Mislocalization and Degradation of Human P23H-Rhodopsin-GFP in a Knockin Mouse Model of Retinitis Pigmentosa

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PURPOSE. To engineer a knockin mouse model that can be used to monitor the effects of treatments on degradation and mislocalization of proline-to-histidine change at codon 23 (P23H) rhodopsin, a common cause of autosomal dominant retinitis pigmentosa (ADRP). The goal was to introduce a gene that expressed rhodopsin at low levels to avoid rapid retinal degeneration, and with a readily visible tag to make it easy to distinguish from wild type rhodopsin.

METHODS. One copy of the endogenous mouse rhodopsin gene was replaced with a mutant human rhodopsin gene that encodes P23H-rhodopsin fused to enhanced green fluorescent protein (GFP) at its C terminus. The gene includes a LoxP site in the sequence corresponding to the 5'-untranslated region, which greatly reduces translation efficiency. Characterized are the resulting heterozygous and homozygous P23H-hRho-GFP mouse lines for mRNA and protein expression, P23H-rhodopsin localization in rod cells, effects on visual function, and retinal degeneration.

RESULTS. The retinas of heterozygous P23H-hRho-GFP mice are morphologically and functionally very similar to those of wild type mice, and they display little cell death over time. P23H-hRho-GFP mice transcribe the knockin gene as efficiently as the endogenous mouse allele, but they contain much less of the protein product than do knockin mice expressing nonmutant hRho-GFP, indicating that substantial degradation of P23H-hRho-GFP occurs in mouse rod cells. The remaining P23H-hRho-GFP mislocalizes to the inner segment and outer nuclear layer, with only approximately 20% in rod outer segments.

CONCLUSIONS. P23H-hRho-GFP mice provide a valuable tool for evaluating the efficacy of potential therapies for ADRP that influence the levels or localization of P23H-rhodopsin.

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Retinitis pigmentosa (RP) is the most common hereditary blinding disease, affecting 1 in 4000 people worldwide. The defining feature of RP is a progressive retinal degeneration that causes patients to experience night blindness early in the course of the disease, suggesting dysfunction of rod photoreceptor cells, followed by loss of peripheral vision and, ultimately, complete blindness. Although mutations in more than 45 genes have been linked to RP, defects in the rhodopsin gene account for approximately 10% of cases.^{1,2} Within the human population, more than 150 different mutations in the rhodopsin gene have been linked to RP, with all but a few causing a dominant form of the disease² (<http://www.sph.uth.tmc.edu/retnet/>; <http://www.hgmd.org/>). Disease-causing dominant mutations are distributed throughout the rhodopsin protein-coding region, with the most frequent in North America being a C to A transversion that encodes a proline-to-histidine change at codon 23 (P23H). P23H was the first RP mutation identified in human patients.³ The pathogenic mechanism of this common mutation remains obscure and effective treatments for the resulting disease are not currently available.

When expressed in mammalian cells, P23H-rhodopsin folds poorly and is transported inefficiently to the plasma membrane, with most being retained in the endoplasmic reticulum (ER), degraded, or incorporated into cytoplasmic aggregates.^{4–8} These observations are largely reproduced in a *Xenopus laevis* model of RP, in which P23H-rhodopsin is mainly confined to the ER in the inner segments of the frog rod cells, but does not form aggregates.⁹ Retention of P23H-rhodopsin in the ER is also supported indirectly by analysis of a transgenic rat model of RP, which shows that the unfolded protein response—an indicator of ER stress—is induced in degenerating retinas.¹⁰ Studies in mouse models of P23H-rhodopsin-induced RP do not paint such a consistent picture. Three mouse models for P23H-rhodopsin have been used to investigate P23H-rhodopsin localization—one with a human transgene,¹¹ one with a mouse transgene,¹² and one with a knockin at the mouse rhodopsin locus.¹³ Studies with these lines agree that expression of P23H-rhodopsin causes retinal degeneration, with features in common with the human disease, and that the severity of retinal degeneration correlates with the level of P23H expression. They differ significantly, however, in their conclusions about the localization of P23H-rhodopsin.

