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Authors
Alavi, MV
Chiang, WC
Kroeger, H
et al.

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In Vivo Visualization of Endoplasmic Reticulum Stress in the Retina Using the ERAI Reporter Mouse

Marcel V. Alavi,1 Wei-Chieh Chiang,2 Heike Kroeger,2 Douglas Yasumura,*1 Michael T. Matthes,1 Takao Iwawaki,3 Matthew M. LaVail,1 Douglas B. Gould,1,4 and Jonathan H. Lin2,5

1Department of Ophthalmology, University of California, San Francisco, San Francisco, California, United States
2Department of Pathology, University of California, San Diego, La Jolla, California, United States
3Advanced Scientific Research Leaders Development Unit, Gunma University, Gunma, Japan
4Department of Anatomy and Institute for Human Genetics, University of California, San Francisco, San Francisco, California, United States
5VA San Diego Healthcare System, San Diego, California, United States

Correspondence: Jonathan H. Lin, Department of Pathology, University of California, San Diego, La Jolla, CA, USA; JLin@ucsd.edu.

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PURPOSE. Endoplasmic reticulum (ER) stress activates inositol requiring enzyme 1 (IRE1), a key regulator of the unfolded protein response. The ER stress activated indicator (ERAI) transgenic mouse expresses a yellow fluorescent GFP variant (Venus) when IRE1 is activated by ER stress. We tested whether ERAI mice would allow for real-time longitudinal studies of ER stress in living mouse eyes.

METHODS. We chemically and genetically induced ER stress, and qualitatively and quantitatively studied the Venus signal by fluorescence ophthalmoscopy. We determined retinal cell types that contribute to the signal by immunohistology, and we performed molecular and biochemical assays using whole retinal lysates to assess activity of the IRE1 pathway.

RESULTS. We found qualitative increase in vivo in fluorescence signal at sites of intravitreal tunicamycin injection in ERAI eyes, and quantitative increase in ERAI mice mated to RboP23H mice expressing ER stress-inducing misfolded rhodopsin protein. As expected, we found that increased Venus signal arose primarily from photoreceptors in RboP23H/+;ERAI mice. We found increased Xbp1S and XBP1s transcriptional target mRNA levels in RboP23H/+;ERAI retinas compared to RboP23H/+;ERAI retinas, and that Venus signal increased in ERAI retinas as a function of age.

CONCLUSIONS. Fluorescence ophthalmoscopy of ERAI mice enables in vivo visualization of retinas undergoing ER stress. ER stress activated indicator mice enable identification of individual retinal cells undergoing ER stress by immunohistochemistry. ER stress activated indicator mice show higher Venus signal at older ages, likely arising from amplification of basal retinal ER stress levels by GFP's inherent stability.

Keywords: ER stress, GFP, funduscopy

THE endoplasmic reticulum (ER) organelle is essential for folding of secretory and membrane proteins, lipid and sterol synthesis, and intracellular calcium storage.1 Diverse environmental and pathologic insults, including protein misfolding, oxidative stress, hypoxia, infection, and inflammation, interfere with ER functions and cause ER stress.2 Chronic ER stress triggers cell death, and has been implicated in the pathogenesis and progression of a wide variety of eye diseases, including age-related macular degeneration, glaucoma, and retinitis pigmentosa.3–7 Transgenic mice expressing fluorescent proteins induced by ER stress8 or protein misfolding9,10 provide a means to track ER stress in real time in live animals at single cell resolution under normal or disease conditions. In principle, these in vivo reporters also could reveal temporal fluctuations in ER stress levels that are too dynamic to detect by in vitro approaches. Here, we qualitatively and quantitatively defined the ability of the ER stress activation indicator (ERAI) mouse, a transgenic line that produces green fluorescent protein (Venus) in response to ER stress, to report changes in ER stress levels in live rodent retina by funduscopic imaging accompanied by histologic, biochemical, and molecular analysis of postmortem retinal tissues.

