The Severe Autosomal Dominant Retinitis Pigmentosa Rhodopsin Mutant Ter349Glu Mislocalizes and Induces Rapid Rod Cell Death*

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Background: The C-terminal rhodopsin mutation Ter349Glu causes rapid degeneration in humans.

Results: Ter349Glu rhodopsin, with an additional C-terminal 51 amino acids, activates normally but is defective in subcellular localization.

Conclusion: Loss of proper rod outer segment morphogenesis likely contributes to the severe human phenotype.

Significance: The results point to a likely pathogenic mechanism for one of the most severe forms of hereditary retinal degeneration.

Mutations in the rhodopsin gene cause approximately one-tenth of retinitis pigmentosa cases worldwide, and most result in endoplasmic reticulum retention and apoptosis. Other rhodopsin mutations cause receptor mislocalization, diminished/constitutive activity, or faulty protein-protein interactions. The purpose of this study was to test for mechanisms by which the autosomal dominant rhodopsin mutation Ter349Glu causes an early, rapid retinal degeneration in patients. The mutation adds an additional 51 amino acids to the C terminus of the protein. Folding and ligand interaction of Ter349Glu rhodopsin were tested by ultraviolet-visible (UV-visible) spectrophotometry. The ability of the mutant to initiate phototransduction was tested using a radioactive filter binding assay. Photoreceptor localization was assessed both in vivo and in vitro utilizing fluorescent immunochemistry on transfected cells, transgenic Xenopus laevis, and knock-in mice. Photoreceptor ultrastructure was observed by transmission electron microscopy. Spectrally, Ter349Glu rhodopsin behaves similarly to wild-type rhodopsin, absorbing maximally at 500 nm. The mutant protein also displays in vitro G protein activation similar to that of WT. In cultured cells, mislocalization was observed at high expression levels whereas ciliary localization occurred at low expression levels. Similarly, transgenic X. laevis expressing Ter349Glu rhodopsin exhibited partial mislocalization. Analysis of the Ter349Glu rhodopsin knock-in mouse showed a rapid, early onset degeneration in homozygotes with a loss of proper rod outer segment development and improper disc formation. Together, the data show that both mislocalization and rod outer segment morphogenesis are likely associated with the human phenotype.

Retinitis pigmentosa (RP)2 comprises a heterogeneous group of blinding diseases caused by photoreceptor cells of the outer retina dying prematurely (1). Serial apoptosis results in a progressive loss of rod cells giving rise to night blindness, progressive tunnel vision, and ultimately blindness due to secondary cone cell death. RP can be inherited in an autosomal dominant (ADR) manner, and ~30% of all ADR cases are due to mutations in rhodopsin (2).

Rhodopsin functions as a photon detector for low luminance conditions. It is expressed primarily in rod photoreceptor cells of the vertebrate retina, where it is packaged into membranous discs located in the rod outer segment (ROS) region at high concentrations ranging from 3 to 9 mM giving the rod cell a low threshold of sensitivity to single photons of light (3). The receptor is expressed in the rod inner segment (RIS) region and trafficked in lipid vesicles from the trans-Golgi network to the ROS via the connecting cilium, a 9 + 0 bundle of microtubules. The connecting cilium is comparable with the transition zone of all other primary cilia acting as a link from the RIS to the ROS for proteins to passage (4). Transport of rhodopsin via the connecting cilium requires the involvement of several trafficking proteins including those for cytoplasmic and intraflagellar/microtubular transports (5).

Most rhodopsin-mediated ADRP (RhoADRP) cases are due to Class II mutations that decrease the fidelity of protein folding. This is thought to result in endoplasmic reticulum retention of misfolded mutant and WT rhodopsins causing congestion or aggregate formation, resulting in an apoptotic phenotype (6). Class I rhodopsin mutations appear to resemble WT functionally and exhibit mislocalization of the receptor as a phenotype. Most of these mutations occur in the region at or near the C terminus. The last four amino acids of rhodopsin, the

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2 The abbreviations used are: RP, retinitis pigmentosa; ADRP, autosomal dominant RP; GPCR, G protein-coupled receptor; HPRT, hypoxanthinephosphoribosyltransferase; IMCD, inner medullary collecting duct; OCT, optimal cutting temperature medium; ONL, outer nuclear layer; RhoADRP, rhodopsin-mediated ADRP; RIS, rod inner segment; ROS, rod outer segment; XOP, Xenopus opsin promoter.
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-VXPX sorting motif, have been implicated in several studies to be involved in apical trafficking of rhodopsin in rod cells (7–9). This is an evolutionarily conserved motif implying a vital function. A number of studies in transgenic animals have shown that these mutant rhodopsins mislocalize to the plasma membrane of the RIS while properly localizing to the ROS discs (10, 11). Nakao et al. have shown that when mutant zebrafish expressing the Class I mutation Gln344Ter are reared in the dark (i.e. a condition free of rhodopsin signaling), the photoreceptors accumulating mislocalized rhodopsin apoptosed more slowly than those reared in cyclic light (12). This result would indicate mislocalized (i.e. inner segment) phototransduction proteins, either transducin, other G proteins, or both are involved with the rapid degeneration seen in these animals.