The first study of P23H-rhodopsin-induced RP in mice established three lines carrying genomic human transgenes for the mutant rhodopsin.¹¹ These lines expressed the human transgene mRNA at different levels, equivalent to 6, 2, or 1/3 the expression from a single endogenous copy of the mouse rhodopsin gene. In the two high expression lines, degeneration was rapid, limiting studies of morphologically normal retinas to

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postnatal day 10 (P10), at which time most of the human rhodopsin—detected by an antibody specific for human rhodopsin—was found in the region of the developing inner and outer segments, with significant levels in the outer nuclear layer and in the outer plexiform layer.^{11,14} In the lower expressing, slower degenerating line, however, most of the human P23H-rhodopsin was found in the rod outer segment, although a small fraction was initially misrouted to the outer plexiform layer and later in the course of degeneration to the outer nuclear layer, as well.^{11,14} Parallel studies with lines carrying normal human rhodopsin transgenes demonstrated that human rhodopsin itself is not toxic in mice unless substantially overexpressed,¹¹ a finding confirmed in subsequent studies that showed human rhodopsin is fully functional in mice.¹⁵

Extensive studies have been carried out with a more slowly degenerating mouse line that contains two to five copies of a genomic mouse transgene, which was modified to include five mutations that led to three closely linked amino acid changes: V20G, P23H, and P27L, which we will refer to as GHL.^{12,16–20} The nucleotide changes provide a means to detect GHL mRNA specifically, which in this line is expressed at a level equal to about one copy of an endogenous mouse gene.¹² The flanking amino acid changes were introduced to provide a potential epitope tag specific for GHL opsin. A GHL-specific antibody was used in one study to show that GHL opsin localizes mostly to the rod outer segment, but with some mislocalization to the outer plexiform layer.¹⁹ The majority of localization studies in GHL mice, however, have been carried out using antibodies that do not distinguish between GHL and wild type mouse rhodopsin. In one study the GHL transgene was bred onto a homozygous rhodopsin null background, so that GHL opsin could be specifically tracked.²⁰ In these mice, which do not develop rod outer segments, the GHL rhodopsin was present at much less than its expected levels, suggesting extensive degradation, and it was localized entirely in the ER around the rod cell nuclei in the outer nuclear layer.²⁰ The different conclusions in these two studies could be due to the presence of normal mouse rhodopsin, which may influence the localization of the GHL rhodopsin. Indeed, it has been shown that increasing the amount of normal rhodopsin up to a limit where it is toxic on its own, improves the retinal health of mice that express a P23H transgene.^{20,21}

Recently, a P23H-rhodopsin knockin mouse model was generated, in which the endogenous mouse rhodopsin locus was modified to carry the P23H mutation.¹³ These mice express roughly equal amounts of P23H and normal mouse rhodopsin mRNAs, as expected for a knockin. In mice heterozygous for the P23H knockin and a knockin of a human rhodopsin-GFP fusion gene,²² which makes a longer protein, 90% or more of P23H-rhodopsin was shown to be degraded relative to rhodopsin-GFP.¹³ In mice heterozygous for P23H and normal mouse rhodopsin, all the rhodopsin detected by an antibody that does not distinguish between P23H and normal mouse rhodopsin was found in the rod outer segment.¹³ Given the extensive degradation of P23H-rhodopsin, it is unclear whether mislocalized P23H-rhodopsin could have been detected in these studies.

Collectively, these studies demonstrate that P23H-rhodopsin in mice causes retinal degeneration that exhibits many features in common with the human disease.^{11–13} In the absence of wild type rhodopsin, P23H-rhodopsin appears to be degraded,^{13,20} but there is no information on its fate in the presence of normal rhodopsin. Similarly, there is disagreement as to whether the majority of P23H-rhodopsin is localized correctly^{11,13,19} or mislocalized to other rod cell compartments.^{14,20} Because these questions are fundamental to the mechanism of P23H-rhodopsin induced RP in humans, we sought to establish a P23H-rhodopsin mouse model that could

address P23H-rhodopsin degradation and localization. To do so, we generated a mouse knockin that carries a human P23H-rhodopsin-GFP fusion gene at one of the endogenous mouse rhodopsin loci. We designed the fusion gene to be expressed at a low enough level so that in a heterozygous mouse it triggered no significant retinal dysfunction, thus allowing us to examine degradation and localization in the presence of normal mouse rhodopsin, but in the absence of retinal degeneration. By comparing this mouse line with a previously generated line with a knockin of a human rhodopsin-GFP fusion gene,²² we show that P23H-rhodopsin-GFP is substantially degraded and is mostly mislocalized to the outer nuclear layer and the rod inner segment.

