ATF6 is required for efficient rhodopsin clearance and retinal homeostasis in the P23H rh retinitis pigmentosa mouse model

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Retinitis Pigmentosa (RP) is a blinding disease that arises from loss of rods and subsequently cones. The P23H rhodopsin knock-in (P23H-KI) mouse develops retinal degeneration that mirrors RP phenotype in patients carrying the orthologous variant. Previously, we found that the P23H rhodopsin protein was degraded in P23H-KI retinas, and the Unfolded Protein Response (UPR) promoted P23H rhodopsin degradation in heterologous cells in vitro. Here, we investigated the role of a UPR regulator gene, activating transcription factor 6 (Atf6), in rhodopsin protein homeostasis in heterozygous P23H rhodopsin (Rho+/P23H) mice. Significantly increased rhodopsin protein levels were found in Atf6−/−Rho+/−/P23H retinas compared to Atf6+/−Rho+/−/P23H retinas at early ages (~ P12), while rhodopsin mRNA levels were not different. The IRE1 pathway of the UPR was hyper-activated in young Atf6−/−Rho+/−/P23H retinas, and photoreceptor layer thickness was unchanged at this early age in Rho+/P23H mice lacking Atf6. By contrast, older Atf6−/−Rho+/−/P23H mice developed significantly increased retinal degeneration in comparison to Atf6−/−Rho+/−/P23H mice in all retinal layers, accompanied by reduced rhodopsin protein levels. Our findings demonstrate that Atf6 is required for efficient clearance of rhodopsin protein in rod photoreceptors expressing P23H rhodopsin, and that loss of Atf6 ultimately accelerates retinal degeneration in P23H-KI mice.

Retinitis pigmentosa (RP) is a group of retinal degenerative diseases that leads to irreversible blindness with a worldwide prevalence of 1:40001,2. RP causes progressive retinal degeneration that first results in the loss of rod photoreceptors which then results in the loss of cone photoreceptors, and gives rise to initial night blindness and loss of peripheral vision. Hundreds of gene mutations have been found to cause heritable forms of RP (RetNet, Retinal Information Network, at https://sph.uth.edu/retnet/disease.htm). Mutations in the rhodopsin gene are a common cause of autosomal dominant RP (adRP). More than 150 distinct rhodopsin mutations have been identified in adRP (RetNet| Retinal Information Network- www.sph.uth.tmc.edu/Retnet|2–4. Most mutations introduce missense changes throughout the rhodopsin protein coding region; the most common mutation in the USA is the substitution of proline to histidine at codon 23 (P23H) in the extracellular N-terminal domain, accounting for 15–20% of all adRP cases in the USA5–7.

The endoplasmic reticulum (ER) is vital for membrane protein packaging, secretion, and folding, and, consequently, protein misfolding in the ER can disrupt ER function and lead to “ER stress”. In response to ER stress, cells activate the Unfolded Protein Response (UPR), a cellular homeostatic mechanism that reduces ER stress by promoting the degradation of misfolded proteins and slowing new protein synthesis9. The UPR is controlled by 3 ER resident transmembrane proteins, inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA-like endoplasmic reticulum kinase (PERK). In response to ER stress, IRE1 activates its kinase and RNase functions, initiating the nonconventional splicing of X-box binding protein 1 (XBP-1) mRNA9–11. Spliced XBP-1 (XBP-1s) mRNA encodes a transcription activator that induces expression of...
ER chaperones and ER-associated degradation (ERAD) components that remove and degrade misfolded proteins via the ubiquitin–proteasome system. The PERK arm of the UPR regulates protein synthesis. Upon ER stress, PERK oligomerizes via its cytoplasmic domain, which leads to phosphorylation of eukaryotic initiation factor 2α (eIF2α) and, as a result, attenuation of protein translation. Under prolonged eIF2α phosphorylation, PERK promotes cell death by activating stress-responsive transcription factors such as activating transcription factor 4 (ATF4) and CCAAT/enhancer-binding protein homologous protein (CHOP). ATF6 undergoes proteolytic processing in response to ER stress, releasing its cytosolic bZIP-containing transcription factor domain (ATF6Δ), which then travels to the nucleus to upregulate genes involved in ER protein folding and ERAD (that significantly overlap with the transcriptional targets of XBP-1s). Thus, activation of the UPR ultimately promotes cell viability and homeostasis by reducing misfolded protein levels.