Whereas the C-terminal mutations tend to alter the sorting motif by truncation (Gln344Ter and Ser334Ter), amino acid substitution (P347L/P347S and V345M), or frameshift mutations (Del341–343) (13), another mutation at the C terminus exists that results in RP. The read-through mutation Ter349Glu extends the C terminus of rhodopsin by 51 amino acids, thereby occluding the VXPX sorting motif (see Fig. 1A) (14). This mutation has been found to cause one of the most severe forms of RhoADRP known in terms of early onset and rate of vision loss; however, no in-depth biochemical studies have been performed on the Ter349Glu rhodopsin mutant. As such, the underlying mechanism by which this mutation causes such a severe disease phenotype has yet to be elucidated.

In this study, through the use of spectrophotometric, biochemical, and cellular localization analyses, we investigated potential mechanisms underlying the severe pathophysiology observed in Ter349Glu rhodopsin patients. We hypothesize that the molecular mechanism of disease pathogenesis is through a dominant negative effect possibly induced or exacerbated by mislocalization of the mutant receptor and/or a loss of proper outer segment morphogenesis as is seen in the RhogFP mouse (15). This is due to the 51-amino acid addition occluding the sorting signal found at the C terminus similar to the way EGFP occludes the C terminus in the RhogFP mouse. Whereas the mislocalizing rhodopsin mutants like Gln344Ter have altered C termini which may diminish binding of trafficking proteins, it is unknown whether these trafficking proteins can interact with the occluded WT C terminus of Ter349Glu rhodopsin.

EXPERIMENTAL PROCEDURES

Generation of Rhodopsin Constructs—The construct containing the cDNA for bovine rhodopsin in the pMT3 vector (provided by Daniel Oprian) was changed via cassette mutagenesis to encode Ter349Glu opsin using phosphorylated and annealed oligonucleotides (Invitrogen). Separate oligonucleotides encoding the amino acids ETSQVAPA (1D4 epitope) were used for the addition of the 1D4 epitope to the 3′ end of the mutant cDNA (Ter349Glu-1D4). pMT3-opsin was mutagenized by QuikChange PCR mutagenesis using Pfu polymerase (Stratagene) to obtain the Gln344Ter and P23H opsin constructs. The cDNAs were also used to create low expression vectors by cassette mutagenesis into the pRevTRE vector (provided by Jay Pieczynski) using phosphorylated and

annealed oligonucleotides (Invitrogen) linking a BamHI to an EcoRI site at the 5′ end of the cDNAs and a NotI to a Sall site at the 3′ end. All DNA-modifying enzymes purchased from New England Biolabs.

Cell Culture and Transfection—COS and inner medullary collecting duct (IMCD) cells were cultured at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin/streptomycin (P/S), 1-glutamine, and fetal bovine serum (FBS) at final concentrations of 100 units/ml/100 μg/ml P/S, 2 mM 1-glutamine, and 10% FBS. IMCD DMEM was substituted 1:1 with Ham’s F-12. COS transfection was carried out using the DEAE-dextran method in either 15-cm dishes to harvest or 12-well plates with coverslips for immunocytochemistry using N-terminal anti-rhodopsin antibodies 72 h after transfection (16). Transfection of IMCD cells was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) in 12-well plates with coverslips.

Spectrophotometric Analysis—COS cells expressing WT, Ter349Glu, or Ter349Glu-1D4 rhodopsin were harvested and the opsins reconstituted with 11-cis-retinal for 1 h on ice at 4 °C in darkness followed by solubilization with 1% dodecyl malto-side in 1× PBS for 1 h at 4 °C in darkness. WT and Ter349Glu-1D4 rhodopsins were purified with a 1D4 immunoaffinity column under dim red light illumination as published previously (16). All rhodopsins were subjected to UV-visible spectrophotometry from 700 to 250 nm using a Varian Cary 50-Bio spectrophotometer. For difference spectra, samples were photo-bleached using bright white light for 30 s and subjected to spectrophotometry and the difference calculated.