METHODS

P23H-hRho-GFP and hRho-GFP Knockin Mice

All animal procedures were carried out according to protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee, and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. The hrhoG(H) mouse line,²² which expresses hRho-GFP, was backcrossed to C57BL/6 mice for more than 10 generations. We generated P23H-hRho-GFP knockin mice by gene targeting in the HPRT⁺ embryonic stem (ES) cell line AB2.2 123, which was derived from mouse strain 129SvEv, essentially as described previously.^{22,23} We introduced the P23H mutation into the targeting vector by site-directed mutagenesis (QuikChange; Stratagene, Santa Clara, CA). An IScel recognition site was engineered into the middle of the first intron in the rhodopsin gene at position 1340 from the start of translation, but it was not used in the experiments described here. The Darwin Transgenic Core Facility, Baylor College of Medicine, electroporated ES cells and selected for HPRT⁺TK[−] cells, and injected correctly targeted ES cells into blastocysts from albino C57BL/6-Tyr^{c-Brd} mice.²⁴ Founder mice carrying the HPRT-P23H-hRho-GFP allele were crossed to GDF-9-iCre mice²⁵ to remove the HPRT minigene. P23H-hRho-GFP mice were backcrossed to C57BL/6 mice for 9 generations.

Mouse Genotyping

We determined mouse genotypes by PCR analysis of tail DNA. For the knockin allele (HPRT-P23H or P23H-hRho-GFP), we used primers on either side of GFP (5'-GTTCCGGAAGTGCATGCTCACCAC and 5'-GGCGTGCTCTCTGGTGGG), which generate a 933-bp product from the knockin alleles and a 194-bp product from the native mouse rhodopsin locus. For the GDF-9-iCre transgene, we used 5'-TCTGATGAAGTCAGGAAGAACC and 5'-GAGATGTCCTTCACTCTGATTC, which generate a 0.5-kb PCR product.²⁵ For the HPRT minigene, we used primers in the mouse 5' untranslated region (5'-GGGAA-GAGATGGGATAGGTGAG) and in human exon 1 (5'-GCCTTCTGTGC-CATTCATG), which generate a 495-bp product if the HPRT gene is absent, but no product if it is present.

Histology

Fixed eyes were sectioned as previously described,²³ with two modifications. We fixed eyes in 4% paraformaldehyde (EM Science, Hatfield, PA), using PBS buffer, and froze eyes in 100% water-soluble glycol and resin compound (Tissue-Tek OCT; Sakura Finetek, Torrance, CA) on dry ice. Sections (10 to 20 μ m thick) were cut parallel to the axis formed by the lens and the back of the eye with a cryostat (Microm HM500; Microm Instruments, Heidelberg, Germany), affixed to glass slides (Superfrost Plus Micro Slides; VWR, Radnor, PA), air-dried and stored at -20°C until use. For nuclear counts, we stained eye sections from the middle of the eye, where the plane of section is orthogonal to the retina, using DAPI (Vector Laboratories, Burlingame, CA) in mounting media (Vectashield; Vector Laboratories), and captured images on a confocal microscope (Leica TCI SP5; Leica, Buffalo Grove, IL) from

FIGURE 1. Gene targeting and analysis of targeted integrants. (A) Strategy for replacing the endogenous mouse rhodopsin gene with P23H-hRho-GFP. The targeting vector contains 4.1 kb of upstream and 6.5 kb of downstream sequences from the mouse rhodopsin locus (dark gray). The P23H-hRho-GFP gene is shown in black, with the exons indicated by rectangles and introns by lines and GFP shown in white. LoxP sites (inverted triangles) flank the HPRT minigene (light gray), which was inserted at the boundary between the human and mouse rhodopsin sequences to serve as a positive selectable marker for ES cell integration. The 3' boundary between human and mouse DNA is marked by a Lox511 site, which is not shown as it played no role in these experiments. We attached the herpes virus TK gene (white) to the end of the targeting vector to allow selection against random integrants. The portion of the mouse rhodopsin gene in ES cells that was replaced in the targeting is shown in white. (B) Southern blot

several different locations in the retina, excluding areas around the optic nerve and the periphery. For the genotypes examined, no regional differences were detected and the data from all locations was averaged.

Northern Blot Analysis

RNA was isolated from retinas of P23-24 mice after homogenization in reagent (TRI Reagent; Ambion, Grand Island, NY), as previously described.²² RNA was extracted from the aqueous phase by using a mini-kit (RNeasy; Qiagen, Valencia, CA). Samples of total RNA were electrophoresed on 1% agarose denaturing formaldehyde gel (0.22 M formaldehyde, 20 mM Mops buffer, pH 7.0, 5 mM sodium acetate, 1 mM EDTA), transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ), and probed with human rhodopsin cDNA (Open Biosystems, Lafayette, CO), which includes the 1047-bp coding region plus 87 bp of the 5' untranslated region (UTR) and 79 bp of the 3' UTR, labeled by random priming in the presence of ³²P-dCTP (DECAprime II Kit, Ambion). Samples were quantified by scanning the storage phosphor screen (Typhoon TRIO Variable Mode Imager; GE Health Care, Piscataway, NJ) and analyzed with software developed by Wayne Rasband (ImageJ; National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Protein Quantification

