA for 20 minutes, blocked with 5% normal goat serum, and permeabilized with 0.1% Triton-X 100. Cells were incubated with rabbit polyclonal ZO-1 antibody (1:100 dilution, #40-2200, ThermoFisher Scientific, Camarillo, CA) overnight, washed with PBS, incubated with a FITC conjugated anti-rabbit secondary antibody (Vector Laboratory), and imaged using a spinning disc confocal microscope (PerkinElmer, Waltham, MA).

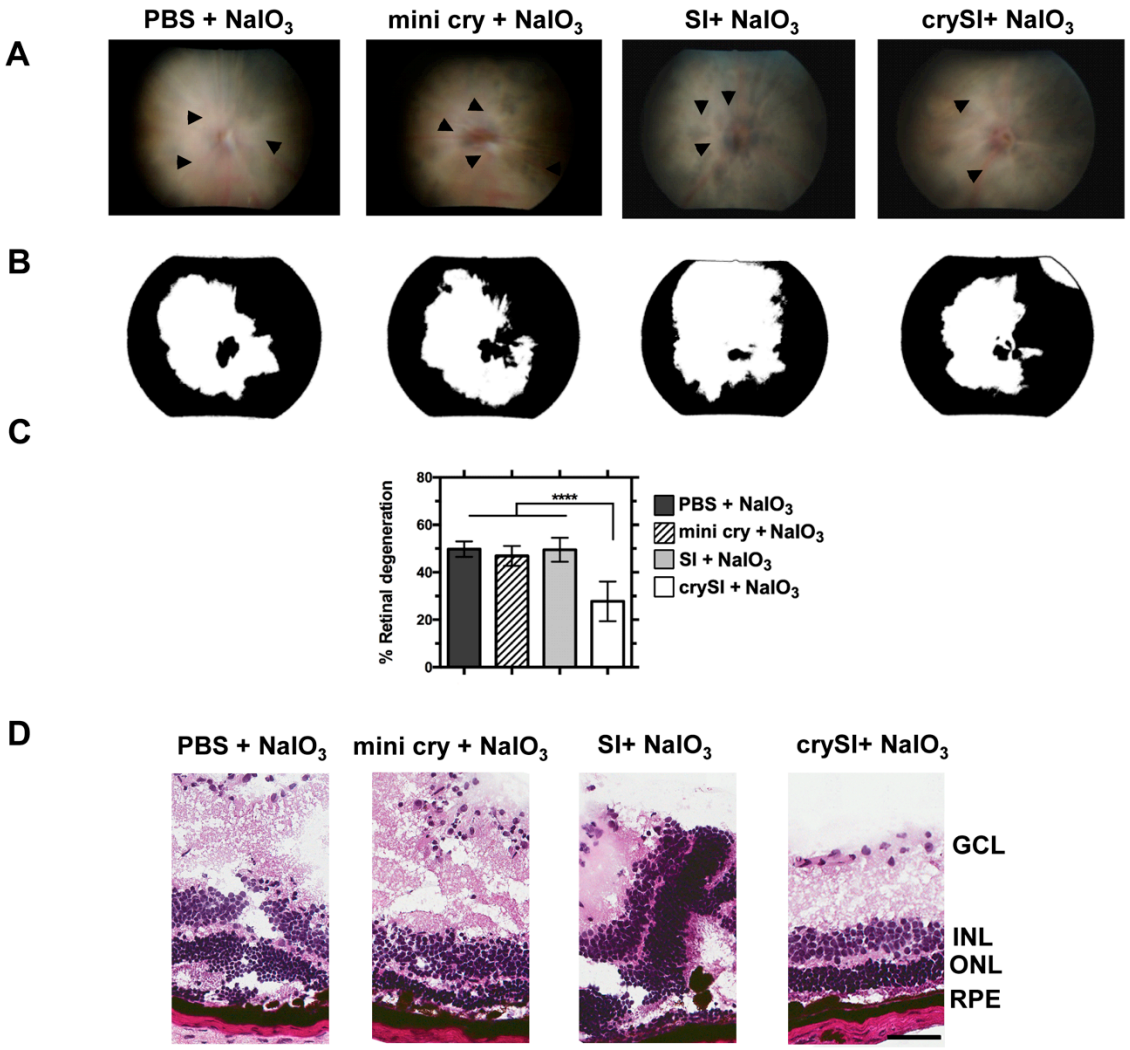

Figure S1. Long-term protection of the retina by intravitreal CrySI administered one week prior to challenge with $NaIO_3$. One week prior to $NaIO_3$ challenge (33 mg/kg) mice were pre-treated with PBS (2 µl), SI (2 µl, 250 µM), crySI (2 µl, 250 µM), or mini cry (2 µl, 250 µM). The retinas were evaluated one month post-challenge **(A)** Color fundus photographs were obtained, and arrowheads indicate areas of retinal degeneration. **(B)** The area of retinal degeneration was thresholded and quantified by image J (NIH, Bethesda, MD). **(C)** $NaIO_3$ induced significant retinal degeneration in mice treated with PBS, mini cry, and SI; however, crySI treatment significantly protected the retina from degeneration. Data are presented as mean ± SD (n= 6-10, ****p<0.0001). **(D)** H&E staining on retinal sections show preservation of retinal layers only in crySI pretreated animals when compared to other experimental groups. Predominant loss of RPE cells, distortion and thinning of ONL and disorganization INL were found. GCL: Ganglion cell layer, INL = Inner nuclear layer; ONL = Outer nuclear layer; RPE = Retinal pigment epithelium. Scale bar: 50 µm.

