Role of Translational Attenuation in Inherited Retinal Degeneration

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Purpose. We reported previously that retinas of mice with inherited retinal degeneration make less protein than retinas of normal mice. Despite recent studies suggesting that diminished protein synthesis rates may contribute to neurologic disorders, a direct link between protein synthesis rates and the progression of neurodegeneration has not been established. Moreover, it remains unclear whether reduced protein synthesis could be involved in retinal pathogenesis. Dysregulation of AKT/mTOR signaling has been reported in the retina during retinal degeneration, but to what extent this signaling contributes to translational attenuation in these mice remains uncertain.

Methods. C57BL/6J and *rd16* mice were subcutaneously injected with anisomycin to chronically inhibit protein synthesis rates. An AAV2 construct encoding constitutively active 4ebp1 was subretinally delivered in wildtype animals to lower protein synthesis rates. *4ebp1/2* were knocked out in *rd16* mice.

Results. Anisomycin treatment lowered retinal translation rates, accelerated retinal degeneration in *rd16* mice, and initiated cell death in the retinas of C57BL/6J mice. AAV-mediated transfer of constitutively active *4ebp1*-4A into the subretinal space of wildtype animals inhibited protein synthesis, and led to reduced electroretinography amplitudes and fewer ONL nuclei. Finally, we report that restoring protein synthesis rates by knocking out *4ebp1/2* was associated with an approximately 2-fold increase in rhodopsin levels and a delay in retinal degeneration in *rd16* mice.

Conclusions. Our study indicates that protein synthesis inhibition is likely not a cell defense mechanism in the retina by which deteriorating photoreceptors survive, but may be harmful to degenerating retinas, and that restoring protein synthesis may have therapeutic potential in delaying the progression of retinal degeneration.

Keywords: integrated stress response, Translational attenuation, retinal degeneration, ciliopathy, neuroprotection

Aberrant proteostasis has become a hallmark in models of neurodegenerative diseases including Alzheimer's disease,[1] prion diseases,[2] and Parkinson's disease,[3] and interventions aimed at restoring translation have proven to be an effective tool for preserving neurons.[2–6] Using three different mouse models of inherited retinal degeneration (IRD; *rd16*, T17M *RHO*, and *rd10*), we previously showed that the retinas of mice with IRD have reduced levels of protein synthesis.[7] Although translation has been implicated in the pathogenesis of several models of neurodegeneration,[1–6] a direct relationship between translation rates and the progression of neurodegenerative disorders has not been established. In addition, whether translational attenuation is protective or detrimental to degenerating neurons remains to be addressed.

Translation is an energetically expensive process, so its control is tightly regulated.[8–10] Cells use many mechanisms to regulate protein synthesis. One way cells regulate translation is through the phosphorylation of eukaryotic initiation factor 2 α (eIF2α) during the integrated stress response. Another mechanism cells use to regulate protein synthesis is by altering the phosphorylation state of translational repressors, eIF4E-binding proteins (4E-BPs).[11,12] Interestingly, both mechanisms have been dysfunctional in the retinas of mice with IRD.[7,13–15] eIF2α phosphorylation prevents the eIF2 complex from bringing the initiator methionine transfer RNA to the preinitiation complex, resulting in translational inhibition. We and other investigators have reported elevated *P*-eIF2α in the retinas of mice with IRD.[7,13,16] Despite these findings, our reported study suggested that the primary point of translation control in retinal degeneration (RD) may not occur through eIF2α.[7]

Cells also control translation through the tight regulation of eIF4E by 4E-BPs. eIF4E is part of the eIF4F complex and is responsible for binding the 5′ methylguanosine cap of an

mRNA, and therefore, is indispensable for initiation of cap-dependent translation.[17] Depending on phosphorylation state, whether hyper- or hypophosphorylated, 4E-BPs can interact with and prevent eIF4E from joining the cap-binding complex, resulting in reduced levels of protein synthesis.[11,12] The affinity of 4E-BPs to eIF4E decreases as more sites are phosphorylated on 4E-BPs.[12] Depending on the metabolic state of the cell, 4E-BPs can be phosphorylated by the mammalian target of rapamycin (mTOR) at multiple sites, dynamically regulating protein synthesis initiation.[11,12] It is worth noting that 4E-BP1 phosphorylation is significantly downregulated in both *rd16* and *rd10* retinas.[7] Despite all of the advances in knowledge regarding translation inhibition in neurodegeneration,[1–6] it is unclear whether a decline in protein synthesis is protective or whether increasing translation rates could be a viable neuroprotective strategy in IRD.

The role of mTOR in photoreceptor health and disease has been highlighted previously in multiple studies.[14,15,18] We previously reported that the mTOR/AKT/4E-BP axis was inhibited in RD,[7] but to what extent this signaling participates in the regulation of protein synthesis in the retina remains elusive. In this study, we first assessed whether chronic inhibition of protein synthesis could postpone RD in mice with IRD, and then investigated if persistent translational attenuation could initiate cell death in the retina of wildtype animals. Finally, we evaluated if restoring protein synthesis, which was achieved by knocking out *4ebp1* and *4ebp2*, could be a viable strategy for the treatment of RD. Our data have not only highlighted major hallmarks of IRD, but have potentially identified new therapeutic strategies aimed at targeting the activity of 4E-BPs.

Methods

Animals

All animal experiments followed a protocol approved by the University of Alabama at Birmingham institutional animal care and use committee (IACUC #21044) and conformed to guidelines set by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed in a facility with a 12-hour light/dark cycle and had unlimited access to a standard lab diet and water. *rd16* mice (BXD24/TyJ-Cep290rd16/J - 000031) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). *4ebp1/2*$^{-/-}$ mice were generated as previously described.[19] *rd16* mice were crossed with *4ebp1/2*$^{-/-}$ mice. Sample sizes ranged from three to eight. At the time points specified in the following sections, mice were euthanized by CO_2 asphyxiation.