Here, we used the P23H−K.I mouse model to evaluate the UPR’s function in rod photoreceptors in the eye and in the pathogenesis/progression of retinal degeneration arising from misfolded rhodopsin proteins. Within two weeks of age, photoreceptor and retinal degeneration begin in the Rho+/P23H mice. At post-natal day 30 (P30) and P60, degeneration continues with outer nuclear layer (ONL) thinning, loss of rhodopsin protein levels, and poorer ERG responses by a-wave and b-wave analysis. In addition, Rho−P23H mice show progressive shortening of the rod outer segment and rod inner segment at P30 and P60. In healthy retinas, rhodopsin is translated at the ER membrane and, when correctly folded, rhodopsin is exported to the outer segment of rod photoreceptor cells to initiate phototransduction in response to light. In contrast, biochemical and cellular studies reveal that P23H rhodopsin is misfolded, retained in the ER, and aggregates; as a result, P23H rhodopsin may be a source of ER stress and cause activation of the UPR. The role of P23H rhodopsin as an ER stress inducer in rod photoreceptors is supported by the activation of the IRE1 pathway in the retina in P23H−K.I mice, which was found using an IRE1-reporter ER stress activated indicator (ERAI) transgenic mouse crossed with P23H−K.I mice. The Rho+/P23H mice showed significant increase of XBP-1s mRNA in the Xbp-1s targets, such as DNA damage inducible transcript 3 (Ddit3), SEC24 homolog d (Sec24d), and homocysteine inducible ER protein with ubiquitin like domain 1 (Herpud1), in Rho+/P23H mice at P30, P60, P90, and P120. These findings demonstrate increased IRE1 activity in rods expressing the P23H misfolded rhodopsin. We therefore proposed that misfolded rhodopsin generated by the P23H mutation activates the UPR, and that in turn may alleviate ER stress caused by P23H rhodopsin through protein clearance mechanisms such as ERAD.

Previously, we demonstrated that chemical-genetic activation of either IRE1 (to produce XBP-1s) or ATF6f expression promoted P23H rhodopsin protein degradation when expressed in heterologous HEK293 cells, while sparing wild-type rhodopsin protein. By contrast, PERK signaling negatively impacted both misfolded and wild-type rhodopsin protein levels. These findings suggest that IRE1, XBP-1s, and ATF6f are important for removing P23H rhodopsin from photoreceptors and, thereby, influence rod photoreceptor survival. IRE1f+/− and Xbp-1s+/− mice develop widespread developmental defects and undergo early embryonic death. In contrast, Atf6f+/− mice are viable and have normal retinal morphology and function at birth and up to 3-month-old, but aged Atf6f+/− mice develop rod and cone dysfunction and retinal degeneration (18-month-old). Here, we test the role of Atf6 in regulating rhodopsin protein levels and influencing retinal degeneration in P23H−K.I mice by crossing Atf6f+/− with P23H−K.I animals.

### Results

#### Loss of Atf6 leads to impaired clearance of rhodopsin protein and hyperactivation of the IRE1-XBP-1s signaling pathway in early age Rho+/−P23H mice.

Previously, we found that P23H rhodopsin protein was rapidly degraded in photoreceptors of P23H−K.I mice at early age (P15). We also found that chemical-genetic activation of Atf6 signaling pathways promoted P23H rhodopsin protein degradation in heterologous HEK293 cells, while sparing wild-type rhodopsin protein. To investigate if Atf6 is important for P23H rhodopsin protein degradation in photoreceptors, we examined retinas of Rho+/P23H mice bred with Atf6f+/− mice.

First, we examined steady-state levels of rhodopsin in retinal protein lysates collected from Atf6f+/−Rho+/P23H and Atf6f−/−Rho+/P23H at P12, an age before morphological defects in photoreceptors or retinal degeneration emerges in Rho+/P23H mice. We saw significantly increased rhodopsin protein levels in Atf6f−/−Rho+/P23H retinas compared to Atf6f+/−Rho+/P23H retinas (191% increase, P = 0.04, Fig. 1a,b). This finding demonstrates that Atf6f+ is necessary for rhodopsin protein homeostasis in Rho+/P23H mice retinas. Rhodopsin mRNA levels were not significantly different between Atf6f−/−Rho+/P23H and Atf6f+/−Rho+/P23H retinas (P = 0.5, Fig. 1b) indicating that the differences seen in rhodopsin protein levels were not due to transcriptional differences. These findings support a role for Atf6f in maintaining rhodopsin protein homeostasis in the retina via post-transcriptional mechanisms such as regulating its protein degradation.

Previously, we found that the IRE1-XBP-1 pathway was activated in Rho+/P23H mice retinas and promoted P23H rhodopsin protein degradation in vitro. To determine how Atf6 affects IRE1-XBP-1 signaling in retinal lysates of P12 Atf6f−/−Rho+/P23H and Atf6f+/−Rho+/P23H mice. Interestingly, Atf6f−/−Rho+/P23H retinas showed significantly increased IRE1a and binding immunoglobulin protein/78-kDa glucose-regulated protein (BiP/Grp78) chaperone protein expression compared to Atf6f+/−Rho+/P23H retinas (P = 0.04, Fig. 1c). To further investigate if IRE1 signaling pathway was increased in these retinas, we measured the mRNA levels of Xbp-1s (i.e. downstream target of IRE1 pathway) and ERAD-associated E3 ubiquitin-protein ligase Hrd1/Synoviolin 1 (Hrd1/Synv1) mRNA levels, an ER-associated protein degradation gene transcriptionally regulated by Xbp-1s. We found significant increase in the mRNA levels of Xbp-1s (P = 0.03) and an Xbp-1s target gene, Synv1, (P = 0.04) in Atf6−/−Rho+/P23H retinas compared to Atf6+/−Rho+/P23H retinas (Fig. 1d). These findings provide evidence that IRE1 is hyper-activated in Rho+/P23H retina in the absence of Atf6 at this age.
By contrast to the changes observed in IRE1 pathway markers, Chop mRNA levels showed no significant differences between Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H mice (Fig. 1e) consistent with prior studies showing no induction of CHOP in mice expressing P23H rhodopsin31. In summary, we find that loss of Atf6 in Rho+/P23H mice leads to rhodopsin protein build-up in the retina at an early age, concomitant with increased activation of the IRE1 pathway.