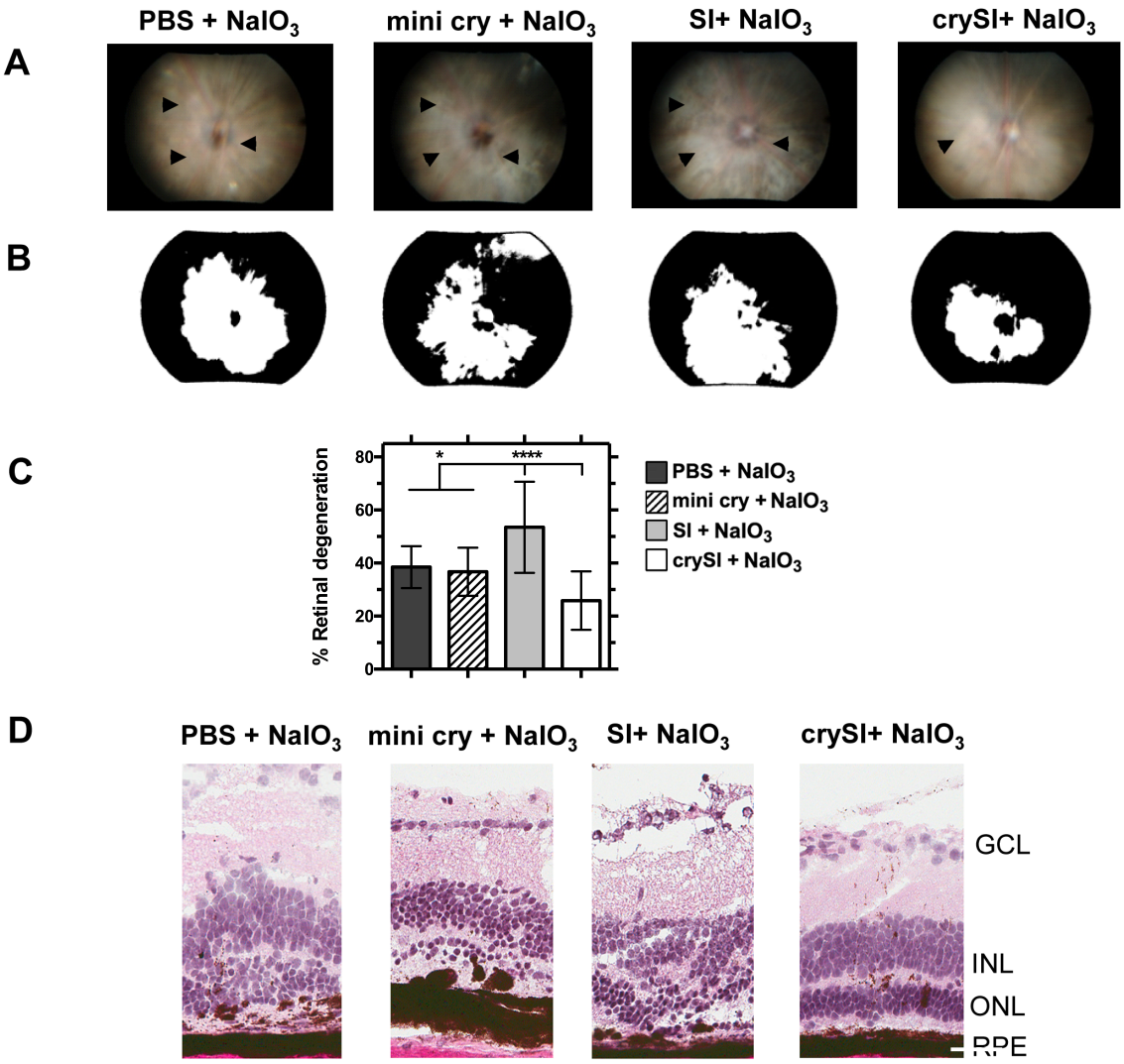

Figure S2. Long-term protection of the retina by intravitreal CrySI administered two weeks prior to challenge with $NaIO_3$. Two weeks prior to $NaIO_3$ challenge (33 mg/kg) mice were pre-treated with PBS (2 µl), SI (2 µl, 250 µM), crySI (2 µl, 250 µM), or mini cry (2 µl, 250 µM). The retinas were evaluated one month post-challenge **(A)** Color fundus photographs were obtained, and arrowheads indicate areas of retinal degeneration. **(B)** The area of retinal degeneration was thresholded and quantified by image J (NIH, Bethesda, MD). **(C)** $NaIO_3$ induced significant retinal degeneration in mice treated with PBS, mini cry, and SI; however, crySI treatment significantly protected the retina from degeneration. Data were presented as mean ± SD (n= 6-10, *p<0.05, ****p<0.0001). **(D)** H&E staining on retinal sections show preservation of retinal layers only in crySI pretreated