Inhibition of Protein Synthesis

To inhibit translation pharmacologically, mice were injected subcutaneously with 30 mg/kg anisomycin (176880; Millipore, Burlington, MA, USA) twice a day for four days. To validate that the drug could lower protein synthesis in the retina, mice were injected with a single dose of anisomycin and 30 minutes later, they were injected with puromycin. C57BL/6J mice were injected from P12-P15 or from P31-P35 for TUNEL and from P31-P35 for electroretinographic (ERG) analysis. For TUNEL analysis, *rd16* mice were injected from P12-P15 and from P14-P18 for ERG analysis. rAAV2-CAG-*4ebp1*-4A (Thr37, Thr46, Ser65, and Thr70) was generated as described previously.[20] Mice were anesthetized and their eyes were injected subretinally with 0.5 µL 1.0×10^{12} GC/ml of either AAV2-*4ebp1*-4A or AAV2-GFP.

Histology

Eyes were enucleated at time points listed in the text and washed with PBS before fixation in 4% paraformaldehyde at 4°C for 4 hours (immunohistochemistry [IHC] and hematoxylin and eosin [H&E]), or overnight (TUNEL staining). Fixed eyes then were washed with PBS and cryoprotected by immersing in 10%, 20%, and then 30% sucrose. Eyes were embedded in tissue-tek O.C.T. compound (VWR: 25608-930) and kept at –80°C for at least 30 minutes. Eyeballs were cut along the vertical meridian using a cryostat tissue sectioning system (Leica CM3050S; Leica, Buffalo Grove, IL, USA). Sections (12 µm) then were stained with H&E (26754-1A, 26762-01, Electron Microscopy Sciences, Hatfield, PA, USA) and the outer nuclear layer (ONL) nuclei were counted by a masked investigator. Digital images were acquired using a Zeiss Axioplan 2 microscope (Carl Zeiss Meditec, Carlsbad, CA, USA). The number of photoreceptor nuclei was measured in increments of 200 µM from the optic nerve head (ONH) in both hemispheres. The number of photoreceptor nuclei were plotted versus distance (200, 400, 600, 800, 1000, 1200, 1400 µM) from the ONH. Groups were compared using 1-way ANOVA in GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

TUNEL-staining (C10617; Click-it plus TUNEL assay; Thermo Fisher Scientific, Waltham, MA, USA) was performed on retinal sections following instructions from the manufacture. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; H-1200; Vector Laboratories, Burlingame, CA, USA). TUNEL-positive nuclei were counted automatically using the RETINA analysis toolkit for ImageJ (available in the public domain at https://imagej.nih.gov/ij/).[21]

For immunohistochemistry, retinal sections were incubated with an anti-rhodopsin antibody (1D4; MA1-722; Thermo Fisher Scientific). The primary antibody then was detected with anti-mouse secondary Alexa-488 conjugate (R37114; Thermo Fisher Scientific). Sections were counterstained with DAPI.

Protein Synthesis Analysis

Two hours before retinal collection, mice were intraperitoneally injected with Click-it azidohomoalanine (C10102; Thermo Fisher Scientific) at a dosage of 1.20 mg/kg. Retinas were collected and 100 µg protein, as determined by the Bio-Rad protein assay (5000001; Bio-Rad Laboratories, Hercules, CA, USA), was subjected to a click-it reaction, following the protocol outlined by the manufacturer (C10276; Thermo Fisher Scientific). Briefly, the reaction involved chemically linking AHA to an alkyne (Biotin-Alkyne; B10185; Thermo Fisher Scientific). After the tagged proteins were precipitated to remove reaction components, they were resolubilized in 1 × SDS-loading buffer (2% SDS, 10% glycerol, 0.005% bromophenol blue, and 5% 2-mercaptoethanol) and heated at 95°C for 10 minutes before half of the reaction (~50 µg) was separated by SDS-PAGE (4568093, 4568096; Bio-Rad Laboratories). Biotinylated proteins then were probed with HRP-conjugated streptavidin (S911; Thermo Fisher Scientific), incubated in ECL substrate (RPN2232; GE Healthcare, Chicago, IL, USA) and then imaged using a LI-COR (Lincoln, NE, USA) Fc imaging system. After imaging, the membrane was stained with coomassie blue G-250 (161-0786; Bio-Rad Laboratories) for 30 minutes and then destained for 30 minutes (water, ethanol, acetic acid at 50:40:10). Experiments using puromycin followed a protocol described previously.[7,22] Briefly, 40 to 60 µg protein were separated by SDS page and detected using an antibody against puromycin (MABE343; Millipore). Densitometry analyses were performed on entire lanes using ImageJ

software. The relative density of biotinylated lanes was normalized to the densities of the coomassie stained lanes. Protein synthesis was analyzed in *rd16,* C57BL/6J, and *rd16 4ebp1/2*$^{-/-}$ mice at P15. Protein synthesis analysis was performed at P51 (3 weeks PI) for C57BL/6J mice subretinally injected with AAV2-*4ebp1*-4A.

To determine the level of rhodopsin protein expression in degenerating retinas, protein extracts from P15 experimental and control groups were subjected to Western blot analysis. Protein extracts (40 µg) were separated by SDS polyacrylamide gel and transferred to membranes as described previously.[23] Membranes were probed with antibodies against rhodopsin (1D4; MA1-722; Thermo Fisher Scientific) and actin (A2228; Sigma-Aldrich, St. Louis, MO, USA). Normalized rhodopsin was expressed in arbitrary units (a.u.).

Electroretinography

Mice were dark adapted overnight. ERG was conducted using a LKC BIGSHOT ERG instrument. Briefly, mice anesthetized with ketamine and xylazine were dilated, then positioned in the instrument. Mice then were exposed to five flashes of 25cd.s/m^2 in 45-second intervals. ERG waveforms were analyzed in LKC EM software.