No gross changes in retinal histology in the absence of Atf6 in young Rho+/P23H retinas. Overexpression of rhodopsin causes photoreceptor cell death and induces retinal degeneration in transgenic animals expressing wild-type rhodopsin or P23H rhodopsin12–34. With that in mind, we asked if the increased steady-state rhodopsin protein levels in Rho+/P23H mice lacking Atf6 corresponded with photoreceptor cell loss (Fig. 1a).

We performed histologic studies to see if photoreceptors or retinal lamination was impacted. A previously published paper has shown that Rho+/P23H retinas have scattered pyknotic nuclei by P1522, with Rho+/P23H mice exhibiting a slight reduction in the ONL thickness. Furthermore, the outer segments and inner segments of rod photoreceptors were shorter compared to the wild-type mice. In both Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H mice, we observed scattered and disorganized nuclei in the ONL of P15 mice. In addition, the thickness of the ONL, outer plexiform layer (OPL), inner nuclear (INL), and inner plexiform layer (IPL) appeared similar (Fig. 2a,b) in retinas (P>0.05, two-way ANOVA analysis). Therefore, the ~2× increase in rhodopsin protein levels found in the absence of Atf6 did not lead to detectable changes in the thickness of photoreceptor ONL or overall retinal anatomy at this age. These findings demonstrate that Rho+/P23H mice can tolerate increased amounts of rhodopsin protein when Atf6 is lost, at least at this young age.

Rhodopsin protein levels stabilize in intermediate age of Rho+/P23H retina in the absence of Atf6. Next, we examined the retina in older Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H mice. At P30, the thickness of retinal layers between Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H mice showed no significant difference (P>0.05, two-way ANOVA analysis, Fig. 3a,b). In both Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H mice, we observed scattered and disorganized nuclei in the ONL. To investigate if absence of Atf6 altered rhodopsin protein levels in P23H...
responses at all light intensities, we were unable to detect differences in photopic cone response between Atf6−/−Rho retinas. (b) The retinal layers of H&E-stained retinal sections through the optic nerve were measured at 8 locations around the retina, four each in the dorsal and ventral hemispheres. Retinal layer thickness between the two genotypes was not significantly different (n = 3–5; Data represents mean ± SEM, Two-way ANOVA, P > 0.5). ONL outer nuclear layer; OPL outer plexiform layer; INL inner nuclear layer; IPL inner plexiform layer. Scale bar = 50 μm.

retina at this age, we performed immunoblot analyses on the retinas of Atf6+/−Rho and Atf6−/−Rho P30 mice. In contrast to the increase in rhodopsin protein levels observed in younger mice (P12), retinal protein lysates of Atf6−/−Rho showed no significant difference in rhodopsin, BiP/Grp78, and IRE1a protein expression compared to Atf6+/−Rho at this age (Fig. 3c,d). We also found no significant increase in the mRNA levels of Xbp-1s in Atf6−/−Rho retinas compared to Atf6+/−Rho retinas (P > 0.05, data not shown). These results show that loss of Atf6 does not alter rhodopsin protein levels or retinal anatomy in Rho+/− mice by this age. We propose that the equalization of rhodopsin protein levels in Atf6+/−Rho mice to levels seen in Atf6−/−Rho+/P23H mice may be a result of the hyperactivation of IRE1-XBP-1s signaling observed in younger mice.

Increased retinal degeneration in the absence of Atf6 in Rho+/P23H retina in older mice. Last, we examined Rho+/P23H mice lacking Atf6 at an older timepoint—P60. In contrast to P15 and P30 mice, morphological analysis of P60 retinas revealed significantly thinner ONL, OPL, INL, and IPL in Atf6+/−Rho+/P23H when compared to Atf6+/−Rho+/P23H retinas (Fig. 4a). Furthermore, the P60 Atf6+/−Rho+/P23H showed shortening of outer segments and inner segments of photoreceptors in the ONL as previously described. In the ONL, the ventral retinal ONL appeared to be selectively degenerated while the dorsal ONL was preserved in Atf6−/−Rho+/P23H retinas (Fig. 4b; P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001). Consistent with the loss of photoreceptors, we observed a reduction in total rhodopsin protein levels in Atf6−/−Rho+/P23H retinas compared to Atf6+/−Rho+/P23H retinas (P = 0.03; Fig. 4c,d). Furthermore, no differences were observed in BiP/Grp78 or IRE1a levels at this age (Fig. 4c,d). These findings demonstrate that, by P60, loss of Atf6 leads to increased retinal degeneration in Rho+/P23H mice. We speculate that in the absence of Atf6, the duration and intensity of ER stress overwhelms the capacity of IRE1 to support ER homeostasis, leading to increased cell death at later ages of Atf6−/−Rho+/P23H mice.