ER stress activation indicator mice carry a Xbp1-Venus fusion transgene expressed under the control of CMV-β actin promoter that drives transcription in all tissues.8 As illustrated in Figure 1A, the endogenous Xbp1 mRNA contains a small intron that is specifically spliced by inositol-requiring enzyme 1 (IRE1) only when IRE1 has been activated by ER stress.8 Spliced Xbp1 mRNA subsequently produces a potent transcription factor XBP1s that upregulates ER protein folding chaperones and ER-associated protein degradation components to reduce misfolded protein levels and thereby alleviates ER stress.11,12 In the Xbp1-Venus reporter, the inhibitory intron is retained so that fluorescent Venus protein is produced only when ER stress has activated the IRE1 protein (Fig. 1B).8 Thus, the production of fluorescent signal in ERAI mice provides a highly specific readout for ER stress. Importantly, the tran-
Endoplasmic Reticulum Stress in the Retina

A

ER stress

IRE1

5' spliced Xbp1 mRNA

5' unspliced Xbp1 mRNA

Cytosol

Gene transcription

*DNajb9

*Sec24d

*Herpud1

XBP1s

XBP1

B

ER stress

IRE1

5' spliced Xbp1-Venus mRNA

5' unspliced Xbp1-Venus mRNA

No transcription activity

Venus

FIGURE 1. Schematic of the mammalian IRE1 pathway and the function of the XBP1-Venus reporter. Unfolded proteins in the ER (ER stress) activate IRE1, which splices out an intron of the Xbp1 mRNA. Spliced Xbp1 encodes the transcription factor XBP1s, which upregulates proteins that alleviate ER stress (A). Upon activation, IRE1 also can remove an intron of an Xbp1-Venus reporter transgene in ERAl mice. Spliced Xbp1-Venus mRNA encodes a transcriptionally inactive, cytosolic XBP1-Venus fusion protein, which allows for monitoring IRE1 activity by its fluorescence signal (B).

METHODS

Animals

Transgenic ERAl mice18 and RboP23H knock-in mice16 have been described. ER stress activation indicator mice were on a C57BL/6JcJ and RboP23H knock-in mice on a C57BL/6J genetic background. We confirmed by DNA sequencing17 that RboP23H mice do not carry the Crb1<sup>1088</sup> allele, which causes recessively inherited retinal degeneration.18 All data were obtained in hemizygous ERAl animals heterozygous for Crb1<sup>1088</sup>. The C57BL/6J genetic background suppresses the retinal degeneration phenotype associated with recessive Crb1 mutations,18 and we did not observe the intraretinal spots characteristic for this phenotype in heterozygous Crb1<sup>1088/+:ERAI</sup> mice (Supplementary Figs. S1A-J). Animals were kept in a 12-hour light/12-hour dark cycle in full-barrier facilities free of specific pathogens with food (standard rodent diet) and water available ad libitum. Mouse breeding, and all experimental studies and procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of California, San Francisco and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In Vivo Imaging

Mice were anesthetized by inhalation of a constant flow of 1.5% to 3.0% isoflurane, and eyes were dilated with one drop of 1% tropicamide and one drop of 2.5% phenylephrine. Corneas were kept moist with regular application of 2.5% methylcellulose. Both eyes of each animal were examined with a Micron III retinal imaging system (Phoenix Research Labs, Pleasanton, CA, USA). Color fundus images were acquired (single frame, medium light intensity) as RGB TIFF images. Fluorescence ophthalmoscopy was done on the same instrument using a BrightLine single-band filter set optimized for yellow fluorescent protein (YFP-2427B-000; Semrock, Lake Forest, IL, USA), and images were acquired with defined settings for light intensity, exposure time, and gain. We quantified fluorescence as the mean intensity of all pixels in the green channel of the unadjusted RGB TIFF images from the fundus camera using ImageJ 1.47m (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). For illustration of the fundus, the native TIFF images were adjusted with levels and sharpened (unsharp mask, 100%, 2 px) using Photoshop CS6 (Adobe, San Jose, CA, USA). Spectral-domain optical coherence tomography (OCT) images were acquired with the Micron Image Guided OCT System (Phoenix Research Labs) by averaging 10 scans, and levels were adjusted to optimize the tonal range of the images using Photoshop CS6 (Adobe).

Intravitreal Injections

Tunicamycin (0.5 μL 20 μg/mL; EMD Millipore, Billerica, MA, USA) or dimethyl sulfoxide (DMSO) was injected into the vitreous of ERAl mice (n = 3) at P120, and eyes were examined by funduscopy, in vivo fluorescence ophthalmoscopy, and OCT at indicated time points after injection.