Statistics

A *t*-test was used for experiments where two groups were compared. One-way ANOVA was used for analysis of three groups. GraphPad Prism 8 software was used for statistical analysis.

Results

Further Reducing Protein Synthesis in Mice Hastens RD

We previously reported that the retinas of mice with RD produce less protein than wildtype animals,[7] but whether this reduction in protein synthesis is protective for RD or contributes to retinal pathogenesis remains uncertain. We hypothesized that the reduced protein synthesis observed in varied models of IRD is a cell defense mechanism activated to slow down RD. Anisomycin is a known inhibitor of protein synthesis that has been tested in multiple studies including one describing duration of anisomycin mediated translational inhibition at certain doses.[24] We first verified that systemic anisomycin administration was able to inhibit translation in the retina. Indeed, anisomycin resulted in an approximately 78% reduction in retinal protein synthesis in postnatal day (P)-35 C57BL/6J retinas (Supplementary Fig. S1) that was similar to the rate of inhibition observed in one of the previously studied RD models.[7] Therefore, we used the compound to inhibit protein synthesis in *rd16* mice, a well characterized mouse model that mimics Leber congenital amaurosis (LCA) and expresses a truncated centrosomal protein of 290 kD (Cep290).[7,25,26] *rd16* mice were treated with anisomycin for 4 consecutive days. Mice were analyzed at P15 or P18 because their retinas degenerate rapidly.[7,26] We next assessed whether anisomycin treatment affects retinal cell viability. To our surprise, treating *rd16* mice with anisomycin resulted in a massive increase in TUNEL-positive nuclei in the ONL and inner nuclear layer (INL) over vehicle-treated littermates at P15 (Figs. 1A, 1B). We next checked if anisomycin treatment also could affect retinal function in *rd16* mice. Interestingly, *rd16* mice treated with anisomycin had undetectable ERG amplitudes (Fig. 1C) at P18, while vehicle-treated pups demonstrated low but detectable ERG amplitudes, suggesting that treatment compromises retinal function. These experiments suggest that it is unlikely that the reduction of protein synthesis observed in degenerating retinas is a protective mechanism. Therefore, we next tested whether inhibiting protein synthesis could trigger RD in wildtype mice.

Treatment With a Protein Synthesis Inhibitor Induces RD in Wildtype Animals

We next assessed if treatment with the protein synthesis inhibitor anisomycin could result in RD in healthy animals. Anisomycin was delivered subcutaneously to C57BL/6J mice at a dose of 30 mg/kg two times a day for 4 days (P12-P15). We first evaluated if treatment resulted in apoptosis in wildtype animals at P15. Indeed, anisomycin resulted in significantly more TUNEL-positive nuclei in the ONL and INL than vehicle-treated controls (Figs. 2A, 2B), indicating that inhibiting protein synthesis may be sufficient to initiate cell death in the retinas of healthy animals. To ensure this observed elevation in cell death was not due to retinal development (P12-P15), C57BL/6J mice were injected with anisomycin from P30-P35. We first assessed if the treatment could cause a change in retinal function. To our surprise, treating adult mice with anisomycin significantly reduced scotopic a- and b-wave ERG amplitudes (Figs. 2C, 2D). Interestingly, the treatment with anisomycin resulted in a larger (400%) increase in TUNEL-positive nuclei in *rd16* retinas than wildtype retinas, indicating that retinas undergoing degeneration may be particularly vulnerable to changes in protein synthesis rates. We next assessed if treatment also could result in cell death in retinas of adult wildtype animals. Counting TUNEL-positive nuclei revealed a significantly higher number of apoptotic cells in the ONL and INL of anisomycin-treated animals than those treated with vehicle (Figs. 2E, 2F). Aside from the apparent different metabolic states of the cells of the INL and ONL, we also proposed that this result could be due to the subcutaneous route of delivery allowing anisomycin to more readily enter cells of the inner retina. Together with results from *rd16*, these experiments indicated that reduced protein synthesis rates could provoke RD and culminate in neuronal cell loss.

Expressing a Constitutively Active 4ebp1 in the Retina is Sufficient to Promote Loss of Photoreceptors

To more definitively discern if a reduction in cap-dependent translation is sufficient to drive RD, we subretinally injected wildtype mice with an Adeno-associated virus (AAV)-construct expressing a constitutively active form of 4ebp1 (rAAV2-*4ebp1*-4A). This construct encodes a product in which four amino acids (Thr37, Thr46, Ser65, and Thr70) were replaced with alanine making 4ebp1 resistant to phosphorylation by mTOR.[20] Left eyes served as a control and were injected with rAAV2-GFP. Mice were analyzed 3 weeks after injection. As expected, retinas subretinally injected with rAAV2-*4ebp1*-4A expressed significantly more (~200%) total 4ebp1 (Supplementary Fig. S2) than mice injected with rAAV2-*GFP*. Protein synthesis was analyzed by the previously validated SUnSET method.[7] Mice injected with rAAV2-*4ebp1*4A displayed a significant reduction (~40%) in levels of protein synthesis compared to rAAV2-GFP injected eyes (Figs. 3A, 3B). Consistent with anisomycin treatment, eyes injected with rAAV2-*4ebp1*-4A had significantly lower ERG amplitudes compared to control eyes (Figs. 3C, 3D), further suggesting the inhibition of protein synthesis may be sufficient to reduce retinal function in wildtype animals. In addition, rAAV2-*4ebp1*-4A injected eyes