Assessment of scotopic and photopic function with ERGs. Last, we evaluated rod and cone function in these P60 Rho+/P23H mice lacking Atf6. For the visual response of rods, we measured scotopic (Fig. 5) responses at P60 in Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H of both oculus sinister (OS) and oculus dexter (OD) by full-field ERG. First, the scotopic ERG was recorded, and the amplitudes of the b-wave were analyzed (Fig. 5). In addition, an example of waveforms of the scotopic ERG responses from P60 Atf6+/−Rho+/P23H (blue) and Atf6−/−Rho+/P23H (red) (Fig. 5a,b) retinas were generated. For the amplitudes of the resulting b-wave responses at all light intensities, we did not detect differences in scotopic rod response between P60 Atf6+/−Rho+/P23H and P60 Atf6+/−Rho+/P23H mice. Based on these data, the reduction of retinal layers in ventral retina observed in P60 in absence of Atf6 in Rho+/P23H retina were likely not detected with full-field ERG due to preservation of the dorsal ONL layer.

The photopic ERGs also demonstrated no noticeable changes between P60 Atf6+/−Rho+/P23H and P60 Atf6+/−Rho+/P23H retinas (Fig. 6). An example of waveforms of the photopic ERG responses from Atf6+/−Rho+/P23H (blue) and Atf6−/−Rho+/P23H (red) (Fig. 6a,b) retinas are shown. For the amplitudes of the resulting b-wave responses at all light intensities, we were unable to detect differences in photopic cone response between
Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H P60 mice. In addition, the pattern of waveforms between the two groups were consistent across all light intensities measured.

Discussion

Many disease variants in the human RHODOPSIN gene found in RP patients introduce missense mutations in the rhodopsin polypeptide that cause rhodopsin protein misfolding, retention in the ER, and inability to bind to 11-cis-retinal. These molecular defects instigate rod photoreceptor decline by incompletely understood mechanisms and, ultimately, lead to the clinical manifestations of RP. Currently, there is no cure for RP caused by these misfolded rhodopsin proteins. We previously found that chemical-genetic activation of the ATF6 signaling pathway significantly reduced protein levels of several misfolded RP rhodopsin variants, such as T17M, Y178C, C185R, D190G, and K296E rhodopsin, while sparing wild-type rhodopsin when expressed in heterologous HEK293 cells. Furthermore, activation of ATF6 reduced misfolded P23H mutant rhodopsin protein levels (monomer, dimer, and multimers) in HEK293 cell in vitro. Here, we investigated how ATF6 signaling affected P23H mutant rhodopsin protein in photoreceptors in vivo. We examined the steady-state levels of total rhodopsin protein in retinal samples collected from Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H mice. The rhodopsin protein species in these heterozygous Rho+/P23H mice consist of wild-type and P23H rhodopsin. We found significantly more (nearly 2x) total rhodopsin protein in Atf6−/−Rho+/P23H compared to Atf6+/−Rho+/P23H while rhodopsin mRNA levels did not significantly change between these strains of mice at 12. These findings provide support that Atf6 is important for rhodopsin protein quality control in rod photoreceptors, because in the Atf6−/−Rho+/P23H mice, steady state rhodopsin protein levels increased almost 2x. ATF6 signaling likely ensures the efficient degradation of mutant P23H rhodopsin protein through transcriptional induction of factors involved in ER protein folding and ERAD. Therefore, this model demonstrates that loss of Atf6 leads to accumulation of P23H rhodopsin protein that contributes to the ~2x increase in steady-state rhodopsin protein levels at early ages. Our
findings may provide mechanistic insight into prior studies demonstrating a protective role for ATF6 activity in RP models. For example, in vivo intravitreal AAV injection of one of ATF6’s downstream targets, the BiP/Grp78 chaperone, into P23H rhodopsin transgenic rats improved ERG responses. This protective response could arise from increased BiP/Grp78 chaperone-mediated increase in ERAD. Taken together, these findings underscore the importance of Atf6 plays in rhodopsin protein homeostasis in rods.

Variants in the human ATF6 gene cause achromatopsia and cone-rod dystrophy carrying bi-allelic disease alleles. Patients with these ATF6 mutations showed malformation of the fovea, dysfunction of photoreceptors, and severe vision loss from infancy. Furthermore, it is reported that abnormal retinal vasculature development may lead to malformation of the fovea. However, none of these findings are apparent in young Atf6−/− mice or in young Atf6−/− Rho+/P23H mice (Supplemental Fig. S1). This difference may reflect a selective function for ATF6 in human cone and/or foveal development. For example, retinal organoids produced from the patients homozygous for ATF6 disease alleles showed significant defects in cone photoreceptor development accompanied by reduction in cone gene expression which included all cone phototransduction genes (CNGB3, CNGA3, PDE6C, PDE6H, and GNAT2) and red and green cone opsin genes. Although cones do not appear to be selectively compromised in Atf6−/− mice or Atf6−/− Rho+/P23H mice, the absence of ATF6 does accelerate degeneration throughout the retina in Rho+/P23H mice. This is consistent with ATF6 expression in all retinal cell types, where it likely functions to ensure cell viability in the face of ER stress throughout life.
In our study, we found that the IRE1 signaling pathway was hyper-activated in Atf6−/− Rho+/P23H mice when rhodopsin steady-state levels were increased at young ages. We propose that this hyper-activity in IRE1 signaling reflects a compensatory response to loss of Atf6. Specifically, the loss of Atf6 leads to reduced degradation of mutant rhodopsin protein in Rho+/P23H. In turn, this accumulation of misfolded rhodopsin hyper-activates the IRE1 signaling pathway to degrade the increased rhodopsin accumulating in P12 Atf6−/− Rho+/P23H. Consistent with a role for IRE1 signaling in rhodopsin degradation, we have previously demonstrated that the IRE1 signaling pathway of the UPR is selectively activated in photoreceptors of Rho+/P23H ERAI−/− compared to