mice when compared to other experimental groups. Predominant loss of RPE cells, distortion and thinning of ONL and disorganization INL were found. GCL = Ganglion cell layer, INL = Inner nuclear layer; ONL = Outer nuclear layer; RPE = Retinal pigment epithelium. Scale bar: 50 µm.

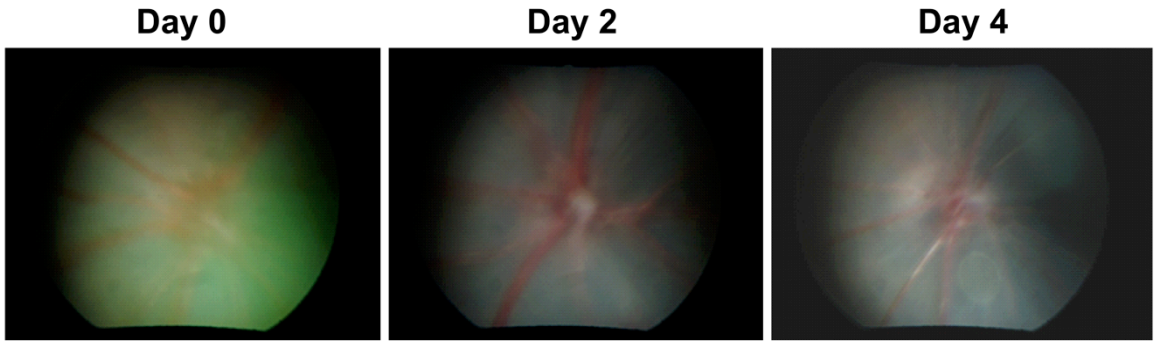

Figure S3. Fluorescein mini cry is rapidly eliminated from the eye following intravitreal administration. Due to a lack of detection at one week after administration (**Fig. 3**), the ocular disappearance of mini cry was evaluated qualitatively in a shorter duration study. 2 µL of fluorescein labeled mini-cry (250 µM) was administered to each mouse eye and fundus photography was performed at 0, 2, and 4 days. The images presented are representative of the study group (n=3). By two days after administration, fluorescence from the mini cry peptide was undetectable above background fluorescence.

