Role of Translational Attenuation in Inherited Retinal Degeneration

Christopher R. Starr,1 Cyril N. A. Nyankerh,1 Xiaoping Qi,2 Yang Hu,3 Oleg S. Gorbatyuk,1 Nahum Sonenberg,4 Michael E. Boulton,2 and Marina S. Gorbatyuk1

1Department of Optometry and Vision Science, School of Optometry, University of Alabama at Birmingham, Birmingham, Alabama, United States
2Department of Ophthalmology, and Vision Sciences, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, United States
3Department of Ophthalmology, School of Medicine, Stanford University, Stanford, California, United States
4Department of Biochemistry, McGill University, Montreal, Quebec, Canada

Correspondence: Marina S. Gorbatyuk, Department of Optometry and Vision Science, School of Optometry, University of Alabama at Birmingham, 1670 University Boulevard, Birmingham, Alabama 35233; mgortk@uab.edu.
Submitted: May 14, 2019
Accepted: October 22, 2019

Citation: Starr CR, Nyankerh CNA, Qi X, et al. Role of translational attenuation in inherited retinal degeneration. Invest Ophthalmol Vis Sci. 2019;60:4849–4857. https://doi.org/10.1167/iovs.19-27512

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 electoretinography 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, 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.4–10 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.\textsuperscript{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.\textsuperscript{11,12} The affinity of 4E-BPs to eIF4E decreases as more sites are phosphorylated on 4E-BPs.\textsuperscript{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.\textsuperscript{11,12} It is worth noting that 4E-BP1 phosphorylation is significantly downregulated in both rd16 and rd10 retinas.\textsuperscript{7} Despite all of the advances in knowledge regarding translation inhibition in neurodegeneration,\textsuperscript{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.\textsuperscript{14,15,18} We previously reported that the mTOR/AKT/4E-BP axis was inhibited in RD,\textsuperscript{7} but to what extent this signaling participates in IRD is less clear.\textsuperscript{12} Depending on the metabolic state of the cell, 4E-BP1 phosphorylation is significantly downregulated in both rd16 and rd10 retinas.\textsuperscript{7} Despite all of the advances in knowledge regarding translation inhibition in neurodegeneration,\textsuperscript{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.

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 (B6D2F1/J-Cep290rd16J-4ebp1/2 - 000031) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). 4ebp1/2\textsuperscript{-/-} mice were generated as described previously.\textsuperscript{20} rd16 mice were crossed with 4ebp1/2\textsuperscript{-/-} mice. Sample sizes ranged from three to eight. At the time points specified in the following sections, mice were euthanized by CO\textsubscript{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. AAV2-CAG-4ebp1/4ebp2 (Thr37, Thr46, Ser65, and Thr70) was generated as described previously.\textsuperscript{20} Mice were anesthetized and their eyes were injected subretinally with 0.5 \(\mu\)L of either AAV2-4ebp1/4ebp2 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\textdegree{}C for 4 hours (immunohistochemistry [IHC] and hematoxylin and eosin [H&E]), or overnight (TUNEL staining). Fixed eyes 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\textdegree{}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 \(\mu\)m) were then 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 \(\mu\)m 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 \(\mu\)m) 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/).\textsuperscript{21}

For immunohistochemistry, retinal sections were incubated with an anti-ridhospin antibody (1D4; MAI-722; Thermo Fisher Scientific). The primary antibody was detected with anti-mouse secondary Alexa-488 conjugate (B377114; 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 \(\mu\)g 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 alkyn (Biotin-Alkyne; B10185; Thermo Fisher Scientific). After the tagged proteins were precipitated to remove reaction components, they were resolubilized in 1 \(\times\) SDS-loading buffer (2% SDS, 10% glycerol, 0.005% bromophenol blue, and 5% 2-mercaptoethanol) and heated at 95\textdegree{}C for 10 minutes before half of the reaction (~50 \(\mu\)g) was separated by SDS-PAGE (4568093, 4568096; Bio-Rad Laboratories). Biotinylated proteins were then 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.\textsuperscript{22} Briefly, 40 to 60 \(\mu\)g 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. 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 were then exposed to five flashes of 25 cd/s m² 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,1 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.25 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).rd16 mice were treated with anisomycin for 4 consecutive days. Mice were analyzed at P15 or P18 because their retinas degenerate rapidly.25-26 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.27 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 = 5) or anisomycin (n = 5) 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 = 5) 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.
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 could restore translation in the retinas of rd16 mice. Consistent with our previous study, the retinas of rd16 mice demonstrated an approximately 30% reduction in protein synthesis compared to
C57BL/6J (Figs. 4A–C). However, translation completely recovered in the retinas of rd16 4ebp1/2/C0 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/C0 was associated with functional or morphologic improvements. Surprisingly, the elevation in translation in rd16 4ebp1/2/C0 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/C0 mice (Figs. 4F, 4G). On average, four more rows of photoreceptor nuclei were found in both retinal hemispheres of rd16 4ebp1/2/C0 retinas. We next checked whether the retinas of rd16 4ebp1/2/C0 mice had less ongoing photoreceptor apoptosis than rd16 mice. Indeed, rd16 4ebp1/2/C0 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/C0 mice at P15. Taken together, these results indicated that
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 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/6J mice with a protein synthesis inhibitor and the subretinal injection with AAV-4ebp1/4-A 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 ciliogenesis26 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 domains.26 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.27,28 Therefore, our strategy proposing a restorative 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.50