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**Figure 5.** Scotopic ERG recordings from Atf6+/− Rho+/P23H and Atf6−/− Rho+/P23H retinas. (a) Representative waveforms generated by scotopic intensity series (− 1.5 to 2 log cd s/m² stimuli) for Atf6+/− Rho+/P23H (blue) and Atf6−/− Rho+/P23H (red) in OS retinas. (b) Representative waveforms generated by scotopic intensity series (− 1.5 to 2 log cd s/m² stimuli) for Atf6+/− Rho+/P23H (blue) and Atf6−/− Rho+/P23H (red) in OD retinas. (c,d) In P60, the b-wave amplitudes of Atf6+/− Rho+/P23H (blue) and Atf6−/− Rho+/P23H (red) in across multiple intensities, ranging from − 1.5 to 2 log cd s/m² showed no significant difference (n = 6, Data represents mean ± SEM, Two-way ANOVA, p > 0.5).
We found that induction of ERAD by IRE1 signaling leads to ubiquitination of P23H rhodopsin in photoreceptors in Rho+/−/P23H mice22. This demonstrated that P23H rhodopsin is rapidly degraded by induction of ERAD in photoreceptors to eliminate misfolded rhodopsin from the ER in vivo. Furthermore, in a 2021 ARVO poster, Rho+/−/ERAI−/− mice in a study that used the ERAI mouse GFP reporter line to indicate IRE1-XBP-1 activation22.

Figure 6. Photopic ERG recordings from Atf6+/−/Rho+/P23H and Atf6+/−/Rho+/P23H retinas. (a) Representative waveforms generated by photopic intensity series (−0.31 to 2.81 log cd s/m² stimuli) for Atf6+/−/Rho+/P23H (blue) and Atf6−/−/Rho+/P23H (red) in OS retinas. (b) Representative waveforms generated by photopic intensity series (−0.31 to 2.81 log cd s/m² stimuli) for Atf6+/−/Rho+/P23H (blue) and Atf6−/−/Rho+/P23H (red) in OD retinas. (c,d) In P60, the b-wave amplitudes of Atf6+/−/Rho+/P23H (blue) and Atf6−/−/Rho+/P23H (red) in across multiple intensities, ranging from −0.31 to 2.81 log cd m² stimuli showed no significant difference (n = 6, Data represents mean ± SEM, Two-way ANOVA, p > 0.5).
Massoudi et al. selectively deleted the gene encoding IRE1α in rod photoreceptor in Rho+/P23H mice and demonstrated that ablation of IRE1α in rod photoreceptors damaged retinal function and increased retinal degeneration in Rho−/+P23H mice. Based on our previous and current study and the recent report by Massoudi et al. (2021), both ATF6 and IRE1α protect against ER stress in photoreceptors in Rho−/+P23H mice. Our current study showed that the levels of Xbp-1s mRNA, BiP/Grp78 protein, and other transcriptional targets were significantly increased in the retinas of Atf6−/−Rho+/P23H mice compared to Atf6+/−Rho+/P23H mice at early age. Many of XBP-1’s target genes encode components of the ERAD pathway, and these genes have been found to be upregulated in the retinas of Rho−/P23H mice. These findings suggest that degradation of P23H rhodopsin via downstream transcriptional activity of the IRE1-XBP-1s pathway and, consequently, ERAD, both work to alleviate ER stress caused by the accumulation of misfolded rhodopsin. Our model is further supported by the findings that E3 ubiquitin ligases, SORDDI1/2, was able to facilitate degradation of Rh P23H (the Drosophila equivalent of P23H rhodopsin) at larval and earlier stages of growth to allow for development of healthy adult eyes. Furthermore, SORDDI1/2 and HRD1/SYVN1 were also able to prevent retinal degeneration in Drosophila with the G69D (glycine to aspartic acid at amino acid residue 69) rhodopsin mutation. The lack of E3 ubiquitin ligases downstream of IRE1-XBP-1s-ERAD to target misfolded rhodopsin in early stages of life. In contrast, Chop mRNA levels (an ER stress gene induced by PERK pathway) were not affected between Atf6+/−Rho+/P23H and Atf6−/−Rho+/P23H mice, which is consistent with previous studies showing that Chop was not induced during retinal degeneration in P23H rhodopsin mice and that the loss of CHOP had no impact on retinal degeneration based on histology or ERG. Activation of PERK signaling also did not lead to greater reduction in rhodopsin protein levels in WT or P23H mice.

There are several lines of evidence suggesting alterations of other degradation systems in Atf6−/−Rho+/P23H mice. We have previously reported in cell culture models that IRE1 relies on functioning proteasomes and lysosomes to degrade the mutated, misfolded rhodopsin. Yao et al. (2018) also reported that P23H mice experience increase in autophagy secondary to ER stress, which leads to proteasome insufficiency and increase retinal degeneration. In contrast, genetic or pharmacologic inhibition of autophagy reduced retinal degeneration and improved proteasome levels. Modulating the ratio between autophagy and proteasome activity (A:P) also helped to improve photoreceptor survival. The authors demonstrated that normalizing the A:P ratio, either by improving folding of P23H rhodopsin or increasing proteasome activity to keep autophagy pathways down, increased photoreceptor survival and preserved retinal function. Taken together, we suggest that autophagy activity is increased as a result of the loss of Atf6.

