Supporting Information

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SI Results

Investigation of the UPR in P23H-3 RHO rats revealed an activation of the three individual pathways in the retina affected by misfolded rhodopsin. Fig. S1 contains images of Western blots obtained from Sprague-Dawley and P23H-3 *RHO* (P23H-3) transgenic rats at P30. We performed subretinal injection of AAV5-BiP (NM_005347). The map is shown in Fig. S2.

We also performed injection of P23H-3 *RHO* rats with AAV-BiP-Flag. At 1 month after injection, animals were euthanized, and eyes were enucleated for immunostaining analysis with anti-FLAG antibody. Distribution of BiP-Flag is shown in Fig. S3.

At 1 month after injection, we isolated retinas to perform RNA and protein analysis. To estimate the proportion of human BiP protein, we conducted reverse transcriptase reaction to obtain total cDNA. Total cDNA then was used for PCR using primers that amplified both rat and human BiP mRNA. The product was digested with NCO1 and loaded on 4–20% polyacrylamide gel. An image of digested PCR product is shown in Fig. S4.

Searching for the mechanism by which overexpressed BiP might rescue the photoreceptors and cause the therapeutic effect, we performed a series of immunoprecipitations allowing us to determine the protein ligands involved in prevention of the apoptosis. We precipitated a protein extract isolated from BiP-Flag-injected retina with procaspase-12 and NBK or BiK. Denatured lysates were run in 12% SDS polyacrylamide gels and blots were treated with anti-Flag primary antibody. To confirm the results we also performed the immunoprecipitation of BiP-Flag with anti-Flag antibody, ran gels with protein complex and treated the blots with anti-caspase-12 and BiK primary antibodies. Results of the experiment are shown in Fig. S6.

To find a link between BiP and rhodopsin proteins in vivo we performed immunoprecipitation of total rhodopsin with 1D4 antibody, which reacts with the C terminus of rhodopsin. Immunoprecipitates were run 12% SDS polyacrylamide gels and transferred to PVDF membranes, which were reacted with anti-BiP antibody. The result of this experiment demonstrated that BiP protein forms a complex with rhodopsin, pointing out the special role for this chaperone in the transgenic retina (Fig. S6*C*).

Despite the link between BiP and rhodopsin protein in transgenic retinas (Fig. S6*C*), we did not observe increased trafficking of P23H RHO to the cell membrane by overexpressing BiP in cultured cells. Therefore, we tested the content of rhodopsin protein in the outer segments of dark adapted photoreceptors. The results of this experiment indicated that overexpression of BiP did not support the increase in the content of rhodopsin in outer segments either and, consequently, did not assist appropriate folding of rhodopsin. The results of the experiment also pointed out that another mechanism is involved in the rescue of photoreceptors besides the chaperoning function of BiP protein. Quantification of rhodopsin content in retinas of the four transgenic rats injected with AAV-BiP and AAV-GFP is shown in Fig. S5.

Therefore, we proposed mechanism by which increasing expression of BiP blocks photoreceptor apoptosis is presented in Fig. S7.

SI Materials and Methods

HeLa Cells Protein Extract for Immunoblot Analysis. Protein concentration was measured by comparison with a known concentration of BSA using a kit (23223; Pierce), and equal concentration of total protein was loaded on 12 or 15% SDS polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Invitrogen) by electrophoresis. Then, primary antibodies were applied. Secondary antibodies were tagged with infrared dyes. The Li-Cor Odyssey system was used to detect bands.

Rat Model, Subretinal Injection of AAV Vectors. P23H-3 RHO homozygous animals were bred with albino Sprague-Dawley rats to produce P23H-3 RHO heterozygous rats with a single P23H mouse *RHO* transgene. All rats were kept in 12-h light/dark cycle of dim light, measuring less than 90 lux at the level of the cages. We injected P23H-3 *RHO* transgenic rats at postnatal day 15 with two viruses: AAV5-CBA-BiP/GRP78 and AAV5-GFP, dividing them into groups of 15 animals. Subretinal injections were conducted with 2 μl virus suspension (8×10^9 viral particles). Then we analyzed animals by scotopic ERG monthly for 3 months after injection. Finally, we humanely euthanized the animals to measure the thickness of the ONL thickness by morphometry (see below).