Analysis of Activation of the G Protein Transducin—COS cells transfected with either WT or Ter349Glu opsins were harvested, and opsin-containing membranes were obtained by ultracentrifugation of lysed cells on a sucrose float as described previously (17). Membranes were reconstituted with 11-cis-retinal for 1 h on ice at 4 °C in darkness. Transducin was purified from bovine ROS using a diethylaminoethanol-cellulose anion-exchange column as described previously (18). Reactions were set up in the dark with GTPγS as published previously with six time points in the dark and six after exposure to bright white light for 30 s (17). Using a Beckman-Coulter LS6500 scintillation counter, GTPγS uptake by transducin was assessed as an indirect measure of rhodopsin activation ability. Graphing was performed using Origin 8.5.1 graphing and analysis software (OriginLab).

Analysis of Cultured Cell Localization of WT and Mutant Opsins—IMCD cells transfected with either high expressing WT, Ter349Glu, Ter349Glu-1D4, or Gln344Ter rhodopsin constructs were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, permeabilized with 1:1 ice-cold methanol:acetone, and blocked in 10% goat serum in PBS for 1 h at room temperature. Cells were subjected to immunocytochemistry using anti-rhodopsin antibody B6-30N (courtesy of W. Clay Smith and Paul Hargrave), an anti-ADP-ribosylation factor-like 13b (Arf13b) antibody (NeuroMab), and an anti-zonule occludens-1 (ZO-1) antibody (courtesy of Bradley K. Yoder) followed by a goat anti-mouse IgG1 secondary conjugated to Alexa Fluor 488 (B6-30N), a goat anti-mouse IgG2a secondary antibody conjugated to Alexa Fluor 568 (Arf13b), and a goat
anti-rat IgG secondary antibody conjugated to Alexa Fluor 647 (ZO-1). IMCD cells transfected with the low expressing pReTRE constructs were immunochemically labeled using anti-rhodopsin antibody K62-82 (provided by W. Clay Smith), anti-Arl13b, and an anti-γ-tubulin antibody (Sigma) followed by a goat anti-mouse IgG2 secondary antibody conjugated to Alexa Fluor 488 (K62-82), a goat-anti-mouse IgG2ε secondary antibody conjugated to Alexa Fluor 568 (Arl13b), and an anti-mouse IgG, secondary antibody conjugated to Alexa Fluor 647 (γ-tubulin). Cell nuclei were labeled using 1.4 μM DAPI. Coverslips were mounted in Prolong Gold antifade reagent. All secondary antibodies, DAPI, and Prolong Gold were purchased from Invitrogen. Fluorescence microscopy was performed using a PerkinElmer Life Sciences Ultraview ERS6 laser scanning confocal microscope.

**Analysis of Ter349Glu Rhodopsin Localization in Xenopus laevis**—Transgenesis of Xenopus laevis embryos was performed using a modified form of the Amaya and Kroll method (19) with the following modification. Frogs were allowed to lay eggs in high salt modified Barth's saline containing 108 mM NaCl, 1 mM KCl, 1 mM MgSO4, 2.5 mM NaHCO3, 0.7 mM CaCl2, and 5 mM HEPES, pH 7.4. The DNA construct containing 0.8 kb of the Xenopus opsin promoter (XOP) was provided by Orson Moritz. The cDNA coding for bovine Ter349Glu rhodopsin was subcloned into the vector using EcoRI and NotI restriction endonucleases immediately following the XOP region. Tadpoles were reared under normal light/dark cycles for 2 weeks and euthanized, fixed in 4% paraformaldehyde in PBS, pH 7.4, overnight at 4 °C, cryoprotected in 30% sucrose in PBS, pH 7.4, for 2 h at 4 °C, frozen in optimal cutting temperature medium (OCT), and cryosectioned into 10-μm sections and washed free of OCT in PBS. Sections were postfixed in 4% paraformaldehyde for 2 h at room temperature in 10% goat serum in PBS, pH 7.4, for 1 h at room temperature. WT and transgenic sections were labeled using either B6-30N (endogenous) or A5-3-12 (transgenic) primary antibodies for rhodopsin for 2 h at room temperature in 10% goat serum in PBS, washed in PBS, labeled with a goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 with DAPI in 10% goat serum in PBS, washed in PBS, and labeled with a goat anti-rat IgG secondary antibody conjugated to Alexa Fluor 647 (K62-82), a goat-anti-mouse IgG2ε secondary antibody conjugated to Alexa Fluor 568 (Arl13b), and an anti-mouse IgG, secondary antibody conjugated to Alexa Fluor 647 (γ-tubulin). Cell nuclei were labeled using 1.4 μM DAPI. Coverslips were mounted in Prolong Gold antifade reagent. All secondary antibodies, DAPI, and Prolong Gold were purchased from Invitrogen. Fluorescence microscopy was performed using a PerkinElmer Life Sciences Ultraview ERS6 laser scanning confocal microscope.