We found increased retinal degeneration and diminished rhodopsin protein levels in P60 Atf6−/−Rho+/P23H retinas compared to P60 Atf6+/−Rho+/P23H. Furthermore, we found that the thickness of retinal layers including OPL, IPL, INL, and IPL were also significantly lower in the ventral part of the Atf6−/−Rho+/P23H retina compared to Atf6+/−Rho+/P23H. The reduction of ONL in the ventral part of the retina is consistent with previous histological data but the thickness of other retinal layers was not measured previously. In Rho−/+P23H mice, approximately half of the rod photoreceptor cells had disappeared between P14-P40 when compared to Rho−/+ retina, which showed no reduction of rod photoreceptors between P40 and P63 as described in previous studies. Our data demonstrate that by P60, loss of Atf6 accelerates retinal degeneration in Rho−/+P23H mice. Why does loss of Atf6 increase retinal degeneration in Rho−/+P23H mice at P60, while not affecting younger animals? Our previous study showed that early wave of photoreceptor cell death and peak induction of the IRE1 reporter occur during the first postnatal month in Rho−/+P23H mice. The activation of IRE1 in Rho−/+P23H is maintained throughout life to regulate proteostatic balance to remove P23H rhodopsin. We hypothesize that hyperactivation of IRE1 (as seen in the younger animals) restored rhodopsin protein homeostasis in the absence of Atf6 beginning at P12 (i.e., early stage), so that P30 Rho−/+P23H retinas looked indistinguishable. Why can’t IRE1 hyperactivation keep rhodopsin and retina healthy at P60? We propose that the capacity of IRE1 to support ER homeostasis may ultimately be overwhelmed in the absence of Atf6, leading to increased rod photoreceptor cell death, and reduction of rhodopsin protein levels at later ages of Atf6−/−Rho+/P23H mice. The ongoing photoreceptor cell death in RP retina likely causes widespread ER stress from oxidative damage, mitochondrial dysfunction, and other metabolic degenerative mechanisms. Other sources of ER stress arising in the degenerating retina include damaged lipids, proteins, carbohydrates, enzymes, and DNA in photoreceptor cells, which ultimately results in further photoreceptor cell death through lipid peroxidation. Thus, P23H rhodopsin-induced cell damage in addition to P23H rhodopsin protein itself could elicit too much ER stress, overwhelming the proteostatic balance maintained by the IRE1 in in the Atf6−/−Rho+/P23H mice.

Here, we observed no detectable difference in the function of rods and cones between P60 Atf6−/−Rho+/P23H and P60 Atf6+/−Rho+/P23H mice. Although, we observed a reduction of ONL and other retinal layers in Atf6+/−Rho+/P23H mice compared to Atf6−/−Rho+/P23H mice, no significant difference was noted in amplitude of either the scotopic or photopic b-wave in the strains of mice. Why did the reduction of retinal layers in Atf6+/−Rho+/P23H mice compared to Atf6−/−Rho+/P23H mice show no functional changes? We propose that the full-field flash ERG is relatively insensitive to detect smaller defects because it represents the global retinal function via summation of electrical response of the whole retina excited by a flash of light. Thus, the reduction of retinal layers in ventral retina observed in P60 in the absence of Atf6 in Rho−/+P23H retina was likely not detected with full-field ERG due to preservation of the dorsal ONL layer.

In recent years, numerous small molecules have been identified that activate or inhibit ATF6 or IRE1. Agonists of IRE1 signaling include Type 1 IRE1 kinase inhibitors, which allosterically activate the RNase function of IRE1, and IRE1 activators, which activate both RNase and kinase function; however, these small molecule candidates (e.g. 474, I4A4, and I4A6), albeit showing no activation of IRE1-dependent cell death pathways, have yet to be fully tested for rhodopsin proteostatic properties. By contrast, ATF6 agonists (e.g. AA147 and AA263) are effective in vivo and may have significant implications for amyloid related diseases and retinal development through ATF6 activation. Furthermore, research from other groups similarly propose that
BiP/Grp78, a prominent target of ATF6 upon ER stress, alleviates P23H RP symptoms\(^{45}\). We propose that ATF6 and IRE1-XBP-1 small molecule agonists are promising agents for further RP clinical studies if their rhodopsin proteostatic properties can be shown in vivo.

**Methods**

**Animals**

Transgenic Atf6\(^{+/+}\) and Atf6\(^{-/-}\)^{30,41} and Rho P23H-K1 mice\(^{23,24}\) on a pure C57BL/6j background were used to generate Atf6\(^{+/+}\)Rho\(^{+/+}\) and Atf6\(^{-/-}\)Rho\(^{+/+}\) mice for the experiments. First, breeding pairs of Atf6\(^{+/+}\)Rho\(^{+/+}\) and Atf6\(^{-/-}\)Rho\(^{+/+}\) mice of C57BL/6j background were crossed to generate Atf6\(^{+/+}\)Rho\(^{+/+}\). Breeding pairs of Atf6\(^{-/-}\)Rho\(^{+/+}\) with Atf6\(^{-/-}\)Rho\(^{+/+}\) mice of C57BL/6j background were crossed to generate Atf6\(^{-/-}\)Rho\(^{+/+}\).