Retinal Protein Extract for Western Blot Analysis. Retinal protein extracts were obtained from dissected retinas by sonication in a buffer containing 25 mM sucrose, 100 mM Tris-HCl, pH = 7.8, and a mixture of protease inhibitors (PMSF, TLCK, aproptinin, leupeptin, and pepstatin). Individual right and left retinas were used to quantify the concentration of total protein (Biorad Protein Assay), 40 μg of total protein was used to detect proteins; 10 μg of total protein was used for detection β-actin as internal control. Detection of proteins was done by using infrared secondary antibody and Odyssey an infrared imager (Li-Cor, Inc.). We used antibodies that detect the stress-induced phosphorylated proteins: anti-peIF2α (Abcam Co.) 1:1,000, anti-ATF6 (Genetech) 1:1,000 and antibody anti-β-actin, anti-Flag (Sigma-Aldrich) 1:5,000, anti-BiP 1:1,000, anti-CHOP 1:1,000, anti-NBK 1:1,000 (Santa-Cruz Biotechnology), anti-caspase-7 and -12 (Cell Signaling), anti-rhodopsin-1D4 1:1,000 (a gift of Dr. Robert Molday of the University of British Columbia, Vancouver, BC, Canada).

Immunostaining Analysis. The 1D4 antibody against rhodopsin, anti-Flag antibody, and pan-Cadherin (Abcam Co.), a plasma membrane marker, were used as primary antibodies for immunostaining of transfected cells. Cy-2 and Cy-3 conjugated secondary antibodies were applied for rhodopsin and Flag tag, pan Cadherin coordinately to visualize the proteins. For immunostaining analysis of retina expressing BiP-Flag protein, eyes were enucleated and the tissue was fixed and prepared for frozen sectioning. 12 μm-sections were obtained by using a cryostat, and detection of fluorescence was recorded by using primary anti-Flag, secondary Cy-2 antibody, and confocal microscopy.

Full Field Scotopic ERG. For ERG, we used overnight dark-adapted rats, anesthetized with keatmine/xylazine. Their eyes were dilated in dim red light with 2.5% phenylephrine solution. Small contact lenses with gold wire loops were placed on each cornea with a drop of 2.5% methylcellulose to maintain corneal hydration. Silver wire reference electrodes were placed s.c. between the eyes, and a ground electrode was connected s.c. in a hind leg. Scotopic ERGs were elicited with 10 μsec flashes of white light at 30, 20, 10, and 0 dB of attenuation. Five to ten scans were averaged at each light intensity. The a-wave amplitudes were measured from the baseline to the peak in the cornea-negative direction, and b-wave amplitudes were estimated from the cornea-negative peak to the major cornea-positive peak. Differences in a- and b-wave amplitudes between simultaneously recorded test and control eyes were the primary measure of outcome.

Retinal Tissue Preparation and Histological Quantification of Retinal Outer Nuclear Layer. We euthanized rats by overdose of carbon dioxide and immediately perfused them intracardially with 2% paraformaldehyde and 2.5% glutaradehyde. Eyes were removed, postfixed in osmium tetroxide, and embedded in epoxy resin. Histological sections 1-μm thick were made along the vertical meridian. Tissue sections were aligned so that the rod outer segments and Müller cell processes crossing the inner plexiform layer were continuous through the plane of section to ensure that the sections were not oblique, and the thickness of the ONL was measured. Fifty-four measurements of the ONL were made at 18 contiguous fields around the entire retinal section that are plotted as a distribution of thickness across the retina. Mean ONL thickness of the entire retina or specific region of the eye were compared between the AAV-BiP and control-injected eyes.