Morphology, Immunohistochemistry, and Microscopy

Analysis of retinal morphology has been described previously.15 For immunohistochemistry, eyes were enucleated and fixed by immersion in 4% paraformaldehyde in PBS for 1 hour at room temperature. After overnight incubation with 30% sucrose at 4°C, eyes were frozen in Optimal Cutting Temperature (O.C.T.)
compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA). Sections (8 μm) were cut through the optic nerve head and labeled with indicated antibodies. Sections were blocked with 5% goat serum in 1% BSA/PBS and 0.1% Triton X-100 for 1 hour, followed by incubation with a primary antibody at 4°C overnight. Primary antibodies used were 1D4 anti-rhodopsin antibody at 1:500 dilution (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and anti-GFP at 1:250 (Invitrogen, Carlsbad, CA, USA). After washing in 0.1% Triton X-100 in PBS three times, sections were incubated with secondary antibodies that included Alexa 488 goat anti-mouse (red) antibody (Molecular Probes, Eugene, OR, USA; Invitrogen) and Alexa 488 goat anti-rabbit (green) antibody (Molecular Probes) used at a dilution of 1:500. After washing in PBS three times, cover slips were mounted in Prolong Gold antifade reagent with 4',6-diamidino-2-phenylendole (DAPI; Invitrogen), and images were collected with an Olympus Fluoview-1000 confocal microscope and processed using Olympus Fluoview Ver.2.0a Viewer software with an Olympus FluoView-1000 confocal microscope and 2-phenylendole (DAPI; Invitrogen), and images were collected using an Olympus Fluoview Ver.2.0a Viewser software (Olympus Corporation, Tokyo, Japan) at the University of California, San Diego (UCSD) microscopy facility.

**Quantitative PCR**

Total retinal RNA was collected with an RNasy mini kit (Qiagen, Hilden, Germany). mRNA was reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For quantitative PCR (qPCR) analyses, cDNA were used as templates in SYBR green qPCR supermix (Bio-Rad Laboratories, Inc.). Primers included: Ddit3, 5’-ACGGAACAGAGTGTCAGTG-C-3’ and 5’-CAGGAGGT GATGCCCACTGTTC-3’; Dna6b, 5’-TAAAAGCCGATTGCT GAAGG-C-3’ and 5’-TCCGACTTTTGCACTCGGA-3’; Herpud1, 5’-ACCCAGTGGAGTTGAGGTCG-3’ and 5’-TCTCGATTTT GGAGGTATCTTC-3’; Hspa5, 5’-CTCGGTTGCGTGTTGTC CAAG-3’ and 5’-AAAGGGTCATCATTCAAGGTCG-3’; Rpl19, 5’-ATGC CAATCCTGCTACAGC-3’ and 5’-TCTACCTCTCTACAGGTC GACC-3’; Sec24d, 5’-TCTTGCTCAGTGGCAAGAC-3’ and 5’- GACCCGAAAGGAAGGACTCC-3’; Xbp1s, 5’-GAGTCGCCG CAGATG-3’ and 5’-GTGTCAAGAGTCTCAGGGA-3’. For all qPCR analysis, Rpl19 mRNA levels, a transcript with levels unaltered by ER stress, served as internal normalization standards. Quantitative PCR conditions were 95°C for 5 minutes, 95°C for 10 seconds, 60°C for 10 seconds, 72°C for 10 seconds, with 50 cycles of amplification.

**Statistical Analysis**

Fluorescence intensities at P120 from at least 8 eyes of wild-type mice, Rho+/− mice, and RboP23H/+ mice were compared by a one-way ANOVA (PRISM; GraphPad Software, Inc., La Jolla, CA, USA) and presented as scatter plot showing the means ± SD. Fluorescence intensities at different ages were plotted as means ± SD for the indicated sample size of Rho+/− mice and RboP23H/+ mice, and a linear regression or nonlinear regression model (f = a[1 − exp(−b*x)]) was calculated for Rbo+/− mice and RboP23H/+ mice, respectively (SigmaPlot 12; Systat Software, Inc., San Jose, CA, USA). For qPCR data, results are presented as means ± SD from at least five mice per experimental condition. All data (unless stated otherwise) were compared by Student’s 2-tailed t-tests and differences were considered statistically significant for P values below 0.05 and highly significant for P < 0.001.