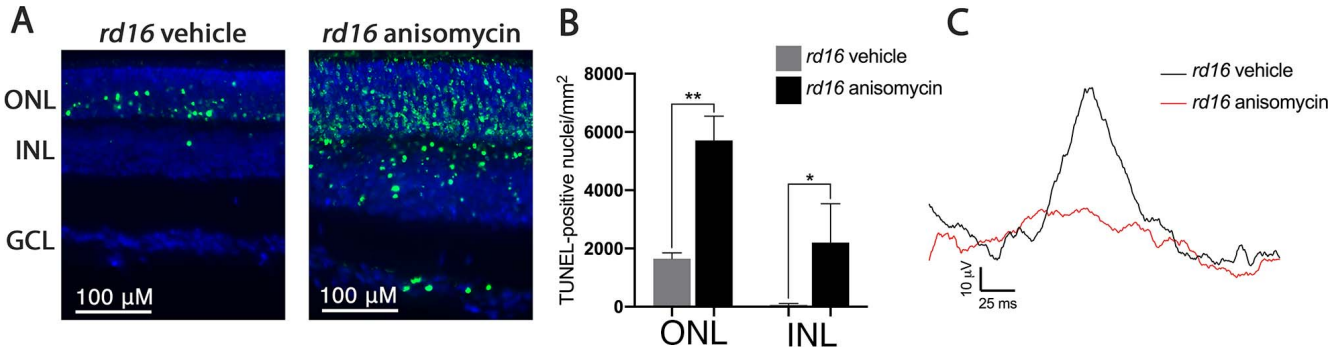

Figure 1. Treatment with a protein synthesis inhibiting compound accelerates RD. (**A**) TUNEL-analysis of retinas of vehicle- and anisomycin-treated *rd16* mice at P15 ($n = 3$). (**B**) Graph demonstrating the results of TUNEL analysis in control and anisomycin treated *rd16* mice. (**C**) Mean ERG waveforms of *rd16* mice treated with vehicle ($n = 3$) or anisomycin ($n = 3$) at P18. *Error bars*: SEM. Statistical significance denoted by $^{*}P < 0.05$, $^{**}P < 0.01$.

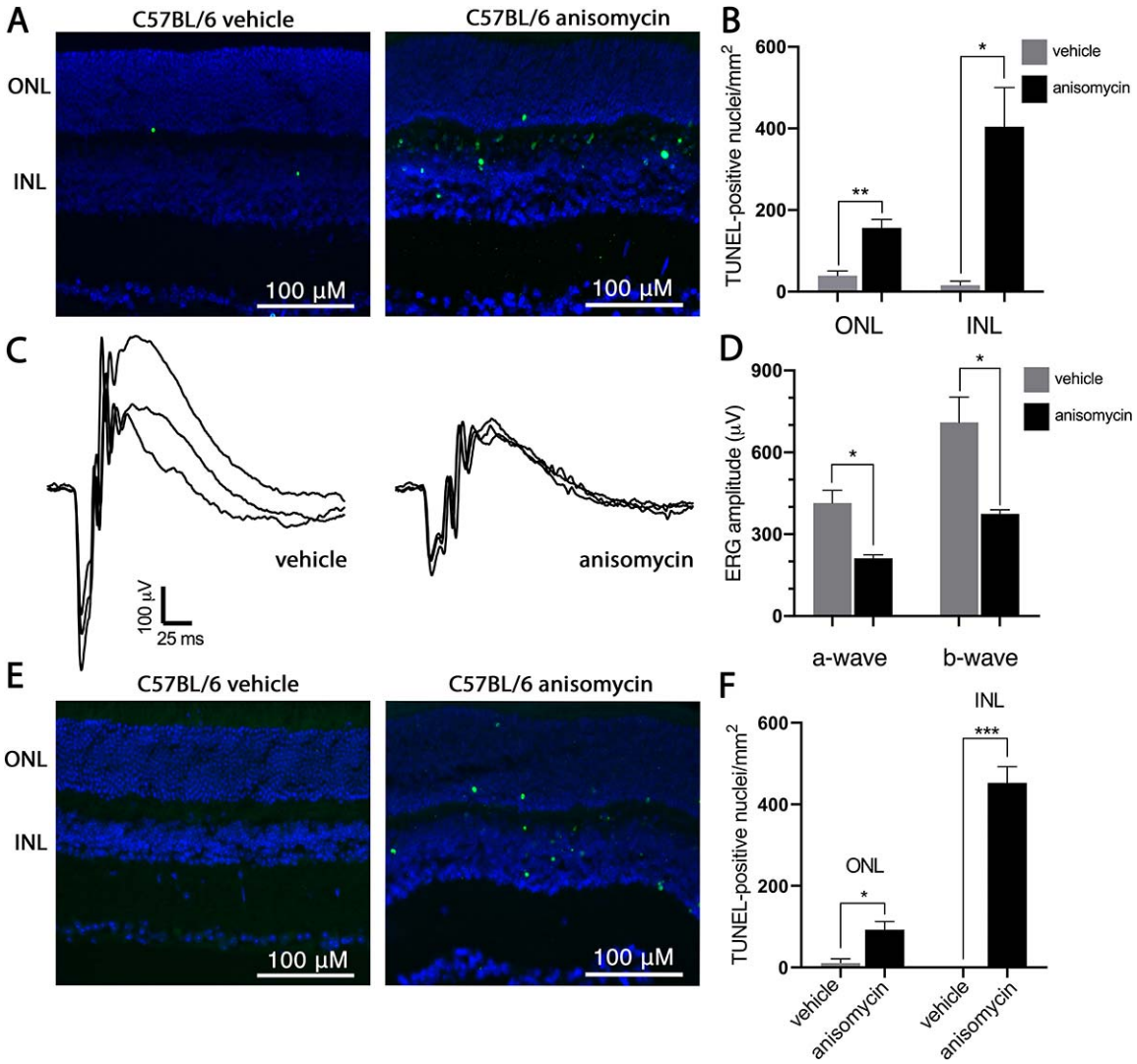

Figure 2. Treatment with a protein synthesis inhibitor, anisomycin, promotes cell death in wildtype retinas. (**A**) TUNEL-analysis of retinas of control ($n = 3$) and anisomycin-treated animals ($n = 3$) at P15. (**B**) Bar graph demonstrating TUNEL-positive nuclei in the ONL and INL of P15 retinas following treatment with vehicle or anisomycin. (**C**) Representative ERG waveforms of vehicle and anisomycin treated mice at P35. (**D**) Graph showing mean A- and B- wave amplitudes of mice treated with vehicle ($n = 7$) or anisomycin ($n = 6$) at P35. (**E**) TUNEL-analysis of retinas of control ($n = 3$) and anisomycin-treated ($n = 3$) animals at P35. (**F**) Bar graph demonstrating TUNEL-positive nuclei in the ONL and INL following treatment with vehicle or anisomycin at P35. *Green*, TUNEL; *blue*, DAPI; GCL, ganglion cell layer. *Error bars*: SEM. Statistical significance denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.001$.