Nucleosome Release Quantification of Apoptosis: We quantified the DNA fragmentation resulting from apoptosis in RP retinas. The DNA fragmentation occurs as a result of Ca^{2+}- and Mg^{2+}-dependent nuclease cleavage of double-stranded DNA that generates release of mono- and oligonucleosomes, complexes tightly associated with the core histones H2A, H2B, H3, and H4. Therefore, we identified nucleosome release levels by a sandwich-enzyme immunoassay using mouse monoclonal antibodies directed against DNA and histones and Cell Death Detection ELISA kit (Roche Diagnostics). At 1 month after viral injection, animals were euthanized, and retinas were harvested and processed according the manufacturer's procedure. Retinas were placed in 200 μL of lysis buffer (provided with the kit) on ice. The BiP-treated and control retinas were individually homogenized for 3 sec with a tissue homogenizer (Polytron; PT 1200). The homogenates were centrifuged at 200 *g* for 10 min, and 10 μL of the resultant supernatant was used for further dilution into 990 μL of lysis buffer. Twenty microliters of this final dilution were used in the assay.

For in vitro experiments, HeLa cells (10^4 cells/well) were seeded into a 96-well plate. The next day, cells were cotransfected with P23H *RHO* and wild-type *RHO* plasmids and a plasmid expressing BiP protein as described in *Materials and Methods*. pcDNA3.1 plasmid was used as a DNA carrier to maintain constant DNA concentration in single transfections with P23H mutant or wild-type rhodopsin. At 48 h posttransfection, the cells were treated with lysis buffer, incubated for 30 min, centrifuged at 200 *g* for 10 min, and 20 μl of the cytoplasmic fraction were used to perform nucleosome release quantitation based on the manufacturer's protocol. Data were normalized to Mock, BiP mean.

RT-PCR Analysis. We performed reverse transcriptase reaction using the first Strand cDNA kit with oligo-dTT(GE Healthcare). Primers for amplification of BiP, β-actin, and XbP1 cDNA are shown in Table S1; 4–20% polyacrylamide gels (Bio-Rad) were used and SYBR green dye was applied to detect individual PCR products.

PCR conditions for amplification of the β-actin cDNA were 94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, 72 °C for 7 min, 21 cycles; for the BiP cDNA conditions were 54 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, 72 °C for 7 min, 23 cycles; for the Xbp1 cDNA conditions were 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, 72 °C for 7 min, 35 cycles. Quantification of the portion of the human BiP was done as described in Fig. S4. Quantification of the spliced portion of the XbP1 cDNA was performed by purification of PCR product with PCR clean up Kit (Sigma-Aldrich) and digestion with the unique for unspliced XbP1 Pst1 endonuclease.