**Results**

We tested whether we could monitor ER stress in vivo with ERAI mice. For this, we injected tunicamycin, an agent that strongly induces ER stress by inhibiting N-linked protein glycosylation,19,20 intravitreally into the superior hemisphere of one eye of hemizygous ERAI mice. The second eye was injected with DMSO as vehicle control (Fig. 2). Two days after injection, funduscopy revealed areas of bright lesions in the superior hemisphere of the hemizygous mice (Fig. 2D), which correlated with attenuation of the well-defined layering of the photoreceptor outer segments and RPE as viewed by OCT (Fig. 2E; compare to DMSO-injected wild-type OCT in Fig. 2B, asterisk). The superior, tunicamycin-injected hemisphere showed increased fundus fluorescence compared to the inferior part of the eye (Fig. 2F) and the contralateral control eye that received DMSO (Fig. 2C). At 7 days post injection, the retina of the tunicamycin-injected eye showed advanced disruption and widespread fluorescence (Figs. 2G–I). By 27 days post injection, the superior region of the tunicamycin-injected eye showed degeneration of the outer retinal layers (Fig. 2K) and decreased fluorescence in the superior hemisphere (Fig. 2L). Still, the remaining areas in the tunicamycin-injected eye showed augmented fluorescence, which further increased over time until the animals were killed 70 days after injection. These results demonstrate that ophthalmic examination is capable of monitoring qualitative changes in chemically-induced ER stress in ERAI mice in vivo.

Next, we tested the specificity and sensitivity of ERAI in monitoring genetic causes of ER stress in vivo. We crossed ERAI mice with the RboP23H+/- knock-in mouse line carrying the p.P23H rhodopsin mutation,16 which is the most common mutation in patients with autosomal dominant retinitis pigmentosa in the United States.11,22 The P23H mutation causes rhodopsin protein misfolding and induces ER stress in heterologous cell culture systems and in rodent models of retinal degeneration.15,25–26 Heterozygous RboP23H+/- mice show progressive photoreceptor loss as they age,16 and by P210, the ONL thickness is only 50% compared to wild-type Rbo+/− mice (see Fig. 6A). We compared Rbo+/− wild-type mice without the ERAI transgene (Figs. 3A–C) to Rbo+/-+ERAI animals (Figs. 3D–F) and RboP23H+/-+ERAI animals (Figs. 3G, 3H) of the same genetic background. Funduscopy of all three lines at P120 appeared unremarkable (Figs. 3A, 3D, 3G). Morphologic analysis of two P120 Rbo+/-+ERAI retinas was without pathologic findings (Supplementary Figs. S1K, S1L) and OCT further confirmed the retinal integrity in the analyzed mice (Fig. 3F). RboP23H+/-+ERAI mice showed advanced photoreceptor loss and shortening of the outer segments by OCT (compare Figs. 3E, 3H). We measured the fluorescence signal coming from the Venus protein as read-out for ER stress by funduscopy imaging. Wild-type mice did not show any detectable fluorescence signal (Figs. 3C, 3I), while ERAI mice of both genotypes showed clearly detectable fluorescence (illustrated in Fig. 5F). When we quantified the fluorescence, we found a significantly stronger Venus signal in RboP23H+/-+ERAI mice compared to Rbo+/-+ERAI animals (P < 0.001; RboP23H+/-, n = 15; Rbo+/-, n = 11; Fig. 3I). These results demonstrated that funduscopy ophthalmic examination of ERAI mice enables monitoring of changes in genetic forms of ER stress in vivo.