We measured total rhodopsin levels by spectral analysis using the differential absorbance of rhodopsin at 500 nm before and after bleaching in the presence of 50 mM hydroxylamine and 1.5% lauryldodecylamineoxide. Whole eyes were homogenized in 240 μ L ROS buffer (1 mM MOPS, pH 7.4; 3 mM NaCl; 6 mM KCl; 0.2 mM MgCl₂; 0.1 mM dithiothreitol; 0.02 mM phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor cocktail (Cat#11460400; Roche Applied Science, Indianapolis, IN). The samples were spun down at 200g and the supernatant was assayed at room temperature using a dual-beam instrument (Olis-modified SLM-Aminco DW-2000; Olis, Inc., Bogart, GA). Rhodopsin concentration was calculated by difference absorbance at 500 nm using the molar extinction coefficient of 42,700 M⁻¹cm⁻¹.²⁶

Electroretinograms

Before ERG testing, we dark-adapted 1-month-old mice overnight and anesthetized them under dim red illumination with an intraperitoneal

analysis of properly targeted ES cells. To identify ES cells carrying the HPRT-P23H-hRho-GFP gene (HPRT-P23H) in place of the mouse gene, we probed for correctly targeted clones at the 5' end, by blotting with the 1.1-kb BamHI-SacI mouse genomic fragment upstream of the 5'-flanking homology used in the targeting vectors. For the 3' end, we used a 1.0-kb BglII-EcoRI DNA probe, which is a subfragment of the 6.5-kb 3'-flanking homology used in the targeting vector. Restriction enzymes used to digest genomic DNA are indicated in parentheses. The positions of the relevant BamHI (B) and HindIII (H) restriction sites are shown in (A), along with the locations of the hybridization probes (short lines). The BamHI site on the left is farther upstream than its position in the diagram would indicate. Correct targeting at the 5' end is indicated by a 8.9-kb band; correct targeting at the 3' end is indicated by a 6.7-kb band. Sizes are in kb. (C) PCR analysis of founder mice carrying the HPRT-P23H allele. We used PCR primers that flanked the GFP gene and amplified both the unmodified mouse allele (+) and the HPRT-P23H allele. Sizes are indicated in kb. (D) Mating scheme to generate mice that are HPRT-P23H and GDF-9-iCre positive. To eliminate the HPRT minigene, we first bred offspring heterozygous for the HPRT-P23H allele to mice with an iCre transgene driven by the GDF-9 promoter, which is expressed in the female germline,²⁵ to generate mice that carried both genes (pup 3). Examples of pups with the expected genotypes are shown. (E) Generation of mice that are P23H-hRho-GFP positive, HPRT negative, and iCre negative. Female HPRT-P23H/GDF-9-iCre mice were crossed to wild type mice and pups were analyzed by PCR to identify those that carried P23H-hRho-GFP (P23H), but lacked HPRT and GDF-9-iCre (pup 1). Examples of mice with the expected genotypes are shown.

A P23H/mRho **B P23H/P23H**

1 Month

2 Months

3 Months

FIGURE 2. Confocal images of retinal sections from heterozygous and homozygous P23H-hRho-GFP mice. **(A)** Retinas from heterozygous P23H-hRho-GFP (P23H/mRho) mice at 1, 2, and 3 months of age. **(B)** Retinas from homozygous P23H-hRho-GFP (P23H/P23H) mice at 1, 2, and 3 months of age. In all cases, retinal sections were stained with DAPI and examined on a confocal microscope (Leica SP5). Retinal sections from various regions across the retina—excluding the periphery and the optic nerve—were examined. As illustrated in these representative images, they show that photoreceptors are preserved across the retinas in P23H/mRho heterozygotes, but are abnormal and rapidly lost in P23H/P23H homozygotes.