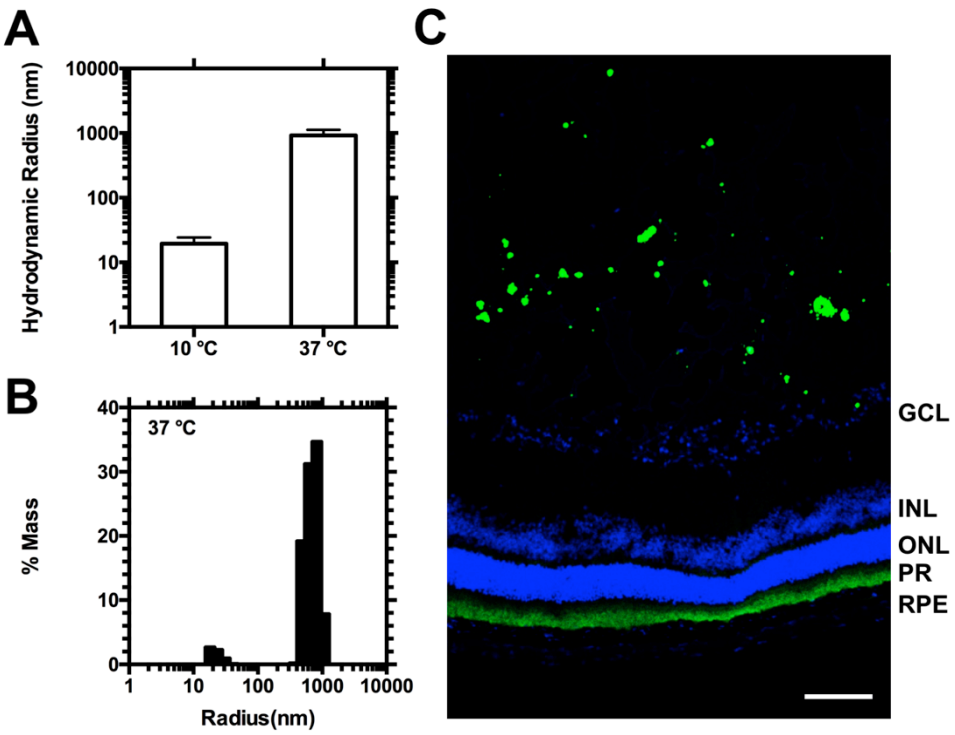

Figure S4. Morphological characterization of crySI in phosphate buffered solution and the mouse vitreous. (A) The hydrodynamic radius of fluorescein-labeled crySI was characterized using dynamic light scattering at 10 ºC and 37 ºC. **(B)** Regularization analysis enabled two populations of particles to be observed for crySI at 37 ºC with the majority forming micron sized coacervates. **(C)** Confocal microscopy was performed for retinal cryo-sections obtained day 7 after mice were given intravitreal fluorescein-labeled crySI (green). Consistent with the hydrodynamic radius observed for crySI coacervates in buffer at physiological temperature, micron-sized fluorescent structures were observed above the retina. Blue: DAPI. Scale bar: 100 μm.

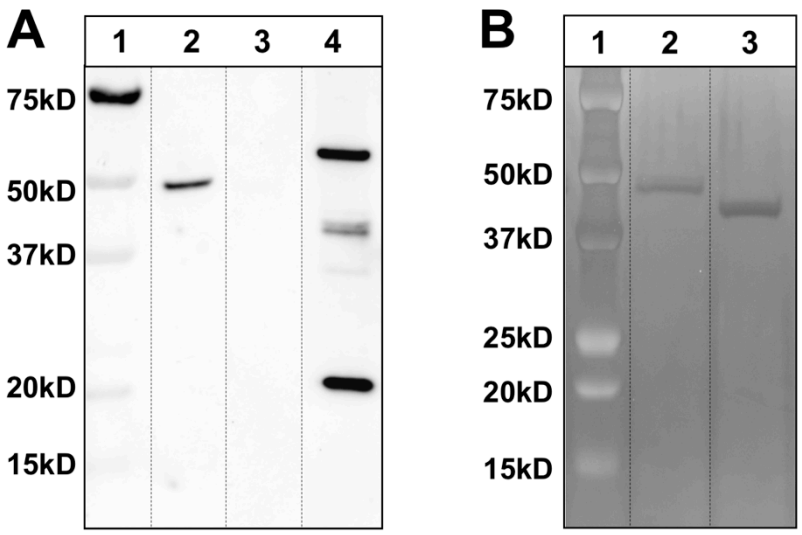

Figure S5. Crossreactivity of an αB crystallin antibody that detects both exogenous crySI (~42 kD) and endogenous αB crystallin (~20 kD). (A) Western blot using a rabbit anti-αB crystallin antibody (Enzo Life Sciences, Farmingdale, NY) Lane: 1) Ladder; 2) purified crySI; 3) purified SI; and 4) cell lysate obtained from human retinal pigment epithelial cells (RPE). **(B)** SDS-PAGE developed with copper chloride. Lane: 1) Ladder; 2) purified crySI; and 3) purified SI.