Next, we investigated temporal changes in Venus fluorescence signal from retinas in Rbo+/-+ERAI and RboP23H+/-+ERAI mice. To this end, we examined animals in vivo by OCT and fluorescence funduscopy from 1 to 9 months of age. The fundus was unremarkable (Supplementary Fig. S1) and OCT revealed no retinal deterioration in Rbo+/-+ERAI mice (Figs. 4A–E). We found progressive thinning of the outer retina in RboP23H+/-+ERAI mice by OCT (Figs. 4F–J). When we examined the same retinas for Venus signal by fluorescence ophthalmoscopy, we found an age-related increase in the fluorescence signal in RboP23H+/-+ERAI animals (Figs. 4P–T). Interestingly, we
also saw a progressive increase in Venus signal in Rbo<sup>p23H/-;ERAI</sup> mice over the same time frame (Figs. 4K–O). These findings indicated that Venus signal in Rbo<sup>p23H/-;ERAI</sup> and Rbo<sup>/-;ERAI</sup> mice increased with age.

To determine which retinal cell type(s) generated the Venus signal, we performed confocal microscopy on immunolabeled mice increased with age. We found that Venus staining in Rbo<sup>p23H/-;ERAI</sup> mice at P30 (Fig. 5A), we saw significantly less Venus labeling against the older animals at P120 (Fig. 5B), Rho<sup>p23H/-;ERAI</sup> mice even though they did not express mutant protein (Fig. 5B). Of note, occasionally cells in the ganglion cell and inner nuclear layers also showed Venus staining in Rbo<sup>/-;ERAI</sup> mice and Rbo<sup>p23H/-;ERAI</sup> mice. Venus is a cytosolic protein, and the labeling in Rbo<sup>/-;ERAI</sup> mice was most prominent in the photoreceptor inner segments and was excluded from the outer segments (Figs. 5A, 5B). When we compared Venus signal from eyes of Rbo<sup>/-;ERAI</sup> mice at P30 (Fig. 5A), we saw significantly less Venus labeling compared to the older animals at P120 (Fig. 5B), consistent with our in vivo imaging results. To rule out that the increased signal in P120 Rbo<sup>/-;ERAI</sup> mice related to funduscopic or confocal imaging artifacts, we performed Western blot analyses against the Venus protein and the Flag-tag at the amino-terminus of the ERAI reporter construct using whole retinal lysates collected from P5 to P270. We found that Venus and FLAG protein levels were significantly higher in older mice. Taken together, the histologic findings revealed that photoreceptors were the predominant retinal cell type expressing Venus. Moreover, our histologic and biochemical analysis further supported the age-related increase in Venus signal that we have discovered in vivo by fluorescence ophthalmoscopy.

Next, we examined quantitative differences in Venus fluorescence between Rbo<sup>/-;ERAI</sup> mice and Rbo<sup>p23H/-;ERAI</sup> mice over time. At P30, we found significant and quantitatively stronger fluorescence in eyes of Rbo<sup>p23H/-;ERAI</sup> mice (n = 9) compared to those in Rbo<sup>/-;ERAI</sup> mice (n = 10, P = 0.037; Fig. 6C). At P60 (Rbo<sup>p23H/-; n = 8, Rbo<sup>/-; n = 4), P90 (Rbo<sup>p23H/-; n = 16; Rbo<sup>/-; n = 13), and P120 (Rbo<sup>p23H/-; n = 15; Rbo<sup>/-; n = 11), we found highly significantly increased Venus fluorescence signal in eyes of Rbo<sup>p23H/-;ERAI</sup> animals (P < 0.001 for all time points; Fig. 6C). However, at P270, we found no significant difference in the fluorescence signal compared to Rbo<sup>/-;ERAI</sup> mice (n = 6 for both genotypes, P = 0.476; Fig. 6C). Our histologic studies identified photoreceptors as the predominant retinal cell type expressing Venus (Fig. 5), and photoreceptors are lost in Rbo<sup>p23H/-;ERAI</sup> mice as they age (Figs. 4, 6A). Therefore, increases in Venus fluorescence in Rbo<sup>p23H/-;ERAI</sup> mice as they aged were likely offset by concurrent loss of the photoreceptors expressing the Venus protein. Indeed, when we normalized our quantification of Venus fluorescence signal to ONL thickness (as a proxy for the number of photoreceptors remaining), we identified substan-
were highly significantly stronger in the wavelength range, while \( \text{Rho}^+/- \) / \( \text{ERAI} \) mice (D-F) and \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice (G-H) showed clearly detectable Venus fluorescence signals, which were highly significantly stronger in \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice (I). *** \( p < 0.001 \).