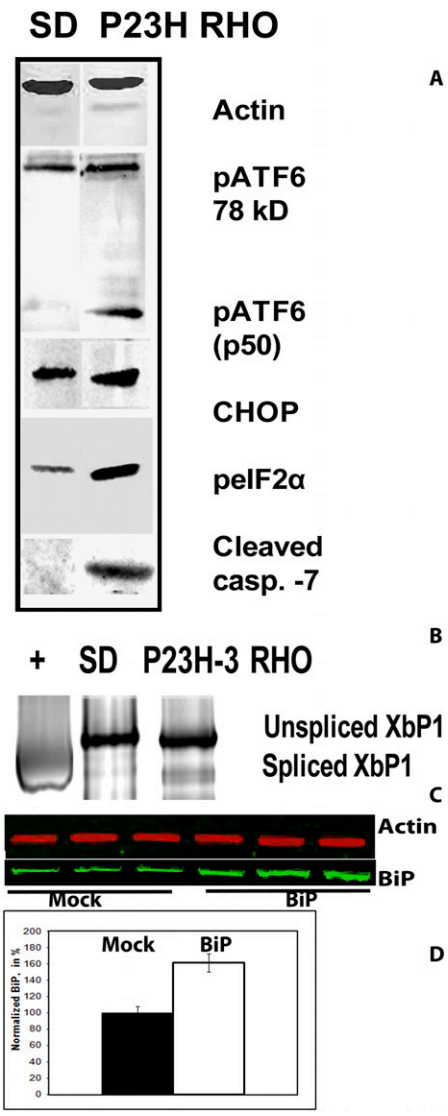

Fig. S1. Study of the UPR in P23H-3 *RHO* transgenic and Sprague-Dawley rats at P30 and BiP overexpression in HeLa cells. (*A*) Images of Western blots obtained from the treatment of the PVDF membranes with primary antibodies against β-actin, phosphorylated ATF6, CHOP, phosphorylated eIF2α, and cleaved caspase-7. (*B*) Images of 4–20% polyacrylamide gel stained with SYBR green. Unspliced XbP1 band is 486 bp, spliced Xbp1 band is 459 bp. "+" — positive control that was obtained by PCR of plasmid expressing spliced XbP1, (*C*) Images of Western blots from HeLa cells transfected with BiP protein were obtained by using a Li-Cor Odyssey Imager, (*D*) Quantification of BiP overexpression in HeLa cells was done by normalizing of BiP to β-actin. First, we detected intensities of individual BiP and β-actin bands and then, the band intensity of BiP was normalized by the β-*actin* signal and the average intensity ratios are presented.

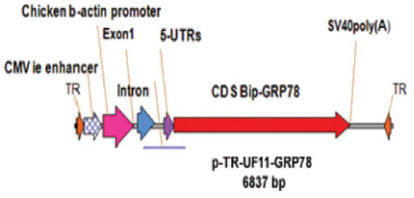

Fig. S2. Design of AAV vector expressing the human BiP/GRP78. The expression of BiP is driven by the chicken β-actin promoter. The processing of the BiP mRNA is facilitated by the insertion of the chicken β-actin exon 1 and intron. The SV40 poly(A) is present for stabilization, maturation and for further BiP mRNA export from the nucleus.

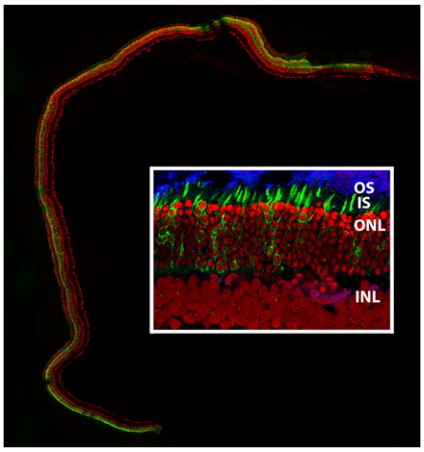

Fig. S3. Delivery of AAV5-BiP-Flag to the retina of P23H-3 *RHO* rats leads to expression of the FLAG epitope at 4 weeks after injection. Immunostaining analysis was performed on cryostat sectioned retina with primary antibody against FLAG and Cy2-conjugated secondary antibody. The analysis shows widespread distribution of virus expressing BiP-Flag (green) all over the retina with more intense region in the inferior hemisphere suggesting the original place of injection. (*Inset*) Propidium iodide-stained nuclei are shown in red and rhodopsin protein stained with primary antibody 1D4 and detected with Cy5-conjugated secondary antibody is shown in blue. The higher magnification demonstrates BiP immunofluorescence in the inner segments and the ONL, as expected for ER localization.

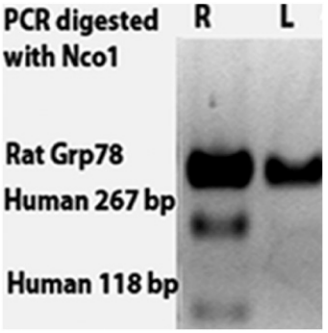

Fig. S4. Quantification of the human BiP mRNA in AAV-BiP-injected retinas. RT-PCR products were obtained and NCO1 digestion was performed. Images of 4–20% polyarcylamide gel stained with SYBR green demonstrated that right eyes expressed the human BiP cDNA that was digested with NCO1 enzyme giving two bands of 267 bp and 118 bp.