There are no reported differences in the phenotypes between Atf6\(^{+/+}\) and Atf6\(^{-/-}\) mice\(^{40,41,42,44}\). Injection of Atf6\(^{+/+}\), Atf6\(^{-/-}\), and Atf6\(^{-/-}\) mice with tunicamycin led to kidney and liver toxicity only in Atf6\(^{-/-}\) animals but not in Atf6\(^{+/+}\) or Atf6\(^{-/-}\) mice; in addition, our previous study has shown normal morphology and normal rhodopsin expression when comparing Atf6\(^{+/+}\) to Atf6\(^{-/-}\) mice\(^{30}\). All experiments used female or male Atf6\(^{-/-}\)Rho\(^{+/+}\) mice in comparison to littermates Atf6\(^{+/+}\)Rho\(^{+/+}\), at the postnatal (P) days 12, 15, 30, and 60 (number (n) = 3 – 6 respectively for each stage). For retinal vasculature assessment in Atf6\(^{-/-}\) mice, female and male P30 Atf6\(^{-/-}\) and P30 Atf6\(^{+/+}\) mice (n = 3 animals per group) on a C57BL/6 J background were used as described in previous studies\(^{30,41}\). For all experiments, animals were kept in cyclic 12-h light/dark conditions with free access to food and water. All mouse care and experimental procedures in this study were approved and conducted in strict accordance with relevant guidelines and regulations by the Institutional Animal Care and Use Committee at the Stanford University and in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines.

**Tissue preparation.**

The animals were euthanized by carbon dioxide euthanasia at P12, P15, P30, and P60. The eyes were enucleated for collection of retinal tissue. For secondary method, we performed cervical dislocation. The lens and the anterior segment were removed, and the eyecups were further dissected to collect whole retinal lysate for biochemistry or molecular biology, or eyecups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), for 60 min at 4 °C. After fixation, the eyecups were processed for hematoxylin and eosin (H&E) staining and cryostat sectioning. For cryostat sectioning, eyecups were transferred from 10% for 1 h to 20% for 1 h to 30% sucrose overnight at 4 °C, then eyecups were embedded in Optimal Cutting Temperature (OCT) medium (Tissue-Tek, Elkhart, IN), frozen in liquid nitrogen and subsequently vertically sectioned on a Leica cryostat (Leica Biosystems Inc, Buffalo Grove, IL) at a thickness of 20 μm. For wholemount retinal preparation, the retinas were isolated from the eyecups and dissected as wholemounts.

**H & E staining.**

The detail protocols for H & E staining in retinal layer was performed as previously published\(^{71}\). Three to five left eyecups from three to five animals (n = 3–5) were sectioned along the vertical meridian on a cryostat at a thickness of 20 μm. Sections were then collected on gelatin-coated slides for H&E staining. Slides were dipped in Harris hematoxylin for 1 min then they were washed in tap water and dehydrated in alcohol. Slides were then dipped in Eosin-Phloxyine for 30 s, then dehydrated in a series of 95% ethanol and 100% ethanol followed by 5 min in xylene, and mounted in Vectashield mounting medium (Vector Labs, Burlingame, CA).

**Immunoblotting analysis.**

The detail protocols for immunoblotting analysis were performed as previously published\(^{27,47}\). Three to five right retinas from three to five animals (n = 3–5) were lysed in lysis buffer (0.5 g/mL n-Dodecyl β-D-maltoside (Calbiochem EMD Bioscience, San Diego, CA) in PBS), protease inhibitor (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor (Thermo Scientific, Rockford, IL). Protein concentrations of the total retinal lysates were determined by BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were applied onto 4–15% Mini-PROTEAN TGX precast gels (Bio-Rad, Hercules, CA) and analyzed by immunoblot. Antibodies B630N anti-rhodopsin 1:1000\(^{39}\) (gift of W.C. Smith, Gainesville, FL); anti-BiP/Grp78 and IRE1α at 1:1000\(^{72}\); and anti-HSP90 at 1:1000\(^{22}\) (GeneTex, Inc., Irvine, CA) were used. After immunoblot, the membranes were incubated in SuperSignal West chemiluminescent substrate (Pierce, Rockford, IL). Antibody bound to the immunoblot was detected by horseradish peroxidase-coupled secondary antibody (Cell Signaling, Danvers, MA). Immunoreactive bands were detected with the SuperSignal West chemiluminescent substrate (Pierce, Rockford, IL).