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 brain33–35 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 autophagy, have reported conflicting data on survival of degenerating retinas.57,58 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-4EBP in degenerating photoreceptors must be explored further. Thus, a longitudinal study by Zhang et al. have demonstrated that sustained activation of mTOR/4EBP pathway does not lead to significant changes in the expression of autophagy genes, an idea further supported by a separate study proposing targeting autophagy to delay cell death in degenerating photoreceptors. Our results also are in agreement with a study proposing deactivation of 4EBPs to promote survival in photoreceptors.

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/C0 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/C0 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. demonstrated that enhancing the mTORC1/4E-BP axis promotes survival in the retinas of Pde6bH620Q/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 4EBPs 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 RD treatment.

Acknowledgments
Supported by National Institutes of Health Grant NIH R01 EY027763.

Disclosure: C.R. Starr, None; C.N.A. Nyankor, None; X. Qi, None; Y. Hu, None; O.S. Gorbatyuk, None; N. Sonenberg, None; M.E. Boulton, None; M.S. Gorbatyuk, None

References
1. Ma T, Trinh MA, Wexler AJ, et al. Suppression of eIF2alpha kinases alleviates Alzheimer’s disease-related plasticity and memory deficits. Nat Neurosci. 2013;16:1299–1305.
2. Moreno JA, Radford H, Peretti D, et al. Sustained translational repression by elf2alpha-P mediates prion neurodegeneration. Nature. 2012;485:507–511.
3. Jan A, Jansonius B, Delaiddeli A, et al. Activity of translation regulator eukaryotic elongation factor-2 kinase is increased in Parkinson disease brain and its inhibition reduces alpha synuclein toxicity. Acta Neuropathol Comm. 2018;6:54.
4. Radford H, Moreno JA, Verity N, Halliday M, Mallucci GR. PERK inhibition prevents tau-mediated neurodegeneration in a mouse model of frontotemporal dementia. Acta Neuropathol. 2015;130:653–642.
5. Sharma V, Onnallah-Saad H, Chakraborty D, et al. Local inhibition of PERK enhances memory and reverses age-related deterioration of cognitive and neuronal properties. J Neurosci. 2018;38:648–658.
6. Mercado G, Castillo V, Soto P, López N, et al. Targeting PERK signaling with the small molecule GSK2606414 prevents neurodegeneration in a model of Parkinson’s disease. Neurobiol Dis. 2018;112:136–148.
7. Starr CR, Pitale PM, Gorbatyuk M. Translational attenuation and retinal degeneration in mice with an active integrated stress response. Cell Death Dis. 2018;9:484.
8. Hinnebusch AG. Structural insights into the mechanism of scanning and start codon recognition in eukaryotic translation initiation. Trends Biochem Sci. 2017;42:589–611.
9. Hinnebusch AG, Lorsch JR. The mechanism of eukaryotic translation initiation: new insights and challenges. Cold Spring Harbor Perspect Biol. 2014;4:a011544.
10. Sonenberg N, Hinnebusch AG. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell. 2009;136:731–745.
11. Gingras AC, Gygi SP, Raught B, et al. Regulation of 4E-BP phosphorylation: a novel two-step mechanism. Genes Develop. 1999;13:1422–1437.
12. Gingras AC, Kennedy SG, O’Leary MA, Sonenberg N, Hay N. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes Dev. 1998;12:502–513.
13. Kunte MM, Choudhury S, Manheim JE, et al. ER stress is involved in T17M rhodopsin-induced retinal degeneration. Invest Ophthalmol Vis Sci. 2012;53:3792–3800.
14. Lin B, Xiong G, Yang W. Ribosomal protein S6 kinase 1 promotes the survival of photoreceptors in retinitis pigmentosa. Cell Death Dis. 2018;9:1141.
15. Punzo C, Kornacker K, Cepek CL. Stimulation of the insulin/mTOR pathway delays cone death in a mouse model of retinitis pigmentosa. Nat Neurosci. 2009;12:44–52.
16. Athanasiou D, Aguila M, Bellingham J, Kanuga N, Adamson P, Cheetham ME. The role of the ER stress response protein PERK in rhodopsin retinitis pigmentosa. Hum Mol Genet. 2017;26:4896–4905.
17. Sonenberg N. elf4E, the mRNA cap-binding protein: from basic discovery to translational research. Biochem Cell Biol. 2008;86:178–183.
18. Venkatesh A, Ma S, Le YZ, et al. Activated mTORC1 promotes long-term cone survival in retinitis pigmentosa mice. J Clin Invest. 2015;125:1446–1458.
19. Le Bacquer O, Petroulakis E, Pargialunga S, et al. Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4EBP1 and 4EBP2. J Clin Invest. 2007;117:387–396.
20. Yang L, Miao L, Liang F, et al. The mTORC1 effectors 4E-BP1 and 4E-BP2 play different roles in CNS axon regeneration. Nat Commun. 2014;5:5416.
21. Mairana DE, Tsoka P, Tian B, et al. A novel ImageJ macro for automated cell death quantitation in the retina. Invest Ophthalmol Vis Sci. 2015;56:6701–6708.
22. Goodman CA, Hornberger TA. Measuring protein synthesis with SunSET: a valid alternative to traditional techniques? Exerc Sport Sci Rev. 2013;41:107–115.
23. Bhootada Y, Kota P, Zolotukhin S, et al. Limited ATF4 expression in degenerating retinas with ongoing ER stress promotes photoreceptor survival in a mouse model of...
1. Autosomal dominant retinitis pigmentosa. *PloS One* 2016;11: e0154779.