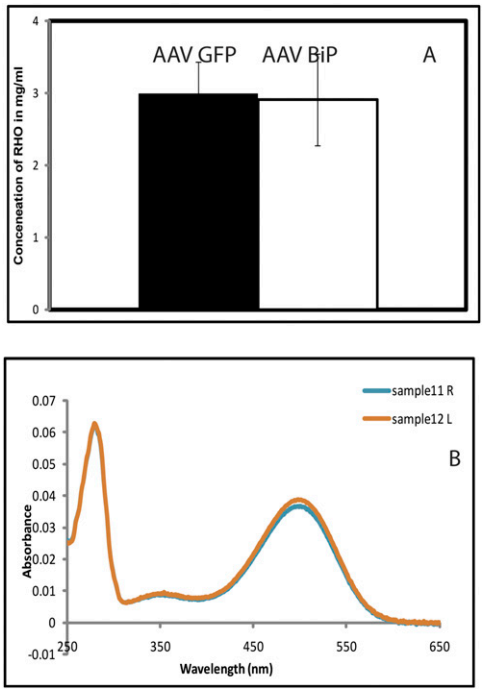

Fig. S5. Quantification of immunoaffinity-purified rhodopsin measured in UV-visible spectrum. Affinity-bound rhodopsin has a λmax of approximately 500 nm in samples from control and BiP treated, the dark adapted P23H-3 *RHO* retinas. (*A*) Immunoprecipitation of the rhodopsin was done by using 1D4 antibody. (*B*) Representative spectrum of the absorption at 500 nm from the immune-purified rhodopsin isolated from right and left retinas.

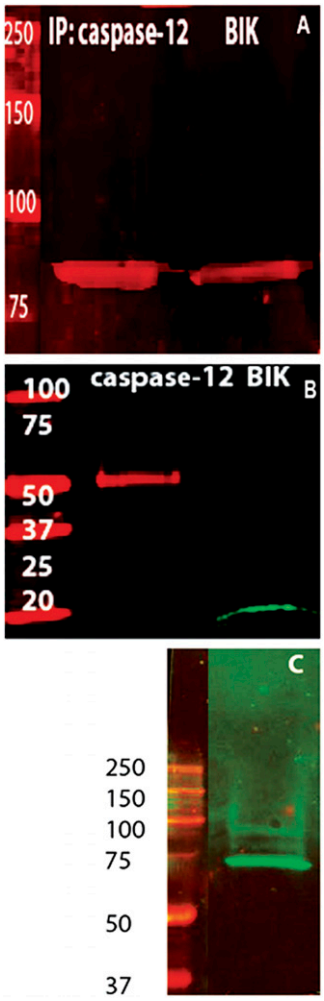

Fig. S6. Immunoprecipitation (IP) of the retinal protein extracts isolated from naïve and the BiP–Flag-treated P23H-3 *RHO* rats. (*A*) Immunoprecipitation was performed by treating protein extract individually with a procaspase-12 (Cell Signaling Technology) and Bik (NBK) antibodies (Santa-Cruz Technology), and protein complexes were further processed to obtain denatured samples by using a Catch-and-Release system (Millipore); 12% SDS-gel was used to perform Western blot analysis. Membranes were treated with secondary antibody (680 W) against Flag sequence (Sigma-Aldrich) to detect BiP size fusion protein (78 kDa) by using Li-Cor Odyssey system. (*B*) IP was performed by treated protein extracts with anti-Flag antibody and samples were separated on 12% SDS gel. The membrane was treated with anti-caspase-12 and anti-BiK antibody. Detection was done by applying secondary antibodies demonstrating procaspase-12 (55 kDa) and Bik (20 kDa). (*C*) By immunoprecipitation assay and immunoblot analysis, we detected that BiP protein is a binding partner for the rhodopsin in naïve P23H-3 RHO retinas. Rhodopsin protein was precipitated by using 1-D4 (N-terminal) antibody. The immunoblot was processed using anti-BiP primary antibody and infra-red labeled secondary antibody.