**Quantitative PCR analysis (qPCR).**

The detail protocols for qPCR analysis were performed as previously published\(^{44,49}\). Three to five right retinas from three to five animals (n = 3–5) were lysed and total RNA was collected with a RNeasy mini kit (Qiagen, Germany) and mRNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio Rad, Hercules, CA). Primers that were used included\(^{22,23}\): mouse Rhodopsin mRNA, 5'-TTC ACCACACCCCTCTACACATCAC-3' and 5'-CGGAAGTTGGCTCATGGGTTG-3'; mouse Rho-1s mRNA, 5'-GAGTCGCGCAGCAAGGTG-3' and 5'-GTGTCAGTGCCATGGGAA-3'; mouse Syvn1, 5'- ACACACTAGTG ATGCTGCG-3' and 5'- CTTCACAGAATTGGTGAGGA-3'; mouse Chop, 5'- ACGGAAACAGAGGTGCTCA GTGC-3' and 5'-CAGGAGGATGTGAGATCAGTCC-3'; and mouse Rpl19, 5'-ATGCCAACCTCCGCATTAG-3' and mouse Rpl19 mRNA levels were used as internal normalization standards for qPCR analysis as they were not altered by ER stress. qPCR conditions were 95 °C for 2 min; 95 °C for 10 s; 60 °C for 10 s; 72 °C for 10 s, with 50 cycles of amplification.

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Electroretinography (ERG) and quantification. Mice were dark adapted for 24 h prior to recordings. ERGs (Diagnosys LLC, Lowell, MA) were recorded from both eyes of Atf6−/−Rho−/+P23H (n = 6) mice and compared to ERGs from eyes of Atf6−/−Rho−/+P23H control littermates (n = 6) at P60 as described previously. Mice were anaesthetized using a combination of ketamine (20 mg/kg; KETASET, Fort Dodge, IA, USA) and xylazine (5 mg/kg; X-Ject SA; Butler, Dublin, OH, USA) using similar procedures as our published protocols. Under a dim red light, the pupils were dilated with Atropine sulfate ophthalmic solution 1% (Akorn Inc, Lake Forest, IL, USA). The recording electrodes attached to two gold wire rings were placed on the cornea of both eyes. The eye lubricant hypropemlose ophthalmic gel, USP 2.5% (HUB pharmaceuticals, LLC, Rancho Cucamonga, CA, USA) was applied to keep the hydration and conductivity between the cornea and recording electrodes. The ground and reference electrodes were placed at the tail and tongue, respectively. The eyes were then given scotopic ERG responses (a series of white light flashes varying from -1.5 to 2 log cd s/m²). After 10 min of light adaptation, photopic ERG responses of -0.31 to 2.81 log cd s/m² were recorded. The amplitudes for the resulting b-wave responses at the series of light flash intensity were plotted.

Retinal vasculature staining in Atf6−/− mice. For wholemount immunohistochemical staining, the same procedures described in our previous studies were used. Three right retinas from three animals (n = 3) were used for wholemount staining. Wholemounts were treated with 1% Triton X-100 in 0.1 M PBS (40 min) before NDS (1 h), and antibody against isoclinet B4-Alexa 488 (IB4, molecular probe, 1:200) was diluted in 0.5% Triton X-100 in 0.1 M PBS (48 h at 4 °C). After incubation, wholemounts were washed for 30 min with 0.1 M PB and cover slipped with Vectashield mounting medium.

Equipment and settings. IB-4 staining wholemount (excitation 488, emission 552, 63 × 1.40 oil objective) images were acquired using a Leica SP8 DLS confocal microscope. Images were processed with the Leica application suite-X software (3.0.11.20652, Leica Mcirosystems—Dimension X × Y—local size 1024 × 1024 pixels, 8 Bit for entire wholemount images). For retinal vasculature assessment in Atf6−/− mice, confocal micrographs of the wholemounts (n = 3, animals per group) were taken at the nerve fiber layer (superficial layer), at the IPL (intermediate layer), and at the OPL (deep layer) of the dorsal regions (1 mm away from optic disc) of the retina. At these regions, serial optical section (Dimension z, 2 μm intervals) was made using a confocal microscope. H & E staining sections (20 × objective) images under brightfield were acquired using a NanoZoomer 2.0-HT slide scanner NDP scan 2.5 and viewed using NDP view 2 (Hamamatsu Photonics). The Hamamatsu NanoZoomer uses 3-chip time-delay integration (TDI) sensor signal. Images were processed with the Image Lab Touch Software version 3.0 (Bio Rad). The retinal layer thickness measurement, thickness of the retina was measured at 0.5 mm intervals beginning from the optic nerve. For each retinal section, three measurements of the ONL, OPL, INL, and IPL thickness were taken for each section (fields covering 350 μm × 350 μm), spaced approximately 100 μm apart, which were then averaged. Layer thickness measurements were collected from three to five retinas from separate Atf6−/−Rho−/+P23H and Atf6−/−Rho−/+P23H mice. The results were plotted as a spider plot with distance from the optic nerve as the x-axis and thickness of retinal layer as the y-axis. Intensity of Immunoreactive bands in all blots were measured with National Institute of Health (NIH) Image J software version 1.50i. For presentation, all Photoshop (Adobe photoshop CC 2020) adjustments (brightness and contrast only) were carried out equally in each figure.

Statistical analysis. All the statistics were expressed as mean ± standard error of the mean (SEM). Student’s t-test was used for comparison. Two-way ANOVA and Fisher’s least significant difference procedure (LSD test) were used to examine the differences among the group of means. All the statistical tests were performed using GraphPad Prism Version 8.3.1. The difference between the means of separate experimental groups was considered statistically significant at P < 0.05.

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Competing interests

The authors declare no competing interests.