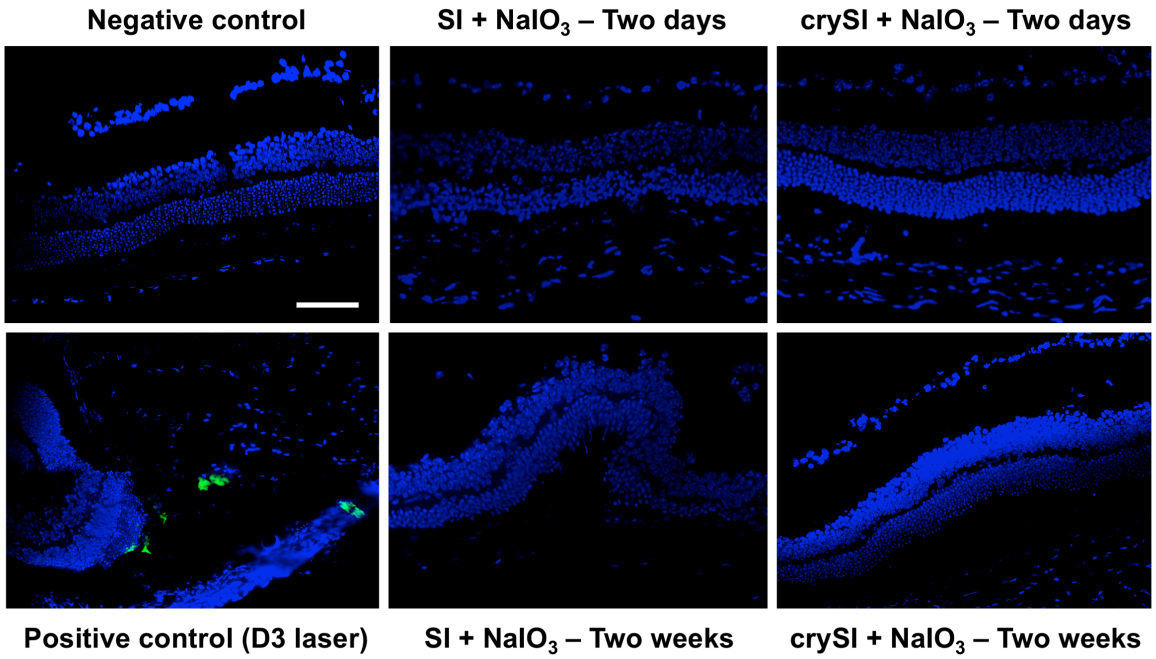

Figure S6. Infiltration of T cells in the mouse retina was assessed using an anti-CD3 antibody (green). Retinal cryo-sections were obtained from mice pre-treated by two days or two weeks with SI or crySI prior to $NaIO_3$ challenge (**Fig. 1a**). Tissues were stained with a FITC anti-mouse CD3 antibody (# 100203, BioLegend, San Diego, CA). Neither SI nor crySI attracted a CD3+ T-cells into the retina. As a negative control, the primary antibody was not incubated with the sample. As a positive control, a laser-induced choroidal neovascular (CNV) model was used to induce CD3+ T cell infiltration into the retina. Scale bar: 50 μm.