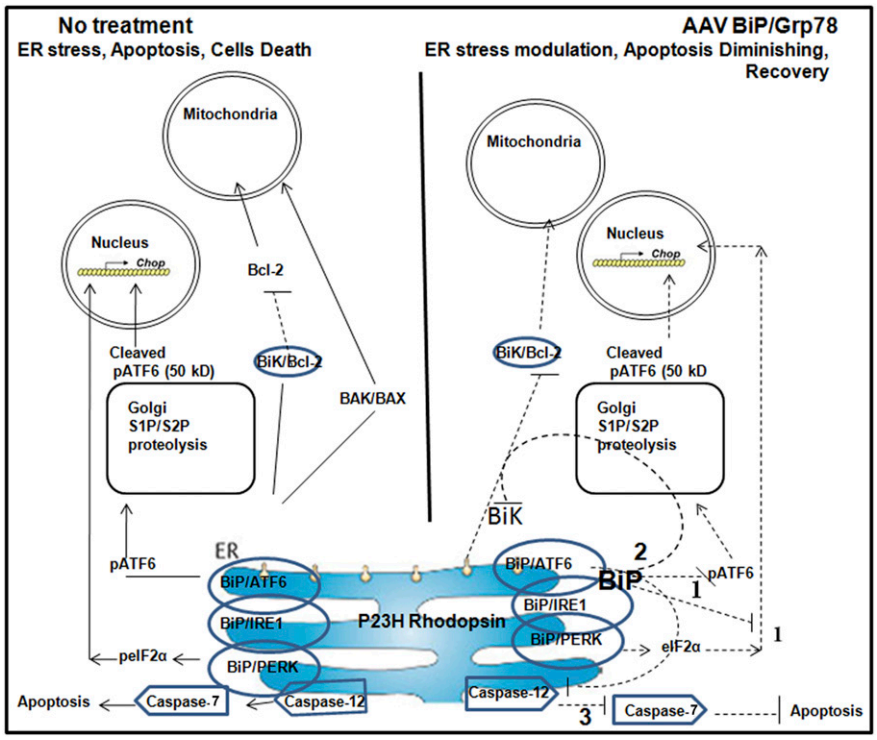

Fig. S7. Proposed mechanisms of the therapeutic effect of AAV-BiP in P23H-3 *RHO* photoreceptors. Pathway 1: Overexpressed BiP modulates the ATF6 and PERK signaling by reducing the cleavage ATF6 product, peIF2α, and CHOP protein. This down-regulation contributes to reduction of the apoptosis. Pathway 2: BiP complexes with BiK (Bcl-2 Interacting Protein). This, in turn, reduces the interaction of BiK with antiapoptotic Bcl-2 protein. Formation of a BiP/BiK complex also affects level of oligomerization and dimerization of BAX/BAK proteins, which leads to reduction of mitochondria-originated apoptosis. Pathway 3: BiP forms a complex with caspase-12 blocking its activation and promotion of ER-associated apoptosis.

Table S1. The sequences of the DNA oligos used in the RT-PCR analysis

Gene	5′-Sence	5′-Anti-sence
b-actin	TGA GACCTTCAACACCCCAGCC	TGGCCATCTCCTGCTCGAAGTC
BiP	AGGAGGAGGACAAGAAGGAGGA	AGCTGCCGTAGGCTCGTGTTGATGAT
X bP1	AGAGTAGCA GCACAGACTGCGCGAGA	GGAACTAGGTCCTTCTGGGTAGACCT