**Figure 3.** In vivo assessment of genetically-induced ER stress in retinas of \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice. Misfolded rhodopsin causes ER stress and progressive photoreceptor degeneration. Nontransgenic wild-type mice (A-C) did not show any detectable fundus fluorescence in the Venus wavelength range, while \( \text{Rho}^+/- \) / \( \text{ERAI} \) mice (D-F) and \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice (G-H) showed clearly detectable Venus fluorescence signals, which were highly significantly stronger in \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice (I). *** \( p < 0.001 \).

A: wild-type

B: CCL, IPL, INL, OPL, ONL, OS/RPE, choroid

C: 

D: \( \text{Rho}^+/- \) / \( \text{ERAI} \)

E: 

F: 

G: \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \)

H: 

I: 

The Figure 3 legend explains the in vivo assessment of genetically-induced ER stress in retinas of \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice. Misfolded rhodopsin causes ER stress and progressive photoreceptor degeneration. Nontransgenic wild-type mice (A-C) did not show any detectable fundus fluorescence in the Venus wavelength range, while \( \text{Rho}^+/- \) / \( \text{ERAI} \) mice (D-F) and \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice (G-H) showed clearly detectable Venus fluorescence signals, which were highly significantly stronger in \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice (I). *** \( p < 0.001 \).

Finally, more Venus signal in \( \text{Rho}^{P23H+/-} \) / \( \text{ERAI} \) mice at all ages compared to \( \text{Rho}^+/- \) / \( \text{ERAI} \) mice (Fig. 6D).

Last, we examined whether endogenous \( \text{Xbp1} \) splicing and transcriptional function also showed the same increase that we observed with the Venus signal (itself produced by processing of the \( \text{Xbp1} \)-\( \text{Venus} \) transgene). For this, we performed molecular assays to quantify endogenous spliced \( \text{Xbp1} \) mRNA and mRNA levels of multiple downstream genes directly transcribed by the XBP1s protein. In \( \text{Rho}^+/- \) mice, \( \text{Xbp1} \) levels at P60, P90, and P120 appeared to be elevated and relatively stable in these animals compared to P30 (Fig. 7A, black bars and solid line). Next, in retinas of \( \text{Rho}^{P23H+/-} \) mice, \( \text{Xbp1} \) levels were significantly elevated compared to \( \text{Rho}^+/- \) mice at ages P30 (\( p = 0.018 \)) and P60 (\( p = 0.016 \)), while at P90 there was only a trend (\( p = 0.070 \)), and at P120 there was no longer a significant difference between the two genotypes (\( p = 0.499 \); Fig. 7A, black bars and dotted line). By contrast, in retinas of \( \text{Rho}^{P23H+/-} \) mice, \( \text{Xbp1} \) levels were significantly elevated compared to \( \text{Rho}^+/- \) mice at ages P30 (\( p = 0.018 \)) and P60 (\( p = 0.016 \)), while at P90 there was only a trend (\( p = 0.070 \)), and at P120 there was no longer a significant difference between the two genotypes (\( p = 0.499 \); Fig. 7A, black bars and dotted line). Next, we measured mRNA levels of \( \text{Sec24d}, \text{Dnajb9}, \text{Herpud1}, \) and \( \text{Hspa5} \), downstream transcriptional targets of XBP1s. For the \( \text{Rho}^+/- \) mice, the increase in \( \text{Dnajb9} \) and \( \text{Herpud1} \) mRNA levels from P30 to P120 also correlated with the increase in the Venus fluorescence signal (Pearson Product Moment Correlation, \( p < 0.05 \)). In summary, our molecular analysis of the \( \text{Rho}^+/- \) mice showed only a trend between these two ages (\( p = 0.055 \); Fig. 6B). At P120, \( \text{Sec24d} \) showed a significant upregulation compared to P30 (\( p < 0.005 \); Fig. 6B). For the \( \text{Rho}^{P23H+/-} \) mice, the increase in \( \text{Dnajb9} \) and \( \text{Herpud1} \) mRNA levels from P30 to P120 also correlated with the increase in the Venus fluorescence signal (Pearson Product Moment Correlation, \( p < 0.05 \)). In summary, our molecular analysis of the \( \text{Rho}^{P23H+/-} \) mice showed relatively stable endogenous \( \text{Xbp1} \) mRNA levels accompanied by a mild increase in mRNA levels of downstream target genes in older mice. These findings raise the question of why does Venus signal increase so much more compared to levels of endogenous spliced \( \text{Xbp1} \) or its downstream target genes in the \( \text{Rho}^{P23H+/-} \) mice? We considered several possible sources for amplification of the Venus signal in the \( \text{Rho}^{P23H+/-} \) mice in the Discussion.