injection of ketamine (70 mg/kg), xylazine (14 mg/kg), and acepromazine (1.2 mg/kg). To dilate the pupils, we applied drops of 1% tropicamide and 2.5% phenylephrine and a single drop of 0.5% proparacaine to each eye for corneal anesthesia. Mice were placed on a heating pad at 39°C inside a Ganzfeld dome. Platinum electrodes mounted on micromanipulators were positioned on each cornea and a small amount of 2.5% methylcellulose was applied to each eye. Platinum subdermal needle electrodes were inserted into the tail and forehead to serve as ground and reference, respectively. Signals were bandpass filtered from 0.1 to 1000 Hz and digitally sampled at 10 kHz. Flashes in the scotopic range were generated by a pair of cyan light-emitting diodes (LEDs; Luxeon K2 LEDs; $\lambda_{\text{peak}} = 505 \text{ nm}$, $\Delta\lambda_{1/2} = 30 \text{ nm}$; Phillips Lumileds, San Jose, CA) wired in series. Square pulses of 0.5 ms duration and varying currents were driven through the LEDs to create flashes of different intensities. The two brightest flashes were generated with 1500 W xenon flash bulbs (Novatron, Dallas, TX). At the lowest intensity, 25 responses were averaged with a delay of 4 seconds between each flash. For analysis of ERG waveforms, the a-wave was measured from baseline to trough of the initial negative deflection (unfiltered) and the b-wave was measured from a-wave trough to peak of the subsequent positive deflection (low-pass filtered, $f_c = 60 \text{ Hz}$).

Statistical Analysis

Statistical analyses were conducted (PASW Statistics GradPack 18; IBM, Armonk, NY). For the analyses of expression of human P23H-rhodopsin-GFP and degradation and localization of human P23H-hRho-GFP (see Figs. 5 and 6), we measured four mice per genotype. For the spectrophotometric analysis of rhodopsin protein concentration (see Fig. 5B), we performed a univariate ANOVA with a least squares difference post hoc analysis. For analyzing the differences in GFP fluorescence intensity in the hRho/mRho and P23H/mRho mice (see Fig. 6B), we conducted a univariate ANOVA. For analyzing the differences in GFP localization (see Fig. 6C), we measured three different sections per mouse and performed a multivariate ANOVA with repeated measures.

FIGURE 3. Counts of nuclei in the outer nuclear layer (ONL) as a function of age. Nuclei in the outer nuclear layer were counted in wild type mice (mRho/mRho, *filled circles*), heterozygous P23H-hRho-GFP mice (P23H/mRho, *open squares*), and homozygous P23H-hRho-GFP mice (P23H/P23H, *open diamonds*). For each retina, we counted 60 to 100 columns of nuclei from several different locations, excluding areas around the optic nerve and the periphery, and averaged them for each time point. Nuclear counts were made in eyes from multiple mice for each time point: three to four eyes for mRho/mRho mice, three to nine for P23H/mRho mice, and two to five for P23H/P23H mice. Error bars indicate standard errors of the mean.

FIGURE 4. ERG analyses of wild type (mRho/mRho), heterozygous P23H-hRho-GFP (P23H/mRho), and homozygous P23H-hRho-GFP (P23H/P23H) mice at 1 month of age. (A) Representative dark-adapted ERGs over a wide range of flash energies. Flash energy units are log (scot cd)(s)/m². (B) A-wave amplitudes showing no statistical difference between mRho/mRho (filled circles) and P23H/mRho mice (open circles). Error bars indicate standard deviations. (C) B-wave amplitudes showing no statistical difference between mRho/mRho and P23H/mRho mice. Error bars indicate standard deviations.

RESULTS

The P23H-hRho-GFP Mouse Model

We generated mice carrying a human P23H-rhodopsin-GFP fusion gene (P23H-hRho-GFP) by homologous replacement of the endogenous mouse rhodopsin gene (Fig. 1), as described previously.^{22,23} We modified the genomic human rhodopsin gene in three ways. First, we fused the coding sequence for enhanced GFP to the C terminus of the rhodopsin gene, so that the encoded rhodopsin protein would be linked to GFP through the peptide APVAT.²² Second, we mutated codon 23 from CCC to CAC to change the encoded amino acid from proline to histidine. Third, at the junction between mouse and human rhodopsin sequences in the upstream, untranslated

region, we inserted an HPRT minigene flanked with LoxP sites to serve as a positive selection marker for manipulation of ES cells. Cre-mediated removal of the marker leaves behind a single copy of LoxP, which reduces translation of the mRNA approximately fivefold.²² This modification was included so that P23H-rhodopsin-GFP could serve as a tracer, allowing us to follow its degradation and mislocalization in the absence of significant retinal degeneration.