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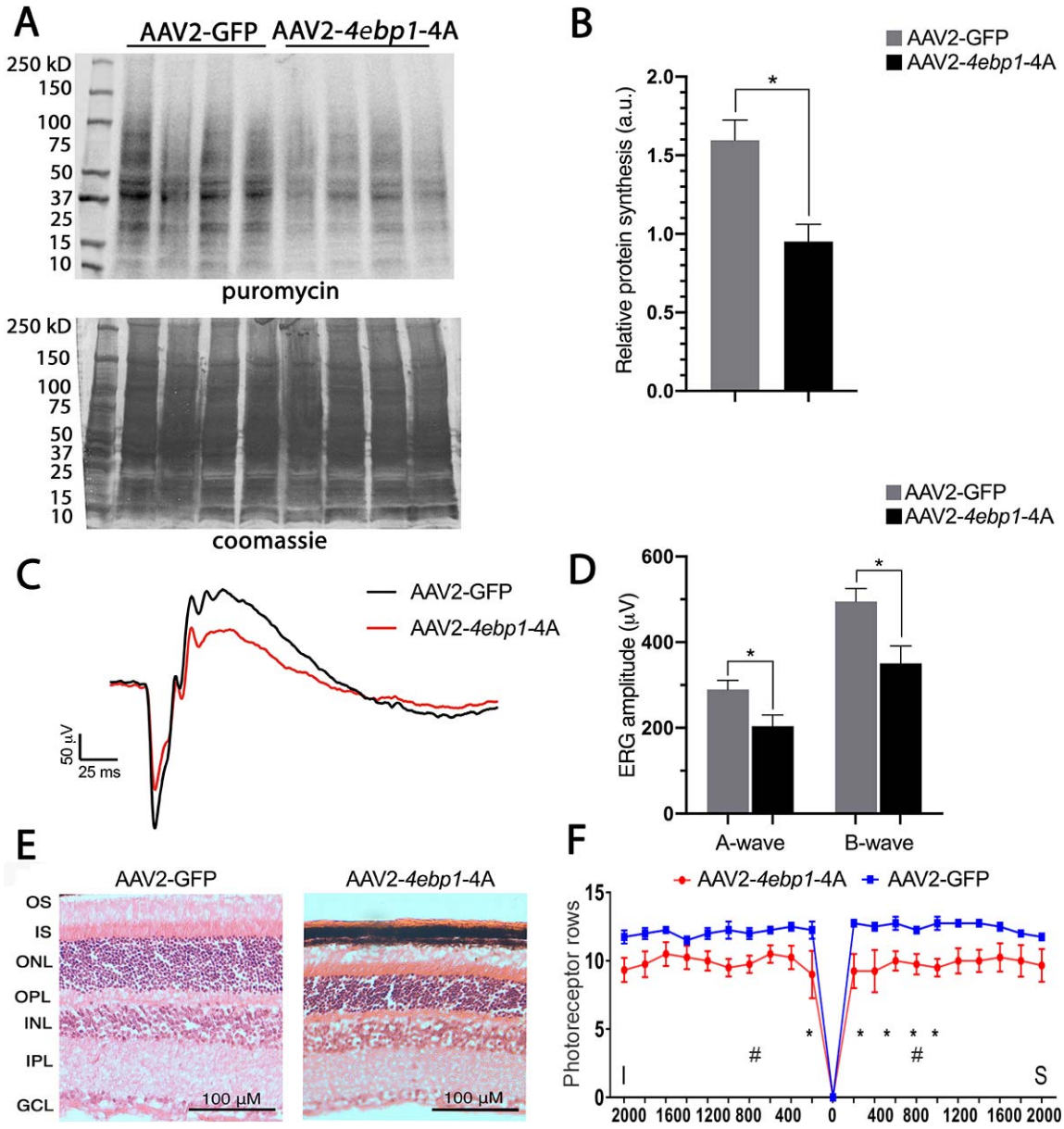

Figure 3. Subretinal injection of an AAV2 construct encoding constitutively active 4E-BP1 is sufficient to initiate cell death in the retina. (**A**) Anti-puromycin Western blot and Coomassie staining of retinal protein from C57BL/6J eyes subretinally injected with AAV2-GFP ($n = 4$) or AAV2-*4ebp1*-4A ($n = 4$). (**B**) Histogram showing relative protein synthesis of AAV2-GFP or AAV2-*4ebp1*-4A injected eyes. (**C**) Averaged scotopic ERG waveforms and (**D**) average ERG amplitude measurements of mice subretinally injected with AAV2-GFP ($n = 8$) or AAV2-*4ebp1*-4A ($n = 8$). (**E**) Representative images of H&E-stained retinal sections of control and experimental AAV2 treated eyes taken in the regions denoted as # in (**F**). (**F**) Spider plots depicting number of photoreceptor nuclei in the ONL as counted by a masked investigator. The spider plots were generated by plotting the number of nuclei using 200 µm step in the distance from the ONH for both hemispheres. Mice were subretinally injected with 0.5 µL 1.0×10^{12} GC/ml AAV. IS, inner segments; OPL, outer plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer. *Error bars*: SEM. Statistical significance denoted by $^{*}P < 0.05$, $^{**}P < 0.01$.

had significantly fewer nuclei in the ONL compared to eyes injected with control AAV2 (Figs. 3E, 3F). Together, these studies indicated that reducing protein synthesis in the retina is likely sufficient to promote retinal cell death, and probably is not a protective mechanism in RD. Therefore, we hypothesized that restoring protein synthesis could delay RD.