For the \( \text{Rho}^{P23H+/-} \) mice, \( \text{Sec24d}, \text{Dnajb9}, \text{Herpud1}, \) and \( \text{Hspa5} \) mRNA levels were significantly increased compared to levels in age-matched \( \text{Rho}^+/- \) mice at all time points analyzed (\( \text{Sec24d}, p = 0.002; \text{Dnajb9} \) and \( \text{Herpud1}, p < 0.001; \text{Hspa5}, p = 0.008 \); Fig. 7C), consistent with the increase in \( \text{Xbp1s} \) found by qPCR in \( \text{Rho}^{P23H+/-} \) mice (Fig. 7A). In \( \text{Rho}^{P23H+/-} \) mice, \( \text{Sec24d} \) and \( \text{Hspa5} \) levels trended higher over time with no significant differences between any two time points (Fig. 7C). For \( \text{Dnajb9} \) and \( \text{Herpud1}, \) we found a significant difference between P30 and P60 (\( p = 0.009 \) and \( p = 0.045 \), respectively;
Fig. 7C), but not at subsequent time points. In summary, our molecular analysis of Rho^P23H/+ mice showed increased levels of XBP1s and downstream target genes at all ages compared to Rho^+/+ mice. As a control, mRNA levels of Ddit3/Chop, another ER stress-induced gene regulated by the PERK pathway, showed no changes over time or between genotypes in retinas of Rho^+/+ mice (Fig. 7B) and Rho^P23H/+ mice (Fig. 7C). This finding suggested that the increases we observed in mRNA levels of XBP1s target genes in Rho^+/+ and Rho^P23H/+ mice do not arise through a universal increase in gene expression or unified protein response (UPR) signaling activity as retinas age.
**DISCUSSION**

Many transgenic reporter mice have been created that produce GFP in response to specific molecular stresses, including ER stress, oxidative stress, and protein misfolding. In these reporter mice, the induction of GFP signal reveals which pathologic and environmental circumstances are associated with a molecular stress, at which point in a disease process this stress emerges, and fluorescently marks the tissues and cell types undergoing the stress. In the eye, the ERAl reporter mouse and a GFP protein misfolding reporter transgenic mouse have identified genetic mutations that trigger ER stress or protein misfolding problems in specific retinal cell types primarily through postmortem enucleation and histologic analysis of retinas from reporter mice crossed with mouse models of retinal disease. In stress-induced GFP reporter mice, fluorescence ophthalmoscopy could provide a way to track rapid and dynamic fluxes in ocular stress levels in the same live mouse over time that would not be possible by postmortem enucleation approaches. In this study, we qualitatively and quantitatively measured GFP fluorescence in ERAl mice undergoing chemical and genetic forms of ER stress conditions lasting up to 9 months. We compared changes in ER stress-induced fluorescence levels with changes in ER stress-induced splicing and gene transcription to determine how in vivo fluorescence ophthalmoscopy detection of ER stress correlates with conventional molecular assays used to detect ER stress.

Here, we found in vivo higher ocular signals by fluorescent ophthalmoscopy in ERAl mice challenged with chemical or genetic forms of ER stress. In parallel, we found increased mRNA levels of ER stress-induced genes by qPCR of whole retina lysates collected from RhoP23H/þ;ERAl mice. We also performed confocal microscopy on enucleated eyes from RhoP23H/þ;ERAl mice and found that fluorescent signal was predominantly confined to photoreceptors, the expected retinal cell type undergoing ER stress in RhoP23H/þ mice. Together, our findings demonstrated that increased fluorescent signal corresponds to increased ER stress in ERAl mice. Based