Retinal Degeneration

To measure the effect of the P23H-hRho-GFP on the development and health of the retina, we used fluorescence microscopy to examine retinal cross sections from mice that were heterozygous or homozygous for the knockin allele. The

TABLE 1. ERG Analyses of Wild Type Mice and P23H-hRho-GFP Mice

Genotype*	n†	Brightest Flash‡		Naka-Rushton Fit§	
		A-Wave (µV)	B-Wave (µV)	B _{max} (µV)	I _{0.5}
mRho/mRho	4	-743 ± 61	1200 ± 100	551 ± 57	-2.68 ± 0.01
P23H/mRho	4	-757 ± 94	1260 ± 200	562 ± 67	-2.72 ± 0.06
P23H/P23H	2	-42 ± 11	389 ± 93	41 ± 1	-3.16 ± 0.18

* Genotypes are wild type (mRho/mRho), heterozygous P23H-hRho-GFP (P23H/mRho), and homozygous P23H-hRho-GFP (P23H/P23H).
† Number of mice examined.
‡ Maximum A- and B-wave ERG amplitudes in response to a flash of 3.45 log (scot cd)(s)/(m²) (rightmost points in Figs. 4B and 4C).
§ Scotopic B-wave data (<0.2 [(scot cd)(s)/(m²)] in Figure 4C were fit using the equation $R = (B_{max} \times I)/(I_{0.5} + I)$, where B_{max} is the maximal scotopic B-wave amplitude, I is the flash intensity, and $I_{0.5}$ is the semisaturation intensity [log (scot cd)(s)/(m²)].

FIGURE 5. Rhodopsin expression in various mouse lines. (A) Northern blot analysis of rhodopsin mRNA from the indicated mouse lines. Membranes were hybridized with radioactive probes prepared from human rhodopsin cDNA. Quantification of the 18S and 28S rRNAs bands from the agarose gels before transfer (shown as a negative below the Northern blots) served as loading controls. Multiple rhodopsin mRNA bands are present; they derive from the normal poly-A sites at the endogenous mouse rhodopsin locus²⁷ and from two human poly-A sites included in the knockin alleles.²² Normalized to the rRNA bands, the levels of RNA relative to the mRho/mRho control (1.0) were 1.0 for homozygous hrhoG(H) mice (hRho/hRho), 1.0 for homozygous P23H-hRho-GFP mice (P23H/P23H), and 1.1 for heterozygous P23H-hRho-GFP mice (P23H/mRho). We used six retinas to prepare mRNA samples. Northern images were obtained on a storage phosphor imaging system (PhosphorImager). Sizes are indicated in kb. (B) Spectrophotometric quantification of rhodopsin protein from the indicated mouse lines. Retinas from the heterozygous hrhoG(H) mice (hRho/mRho) contained 64% as much rhodopsin as the mRho/mRho control, and retinas from the heterozygous P23H-hRho-GFP mice (P23H/mRho) contained 45% as much. For heterozygous mice with these knockin alleles, we expected roughly 58% of the amount of total rhodopsin present in mRho/mRho mice: 50% from the mRho allele and approximately 8% from the knockin allele.²² Statistical significance was evaluated using a univariate ANOVA with a least-squares difference post hoc analysis: ** $P < 0.001$.

morphology of heterozygous P23H-hRho-GFP retinas was normal (Fig. 2A), but the retinas of the homozygous mice were clearly abnormal (Fig. 2B). By counting the number of nuclei in the outer nuclear layer at various ages, we showed that the retinas of homozygous P23H-hRho-GFP mice rapidly degenerated (Fig. 3). By contrast, the retinas of heterozygous mice degenerated very slowly, at a rate that was indistinguishable from that of hrhoG(H) mice that express one allele of wild type human rhodopsin-GFP (hRho-GFP) (Fig. 3).²² Thus, mice heterozygous for the P23H-hRho-GFP allele should serve as a suitable model for analyzing degradation and mislocalization of mutant opsin.

Retinal Function

As an additional measure of the health of the P23H-hRho-GFP retinas, we assayed their response to flashes of light at various intensities. As shown in the electroretinograms (ERGs) in Figure 4 and the summary of results in Table 1, the responses from one-month heterozygous P23H-hRho-GFP mice and wild type mice were not significantly different. By contrast, homozygous P23H-hRho-GFP mice at the same age lacked an appreciable A-wave signal, indicating serious photoreceptor dysfunction, consistent with the observed structural abnormalities (Fig. 2B). Thus, a single P23H-hRho-GFP allele does not interfere appreciably with the development, health, or function of the mouse retina at one month of age.