Restoring Protein Synthesis Delays RD

Currently, to our knowledge there are no pharmacologic compounds that restore protein synthesis. In our previous study, we demonstrated that endoplasmic stress-mediated phosphorylation of eIF2α in the retinas of mice with IRD; however, we also proposed that eIF2α may not be the primary point of translational control in the retinas of these mice.[7] In addition, we reported that the mTOR/AKT/4E-BP axis is downregulated in mouse models of RD,[7] but to what extent translation is regulated by 4E-BPs in RD remains unknown. We first examined what impact the loss of *4ebp1/2* had on translation rates in healthy retinas. To that end, we first assessed whether knocking out *4ebp1* and *4ebp2*[19] could restore translation in the retinas of *rd16* mice. Consistent with our previous study,[7] the retinas of *rd16* mice demonstrated an approximately 30% reduction in protein synthesis compared to

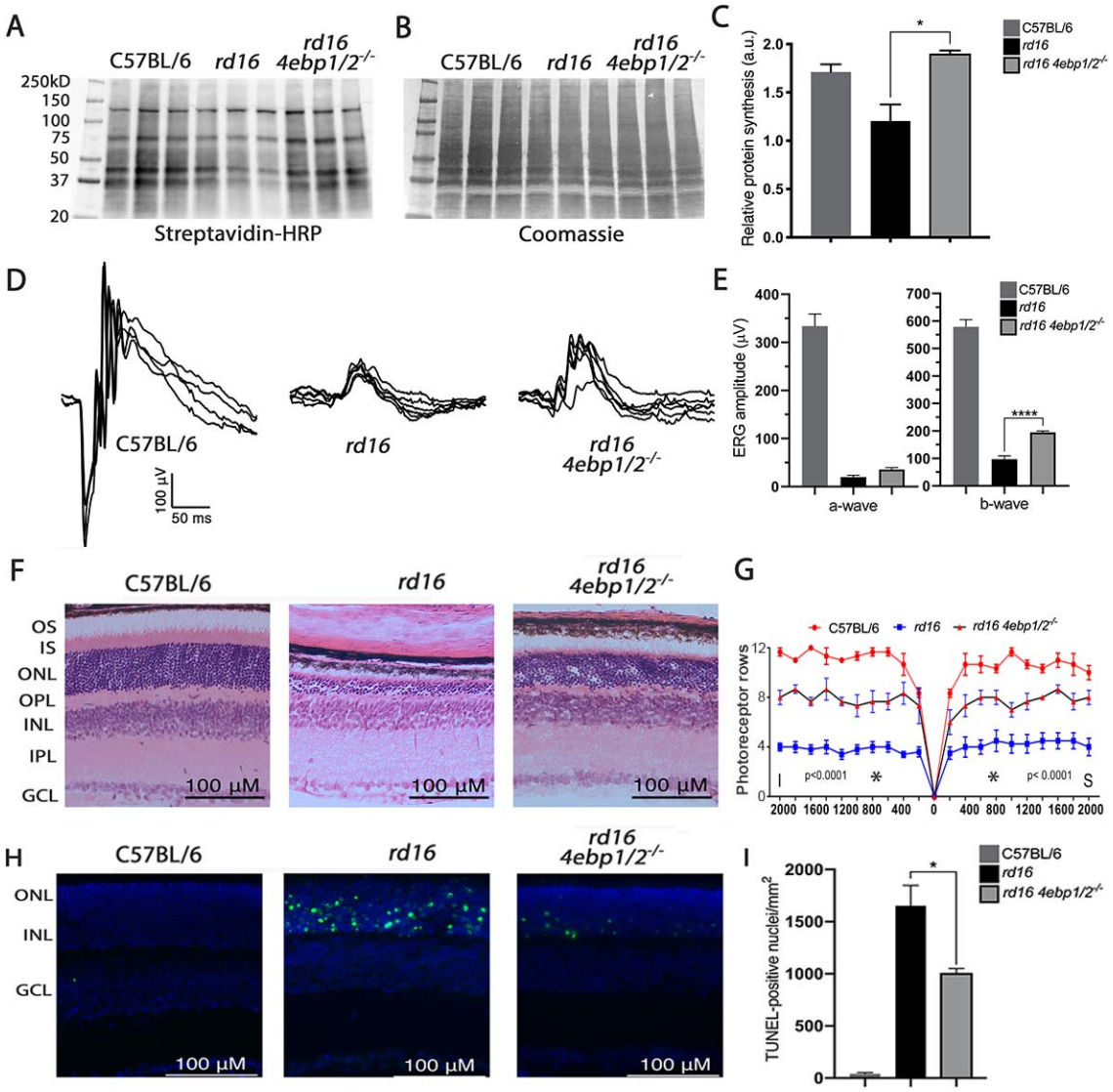

Figure 4. Restoring protein synthesis delays retinal degeneration. (**A**) Protein synthesis levels depicted by the detection of azidohomoalanine incorporation into proteins with HRP-conjugated streptavidin and (**B**) coomassie staining for normalization of the membrane. (**C**) Graph showing relative levels of protein synthesis in the retinas of C57BL/6J ($n = 3$), *rd16* ($n = 3$), and *rd16 4ebp1/2*$^{-/-}$ ($n = 3$) mice at P15. (**D**) ERG waveforms of three groups and (**E**) graph showing mean ERG a- and b- wave amplitudes of C57BL/6J ($n = 4$), *rd16* ($n = 6$), and *rd16 4ebp1/2*$^{-/-}$ ($n = 6$) mice at P17. (**F**) Representative images of H&E-stained retinal sections taken at P18 in the regions denoted as * in (**G**). (**G**) Spider plots depicting number of photoreceptor nuclei in the ONL as counted by a masked investigator. The spidergram was generated by plotting the number of nuclei using 200 µm step in the distance from the ONH for both hemispheres. (**H**) TUNEL staining of retinal sections from C57BL/6J ($n = 3$), *rd16* ($n = 4$), and *rd16 4ebp1/2*$^{-/-}$ ($n = 3$) eyes at P15. (**I**) Graph showing TUNEL-positive nuclei per square millimeter in the three groups. *Green*, TUNEL; *blue*, DAPI; a.u, arbitrary units; kD, kilodaltons. *Error bars*: SEM. Statistical significance denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.001$.