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**FIGURE 5.** Immunohistochemistry against Venus and rhodopsin in RhoP/þ;ERAl and RhoP23H/þ;ERAl mice. RhoP/þ;ERAl eyes (A, B) and RhoP23H/þ;ERAl eyes (C) showed Venus labeling mainly in the photoreceptor layers, as well as single cells in the INL and GCL. Note the increased Venus labeling from P30 to P120 in RhoP/þ;ERAl mice (A, B). Rhodopsin (red), Venus (by anti-GFP, green), nuclear stain (DAPI, blue). Scale bar: 20 μm.
on our experience, we propose that ERAI mice can be used reliably for in vivo identification of conditions that induce ER stress in the eye, although we cannot exclude functional electroretinographic deficits arising in the heterozygous Crb1rd8/+ background, despite histologically normal retinal anatomy in these mice. Histochemical tissue analysis can subsequently identify the precise ocular cell type producing the signal, and with technologic advancements, in the near future, superresolution ophthalmoscopy may do this in vivo. Also as more genetically engineered animals carrying fluorescence reporter become available, standardized quantification of fluorescence signals across different imaging devices and platforms becomes an important issue. Delori et al. resolved this problem by incorporating a small fluorescent plastic piece into the light path of the imaging device serving as internal fluorescent reference.

Quantification of fluorescent signal from ERAI mice in the absence of chemical or genetic sources of ER stress also showed a significant increase in fluorescence as these mice got older. What factors account for the increased fluorescence seen in aging ERAI mice? In lower organisms, ER stress levels increase as a function of age due to a decline in the fidelity of cellular protein quality control and protein homeostasis regulatory mechanisms concomitant with a build-up of misfolded proteins. Age-related increase in retinal ER stress could be one factor contributing to the production of more fluorescent protein and signal seen in the ERAI mice. Indeed, we saw a mild increase in levels of ER stress-induced genes at P120 compared to P30 mouse retinas, but quantification revealed that the magnitude of increase in these molecular ER stress markers was much lower than the magnitude of fluorescence signal increase detected by ophthalmoscopy during the same period. A second factor that likely contributes to the larger increase in fluorescence signal relative to the increase observed in levels of molecular markers of ER stress is the inherent stability of fluorescent proteins. For example, the protein half-life of GFP—of which Venus is a variant—is approximately 26 hours, while the half-life of ER stress-induced proteins typically is less than an hour. The pronounced stability of the XBP1-Venus fusion protein magnifies small increases in ER stress and facilitates sensitive identification of retinal cells afflicted with ER stress in the ERAI mouse. However, Venus' stability likely results in the ongoing presence and accumulation of fluorescent protein/signal in situations where ER stress levels have plateaued or are in decline. Based on our findings, we recommend that quantitative measurements of

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/Journals/IOVS/934564/)
ocular ER stress levels in living ERAI mice using fluorescence ophthalmoscopy take into account GFP's half-life and also be accompanied with independent molecular assays for ocular ER stress levels.

Our study is useful in guiding the development of next-generation transgenic mouse GFP reporters of stress. Destabilized GFPS with short half-lives may provide an opportunity to create transgenic mouse stress reporters with fluorescent signals that better reflect dynamic and rapid changes in stress levels. However, stable and strong fluorescent signal also is necessary to visualize the signal in vivo. Finding the right balance between fluorescent signal amplification and dynamic properties will require careful study. Recently, fundus autofluorescence lifetime imaging was performed successfully in eyes of living mice and may provide more sensitive tools to analyze GFP reporters in the eye. In vivo imaging of fluorescence signal from the retinas of GFP reporter mice also may provide a way to rapidly test the clinical efficacy of candidate pharmacologic agents in modulating ER stress levels, especially the growing number of small molecules that target the IRE1 protein.

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**References**

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supplemental Figure 1
Supplemental Figure 1: Unremarkable fundus of $Rho^{+/+};ERAi$ mice and $Rho^{P23H/+};ERAi$ mice.

Fundus of $Rho^{+/+};ERAi$ mice (A-E) and $Rho^{P23H/+};ERAi$ mice (F-J) at the investigated ages presented without pathological findings. The morphology of two P120 $Rho^{+/+};ERAi$ mice was also unremarkable (K and L).