24. Wanisch K, Wotjak CT. Time course and efficiency of protein synthesis inhibition following intracerebral and systemic anisomycin treatment. *Neurobiol Learn Mem*. 2008;90:485–494.

25. Subramanian B, Anand M, Khan NW, Khanna H. Loss of Raf-1 kinase inhibitory protein delays early-onset severe retinal ciliopathy in Cep290rd16 mouse. *Invest Ophtalmol Vis Sci*. 2014;55:5788–5794.

26. Chang B, Khanna H, Hawes N, et al. In-frame deletion in a novel centrosomal/ciliary protein CEP290/NPHP6 perturbs its interaction with RPGR and results in early-onset retinal degeneration in the rd16 mouse. *Hum Mol Genet*. 2006;15: 1847–1857.

27. Young RW. The renewal of photoreceptor cell outer segments. *J Cell Biol*. 1967;33:61–72.

28. Mookherjee S, Chen HY, Isgrig K, et al. A CEP290 C-terminal domain complements the mutant CEP290 of Rd16 mice in trans and rescues retinal degeneration. *Cell Rep*. 2018;25: 611–625.

29. Young RW. The daily rhythm of shedding and degradation of rod and cone outer segment membranes in the chick retina. *Invest Ophtalmol Vis Sci*. 1978;17:105–116.

30. Valdés P, Mercado G, Vidal RL, et al. Control of dopaminergic neuron survival by the unfolded protein response transcription factor XBP1. *Proc Natl Acad Sci U S A*. 2014;111:6804–6809.

31. Guo X, Snider WD, Chen B. GSK3beta regulates AKT-induced central nervous system axon regeneration via an elF2Bepsilon-dependent, mTORC1-independent pathway. *Elife*. 2016; 5:e11903.

32. Mao D, Sun X. Reactivation of the PI3K/Akt signaling pathway by the bisperoxovanadium compound bpV(pic) attenuates photoreceptor apoptosis in experimental retinal detachment. *Invest Ophtalmol Vis Sci*. 2015;56:5519–5532.

33. Griffin RJ, Moloney A, Kelliher M, et al. Activation of Akt/PKB, increased phosphorylation of Akt substrates and loss and altered distribution of Akt and PTEN are features of Alzheimer’s disease pathology. *J Neurochem*. 2005;93:105–117.

34. Timmons S, Coakley MF, Moloney AM, O’Neill C. Akt signal transduction dysfunction in Parkinson’s disease. *Neurosci Lett*. 2009;467:30–35.

35. Colin E, Régulier E, Perrin V, et al. Akt is altered in an animal model of Huntington’s disease and in patients. *Eur J Neurosci*. 2005;21:1478–1488.

36. Jomary C, Cullen J, Jones SE. Inactivation of the Akt survival pathway during photoreceptor apoptosis in the retinal degeneration mouse. *Invest Ophtalmol Vis Sci*. 2006;47: 1620–1629.

37. Xu L, Kong L, Wang J, Ash JD. Stimulation of AMPK prevents degeneration of photoreceptors and the retinal pigment epithelium. *Proc Natl Acad Sci U S A*. 2018p;115:10475–10480.

38. Athanasiou D, Aguil M, Opefi CA, et al. Rescue of mutant rhodopsin traffic by metformin-induced AMPK activation accelerates photoreceptor degeneration. *Hum Mol Genet*. 2017;26:305–319.

39. Zhang L, Justus S, Xu Y, et al. Reprogramming towards anabolism impedes degeneration in a preclinical model of retinitis pigmentosa. *Hum Molec Genet*. 2016;25:4244–4255.

40. Moreno ML, Merida S, Bosch-Morell F, Miranda M, Villar VM. Autophagy dysfunction and oxidative stress, two related mechanisms implicated in retinitis pigmentosa. *Front Physiol*. 2018;9:1008.