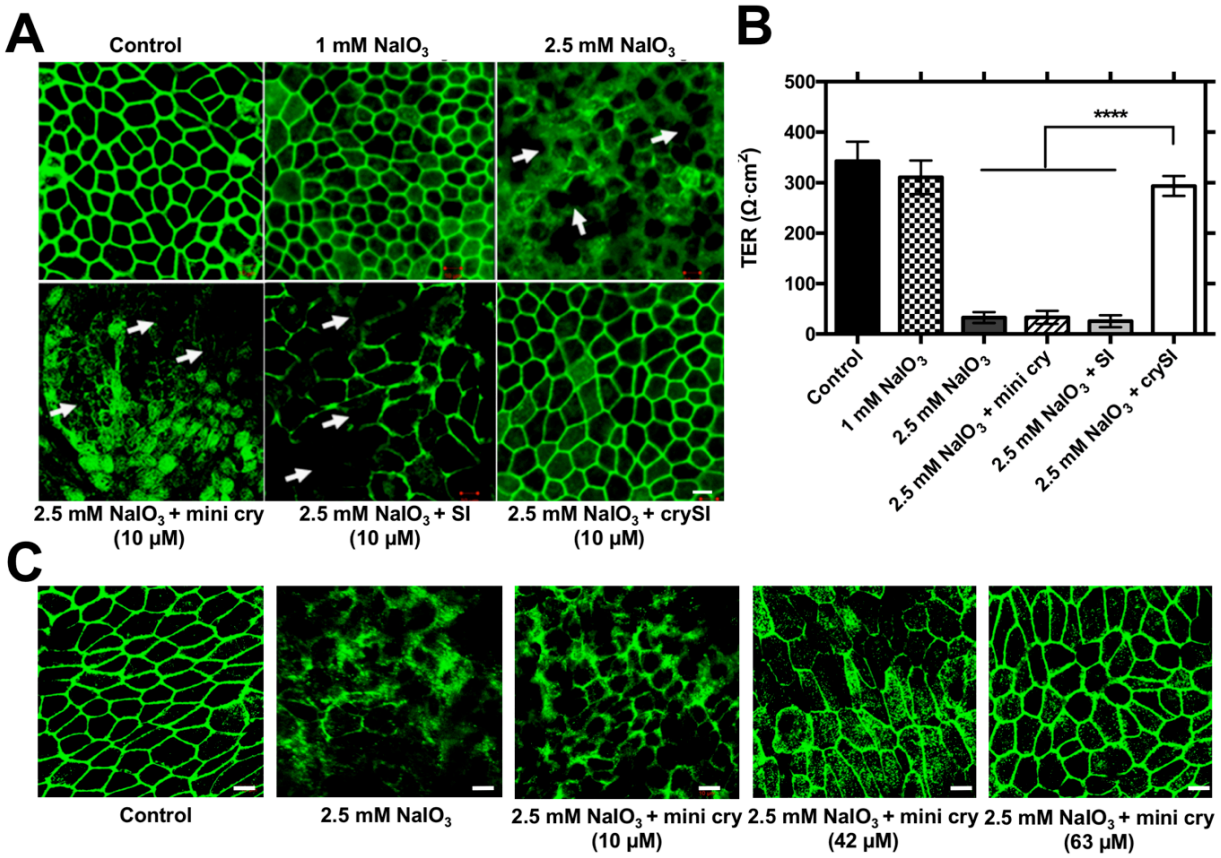

Figure S7. *In vitro* crySI protects human retinal pigment epithelial (RPE) cells from disruption of tight junctions upon $NaIO_3$ challenge. To explore the clinical relevance of crySI, primary human fetal RPE cells were cultured as a polarized monolayer and assessed for Transepithelial Electrical Resistance (TER) and ZO-1, both of which are markers of tight junctions. **(A)** Confluent monolayers were challenged with $NaIO_3$ (1 and 2.5 mM) and treated with mini cry, SI, or crySI (10 µM) for 24 h and the localization of the tight junction marker ZO-1 was determined by secondary immunofluorescence. The $NaIO_3$ challenge resulted in severe breaks (arrows) in ZO-1; furthermore, only crySI treatment prevented the disruption of tight junctions. Scale bar: 10 µm. **(B)** Confluent monolayers were challenged with $NaIO_3$ (1 and 2.5 mM) in the presence of mini cry, SI, or crySI (10 µM) for 24 h and TER was measured. TER was significantly reduced by challenge with 2.5 mM $NaIO_3$. Treatment with crySI protected TER significantly compared to control treatments. Data are shown as mean ± SD (n=8-9, ****p<0.0001). **(C)** Confluent monolayers were challenged with 2.5 mM $NaIO_3$ and treated with varying doses of mini cry (10, 42, and 63 µM) for 24 h and the localization of the tight junction marker ZO-1 was determined by secondary immunofluorescence. The $NaIO_3$ challenge resulted in severe breaks (arrows) in ZO-1; however, mini cry at higher doses (42 and 63 µM) prevented the disruption of tight junctions suggesting its protective role at elevated concentrations. Scale bar: 10 µm.