Expression of Human P23H-Rhodopsin-GFP

We measured transcription in P24 heterozygous and P23 homozygous P23H-hRho-GFP mice by Northern blot analysis, using as controls, wild type mice and homozygous knockin hrhoG(H) mice, which express hRho-GFP (Fig. 5A). P23H-hRho-GFP homozygous mice gave expression levels and patterns like those from homozygous hrhoG(H) mice, whereas P23H-hRho-GFP heterozygous mice gave a pattern that was a mixture of the endogenous mouse and knockin human patterns, as expected.²⁷ Quantification of rhodopsin mRNA levels in these various mice showed that they are all about equal, as might be expected for genes at the endogenous location, driven by the natural promoter. In contrast, the amount of rhodopsin protein, as measured spectrophotometrically, was significantly reduced in the knockin mice relative to normal mice ($P < 0.001$) (Fig. 5B). Reduced expression of rhodopsin in the knockin mice was expected, due in part to the presence of the loxP site in the 5' untranslated region of the mRNA, which has been shown to reduce translation of rhodopsin mRNA about fivefold.²² Although it does not reach statistical significance, the additional reduction of expression in mice heterozygous for the P23H-hRho-GFP allele (P23H/mRho mice) relative to mice heterozygous for the hrhoG(H) allele (hRho/mRho mice) suggests that P23H-opsin may bind 11-*cis*-retinal inefficiently,⁴ and thus would not be detected spectrophotometrically, or that P23H-hRho-GFP may be degraded.

Degradation and Localization of Human P23H-hRho-GFP

To determine whether P23H-hRho-GFP is degraded, we compared fluorescence intensities in P30 heterozygous P23H-hRho-GFP mice and P30 heterozygous hrhoG(H) mice viewed at the same, nonsaturating microscope settings. While intense fluorescence was observed in hrhoG(H) mice, which express hRho-GFP, fluorescence was barely discernable in P23H-hRho-GFP mice (Fig. 6A; compare left and middle images). Quantification showed that fluorescence in P23H-hRho-GFP mice was

FIGURE 6. Distribution and quantification of GFP fluorescence in heterozygous mice expressing hRho-GFP or P23H-hRho-GFP. (A) Age-matched hrhoG(H) (hRho) and P23H-hRho-GFP (P23H) retinas from heterozygous mice were sectioned and stained with DAPI. Sections were imaged on a confocal microscope (Leica SP5) using the heterozygous hrhoG(H) sections (hRho/mRho) to establish gain and laser power settings that gave fewer than 10 saturated pixels. The hRho/mRho image and the adjacent P23H/mRho image were obtained using the same settings. The P23H/mRho image on the right was obtained by increasing the laser power to observe and quantify localization. (B) Quantification of average GFP fluorescence intensity for the hRho/mRho and P23H/mRho eyes. Quantification was performed using software developed by Wayne Rasband (ImageJ; National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) on unsaturated images captured with the same microscope settings, like the first two in (A). (C) Quantification of GFP localization in hRho/mRho and P23H/mRho retinas. Quantification was performed using software (ImageJ) on unsaturated images captured with the same microscope settings, like the third one in (A). For (B) and (C), 15 sections from four hRho/mRho mice and 15 sections from four P23H/mRho mice were examined. Retinal sections were derived from various regions across the retina—excluding the periphery and the optic nerve. In all cases, error bars indicate standard deviations. For analyzing the differences in GFP fluorescence intensity in the hRho/mRho and P23H/mRho mice (B), we conducted a univariate ANOVA. For

only 25% the level of hRho-GFP in hrhoG(H) mice, suggesting that the majority of P23H-hRho-GFP is degraded (Fig. 6B). At increased exposures of the P23H-hRho-GFP retinas (Fig. 6A, right image), we could readily compare the distributions of P23H-hRho-GFP and hRho-GFP in rod photoreceptor cells. As summarized in Figure 6C, >85% of hRho-GFP was properly localized in rod outer segments, whereas only 20% of P23H-hRho-GFP was located in rod outer segments. The majority of P23H-hRho-GFP was mislocalized in inner segments (20%) and the outer nuclear layer (60%). At later time points, a small amount of fluorescence was also visible in the outer plexiform layer in P23H-hRho-GFP heterozygous mice (see Fig. 2).