C57BL/6J (Figs. 4A–C). However, translation completely recovered in the retinas of *rd16 4ebp1/2*$^{-/-}$ mice (Figs. 4A–C), indicating that eIF4E could be the major point of translational control in *rd16* mice. We then checked if the restoration of translation in *rd16 4ebp1/2*$^{-/-}$ was associated with functional or morphologic improvements. Surprisingly, the elevation in translation in *rd16 4ebp1/2*$^{-/-}$ was associated with a significant increase in scotopic ERG b-wave amplitudes (Figs. 4D, 4E). This small, but significant increase in ERG b-wave amplitude came as a surprise, since *rd16* photoreceptors have a ciliary defect, making their functional rescue challenging. Furthermore, compared to *rd16*, there was an improvement (~67%) in the number of nuclei in the ONL of *rd16 4ebp1/2*$^{-/-}$ mice (Figs. 4F, 4G). On average, four more rows of photoreceptor nuclei were found in both retinal hemispheres of *rd16 4ebp1/2*$^{-/-}$ retinas. We next checked whether the retinas of *rd16 4ebp1/2*$^{-/-}$ mice had less ongoing photoreceptor apoptosis than *rd16* mice. Indeed, *rd16 4ebp1/2*$^{-/-}$ mice had significantly fewer (~39%) TUNEL-positive nuclei in the ONL than *rd16* at P15 (Figs. 4H, 4I). Lastly, by detecting rhodopsin in retina sections, we assessed if knocking out *4ebp1/2*$^{-/-}$ in *rd16* mice resulted in any improvement in rod photoreceptor integrity (Figs. 5A–C). Interestingly, by Western blot analysis, we found that rhodopsin level was increased 2-fold in *rd16* retinas deficient in *4ebp1/2* (Figs. 5A, 5B) and this increase was in agreement with much stronger labeling of rhodopsin detected by immunohistochemical analysis in *rd16* retinas deficient in *4ebp1/2* (Fig. 5C). Overall, these data indicated healthier photoreceptor cells in *rd16 4ebp1/2*$^{-/-}$ mice at P15. Taken together, these results indicated that

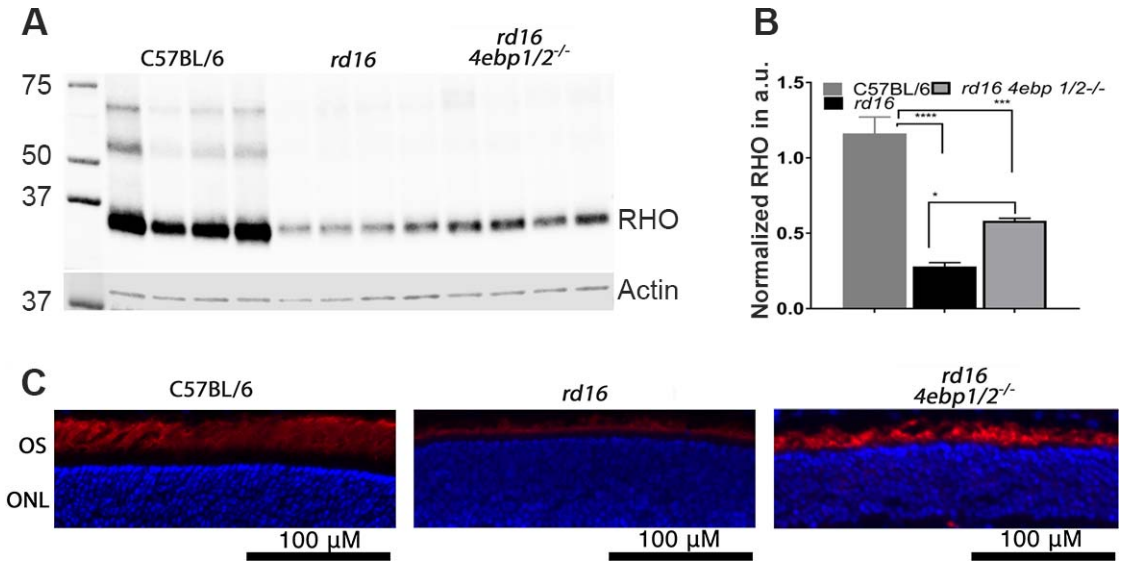

Figure 5. Detection of rhodopsin levels in the retinas of all three mouse groups. (**A**) Western blot analysis was performed at P15 and the retinal protein extracts were run in SDS polyacrylamide gel and transferred to membrane. Images of membrane probed with anti-rhodopsin (*upper*) and anti-actin (*lower*) antibodies are shown. (**B**) The level of rhodopsin was calculated in all three groups. (**C**) Rhodopsin staining of retinal sections from the three groups at P15. *Red*, rhodopsin; *blue*, DAPI. *Error bars*: SEM. Statistical significance denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.001$.

restoring protein synthesis delayed RD in *rd16* mice and could be a valid neuroprotective approach.