DISCUSSION

Here we describe the generation and characterization of a knockin mouse line that carries a P23H-hRho-GFP fusion gene in place of the endogenous mouse rhodopsin gene. Our goal was to generate a mouse model in which the fate of P23H-rhodopsin could be readily determined in the absence of retinal degeneration and in the presence of wild type rhodopsin—two complicating features of previous studies.^{11–14,19,20} To accomplish this, we incorporated two modifications into the P23H knockin gene. First, we engineered a loxP site into the portion of the gene corresponding to the 5' untranslated region of the mRNA. In a previous knockin mouse model of human rhodopsin-GFP, we showed that this alteration reduced translation of the mRNA to 15% of one mouse allele.²² Our aim was to express mutant rhodopsin protein at a sufficiently low level to prevent retinal degeneration, so that we could follow its fate in a healthy retina, thereby eliminating the potential complication that the observed behavior of P23H-rhodopsin might be secondary to retinal disease. As demonstrated here, the P23H-hRho-GFP knockin allele, when paired with a normal mRho allele, gives rise to retinas with normal morphology (Fig. 2) and function (Fig. 4) that degenerate at the same slow rates (Fig. 3) characteristic of hrhoG(H) mice heterozygous for the hRho-GFP knockin allele²² and mice heterozygous for a null allele.^{28,29} Thus, the P23H allele in our knockin mice does not contribute significantly to retinal degeneration.

The second modification was to tag human rhodopsin with GFP to provide a visible marker for rhodopsin. This modification allowed us to follow P23H-hRho-GFP in the presence of wild type rhodopsin in a way that is independent of the properties of specific rhodopsin antibodies, on which the usual methods for detecting rhodopsin in rod cells rely. Our previous studies with an analogous knockin mouse that expresses hRho-GFP [hrhoG(H) mice] showed that most of the fusion protein was correctly targeted to rod outer segments when expressed in the company of an endogenous copy of mouse rhodopsin,^{22,30} an observation reproduced in Figure 6. The correct targeting of a rhodopsin fused to GFP at the C terminus is somewhat surprising because the C-terminal 5 amino acids have been implicated in the trafficking of rhodopsin to the rod discs.³¹ In any case, because hRho-GFP is correctly targeted in mice carrying a normal mouse rhodopsin allele, we were able to compare the distribution and amount of P23H-hRho-GFP relative to hRho-GFP, allowing us to conclude that the differences are

analyzing the differences in GFP localization (C), we measured three different sections per mouse and performed a multivariate ANOVA with repeated measures. ^{**}*P* < 0.001. RIS, rod inner segment; ROS, rod outer segment.

due to the P23H mutation. We found that P23H-hRho-GFP is present at only 25% the level of hRho-GFP, suggesting that 75% is degraded. The remainder is largely mislocalized to inner segments (20%) and the outer nuclear layer (60%), with only 20% correctly targeted to rod outer segments (Fig. 6).

Our measurements of P23H-hRho-GFP degradation agree with previous studies in mouse models, which showed that the majority of P23H-rhodopsin was degraded when it was expressed in the absence of a normal rhodopsin allele.^{13,20} In addition, our results show that P23H-rhodopsin is degraded to a similar extent in the presence of wild type rhodopsin. The fate of the remaining P23H-rhodopsin in mouse rod cells has been a matter of some controversy, with some studies stressing correct localization^{11,13,19} and others emphasizing mislocalization.^{14,20} Our results lend some support to each of these studies, but they show clearly that the majority of P23H-rhodopsin does not reach the rod outer segment. Although we have not determined the subcellular location of mistargeted P23H-hRho-GFP, others have shown that P23H-rhodopsin in these regions is associated with the ER,²⁰ consistent with the sites of accumulation of P23H-rhodopsin in cultured cells and *Xenopus*.^{4–9}

The location of P23H-rhodopsin in rod cells undoubtedly underlies the mechanism by which it induces RP. Previous studies in rodent models of P23H-rhodopsin have variously concluded that retinal pathology arises due to ER stress in rod cells caused by the presence of mutant rhodopsin,^{10,20} due to aberrant function of rod disc membranes caused by incorporation of mutant rhodopsin,^{13,18,19} or due to interference with rod cell synaptic function caused by abnormal accumulation of mutant rhodopsin at the synaptic terminals.¹⁴ Even though we demonstrate that the majority of P23H-hRho-GFP likely associates with the ER, we cannot rule out that retinal pathology is caused by the lesser amount that makes it to the rod outer segment, or even by the tiny fraction that is misrouted to the outer plexiform layer. Our mouse knockin model does, however, provide a powerful tool to address the mechanism of disease, by allowing the fate of P23H-rhodopsin to be tracked in response to treatments that alleviate or exacerbate retinal pathology. For example, additional copies of wild type rhodopsin improve the health of the retina in P23H mouse models.^{20,21} Changes in the distribution (or stability) of P23H-hRho-GFP under such conditions may reveal sensitive sites associated with P23H-rhodopsin-induced retinal pathology. Aside from providing insights into the mechanism of pathogenesis, our P23H-hRho-GFP knockin mice offer a visible target for gene therapeutic approaches designed to suppress, correct, or knock out dominant mutations.³²

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