Discussion

Although we found that mice with RD have reduced translation rates,[7] before the current research, to our knowledge there have been no studies to examine whether chronically diminished global translation could be involved in the pathogenesis of IRD. Recently, studies conducted with animal models mimicking varied CNS diseases in humans have drawn the attention of the scientific community to the importance of restoring protein synthesis in neurons as a therapeutic strategy to slow the rate of neurodegeneration.[1,2,4] While highly significant to our molecular understanding of the pathogenesis of neurologic disorders and, therefore, moving translational research to the clinic, these studies have not identified: (1) whether global translational attenuation is a pathologic event occurring in degenerating neurons and (2) whether restoration of specific neuronal proteins[4] is accompanied by restoration of general protein synthesis. Our study demonstrate for the first time to our knowledge that translational attenuation in degenerating photoreceptors contributes to retinal pathogenesis and has an impact on photoreceptor viability. The treatment of C57BL/6/J mice with a protein synthesis inhibitor and the subretinal injection with AAV-*4ebp1*–4A resulted in elevated retinal cell death, suggesting that chronic translational inhibition can initiate RD. Moreover, as we demonstrated by our use of anisomycin to inhibit translation in *rd16* mice, protein synthesis inhibition also can accelerate RD. Our study also proposed anisomycin treatment as a model of neurodegeneration although its doses must be carefully validated to control the rate of retinal pathogenesis.

Similar to T17M *RHO* and *rd10*, the retinas of *rd16* mice experience translational attenuation.[7] However, the *rd16* retinas degenerate very rapidly over the course of a few days.[7] Defective photoreceptor ciliogenesis[26] makes it very challenging to rescue *rd16* mice. Moreover, due to the sheer size of the *Cep290* gene, delivering this gene by gene therapy requires special tools.[27] Although not fully restoring vision, any cellular therapy aimed at supporting surviving photoreceptors could be a potential strategy to postpone the onset of ciliopathy in individuals with Bardet-Beidl and Senior Loken syndromes, and LCA. Recently, a group of investigators had some success partially rescuing *rd16* mice using AAV-mediated delivery of a portion of the *Cep290* gene expressing the missing myosin tail homology domain.[28] Our results with *rd16 4ebp1/2*$^{-/-}$ mice showed that even without directly targeting the ciliary defect, retinal degeneration was delayed following the restoration of protein synthesis. This could be due to enhanced synthesis of prosurvival factors that at this stage of retinal degeneration are vital for photoreceptor survival. Together, this would suggest that by combining the recovery of protein synthesis with gene therapy, we could perhaps achieve even more efficient neuroprotection.

Next, as photoreceptors constantly renew the membranous disks in their outer segments (OS), they are very energetically demanding.[27,29] Therefore, our strategy proposing a restoration of protein synthesis could enable enhanced disk morphogenesis and promote a survival of photoreceptors. The latter is in agreement with the study proposing that promoting anabolism in degenerating photoreceptors ameliorates neurodegeneration in retinitis pigmentosa by upregulating the mTORC1 pathway.[30]

Activation of AKT/mTOR signaling has been shown to benefit survival during neurodegeneration.[31,32] Dysregulation of AKT/mTOR signaling has been well documented in neurodegenerative diseases of the brain[33–35] and retina.[7,32,36] Activation of AKT/mTOR signaling has been shown to benefit photoreceptor survival in degenerating retinas as well.[15,18] Additionally, recently Guo et al.[31] proposed that AKT/mTOR-mediated neuroprotection likely involves the stimulation of protein synthesis through its activation of mTORC1. Despite the mounted evidence on therapeutic targeting p-AKT/p-mTOR, the controversy on the efficacy of such therapy remains. For example, recently conducted studies with adenosine monophosphate (AMP)-activated protein kinase, an enzyme known to inhibit mTORC1 and, therefore, stimulate autophagy, have reported conflicting data on survival of degenerating retinas.[37,38] While discrepancies in these studies could stem from the use of two different RD animal models,

the overall safety of the approach aimed at sustained activation of p-AKT/p-MTOR/p-4E-BP in degenerating photoreceptors must be explored further. Thus, a longitudinal study by Zhang et al.,[39] and research conducted by Venkatesh et al.[18] have demonstrated that sustained activation of mTOR/4E-BP pathway does not lead to significant changes in the expression of autophagy genes, an idea further supported by a separate group that proposed targeting autophagy to delay cell death in degenerating photoreceptors.[40] Our results also are in agreement with a study proposing deactivation of 4E-BPs to promote survival in photoreceptors.[39]

Outside of protein synthesis regulation, there are no well-studied functions of 4E-BPs, so it is highly probable that these results are due to the change in translation. Therefore, the observed translational restoration in *rd16 4ebp1/2*$^{-/-}$ mice is a consequence of deleting the genes encoding these translational repressors. It is worth mentioning that special precautions should be taken before using this strategy for RD. For example, exactly when to use this strategy in degenerating retinas, whether before or during degeneration, should be determined experimentally. In addition, elevating protein synthesis not only will boost the production of prosurvival proteins, but also will elevate synthesis of misfolded proteins, which could exacerbate certain retinal degenerative diseases. For example, we observed a 2-fold elevation of rhodopsin level in *rd16 4ebp1/2*$^{-/-}$ mice which was in agreement with overall healthier photoreceptor cells. Therefore, the study with mice expressing misfolded rhodopsin proteins would be necessary to conduct to validate this approach. The balance between photoreceptor demands and expenses should be monitored carefully. Despite this concern, Zhang et al.[39] demonstrated that enhancing the mTORC1/4E-BP1 axis promotes survival in the retinas of *Pde6b*$^{H620Q/H620Q}$ mice. Future studies using a mouse model with less severe RD may grant more valuable insight into how large a role the inhibition of protein synthesis has in retinal cell death.

Our results demonstrated that reduced translation has a never before appreciated role in RD. In addition, restoring protein synthesis may be a valuable therapeutic strategy that could be deployed to delay RD. Although we demonstrated that inhibition of translation through 4E-BPs may be the primary means degenerating retinas use, whether the inhibition of eIF2 contributes to retinal pathogenesis remains unclear. Future experiments using the same models of neurodegeneration would be necessary to modulate both translational modes to come up with the best therapeutic strategy for IRD treatment.

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