**Generation of Ter349Glu Rhodopsin Knock-in Mice**—The human rhodopsin Ter349Glu knock-in plasmid was constructed using the human rhodopsin Gln344Ter (pBS-hrhoQ344Ter) knock-in plasmid as a template for PCR (courtesy of Theodore G. Wensel and John H. Wilson). Forward and reverse primers with the sequences 5’-CAACCAAGTCTTATTTCTAGGGG-3’ and 5’-CTCAAATGTCTCAGTCCAGG-3’, respectively, were used to amplify exon 5 of Gln344Ter rhodopsin, the EGFP sequence, and the 3’-untranslated region (UTR) spanning from a KpnI site in intron 4 to an SpeI site in the 3’-UTR. This PCR product was inserted into the pTOPO plasmid using the TOPO Cloning Reaction (Invitrogen) and the product vector digested with AatII and SpeI restriction endonucleases and subcloned into the pGEM-5zf(+) vector (Promega) using the same restriction sites. One of two BsrGI restriction sites in the resulting plasmid was mutagenized by QuickChange to leave a single BsrGI site at the 3’ end of the EGFP cDNA. The plasmid was digested using AgeI and BsrGI endonucleases to remove the EGFP cDNA. Phosphorylated and annealed oligonucleotides abolishing both sites and inserting a unique Xhol site were ligated into the plasmid. The plasmid was transformed into the dam- chemi-competent Escherichia coli strain INV110 (Invitrogen), and the resulting unmethylated plasmid was digested with BspEI and Xhol endonucleases. Five sets of phosphorylated and annealed oligonucleotides containing the entire DNA sequence of exon 5 and the 51 amino acid addition were ligated into the plasmid. Subsequent digestion of the resulting plasmid as well as the pBS-hrhoQ344Ter was performed with KpnI and SpeI endonucleases and the Ter349Glu rhodopsin mutagenized fragment ligated into the pBS-hrhoQ344Ter plasmid, creating pBS-hrhoTer349Glu. This plasmid, along with the final vector containing the hypoxanthinephosphoribosyltransferase (HPRT) mini-gene for positive selection and thymidine kinase-negative selection marker were digested using NotI endonuclease and ligated together to form the final 27-kb knock-in plasmid. After sequence verification, the plasmid was linearized using SalI endonuclease and purified postdigestion using ultracentrifugation on a CsCl gradient. Once purified, the linearized DNA was shipped to the Mouse Embryonic Stem Cell Facility at Baylor College of Medicine, Houston, TX, for electroporation into Ab2.1 mouse embryonic stem (ES) cells, followed by thymidine kinase and HPRT selections. Southern blotting was performed on the ES cell genomic DNAs using alkaline transfer to HyBond XL membrane (GE Life Sciences) and [α-32P]dCTP-labeled probes for DNA hybridization. After identification of positive clones, microinjection into blastocysts and implantation into pseudopregnant female C57B6 mice were performed at the Mouse Embryonic Stem Cell Core Facility at Baylor College of Medicine. The resulting male chimera mice were bred with BALB/c females. Pups were selected for coat color and subsequently genotyped using forward and reverse primers with the sequences 5’-CGGAACCTGCTTCTACC-3’ (forward primer), 5’-GCTGTCCCATTTGAGTGG-3’ (reverse primer 1), and 5’-GTGGATGTCCTTCTCAGG-3’ (reverse primer 2). The offspring were bred to C57BL/6 mice carrying the gene for Cre recombinase under the EIIA promoter (20) to excise the LoxH-flanked HPRT mini-gene, which prevents rhodopsin expression until removed. Pups were tested by Southern blotting for removal of HPRT, and HPRT-negative mice were bred to WT BALB/c to generate heterozygotes and subsequent homozygotes.

**Analysis of Rhodopsin Localization, Degeneration Rates, Photoreceptor Apoptosis, and Photoreceptor Ultrastructure in the Ter349Glu Rhodopsin Knock-in Mouse Retina**—WT (+/+), Ter349Glu heterozygote (Ter349Glu/+), and Ter349Glu homozygote (Ter349Glu/Ter349Glu) mice were euthanized at 2, 3, 4, 5, 6, 8, 10, and 12 weeks of age. Their eyes were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 2 h at 4 °C and cryoprotected in 30% sucrose in PBS, pH 7.4, at 4 °C for a minimum of 4 h. Eyes were frozen in OCT and cryosectioned into 10-μm sections and washed free of OCT in PBS. Sections were post-
fixed in 1:1 ice-cold methanol:acetone for 5 min and blocked in 10% goat serum with 0.2% Triton X-100 in PBS, pH 7.4, for 30 min at room temperature. Sections were labeled with B6-30N primary antibody for 2 h at room temperature in 10% goat serum in PBS, washed in PBS, and probed with a goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 or 568 with DAPI in 10% goat serum in PBS for 1 h at room temperature. Sections were labeled with 1D4 (recognizes WT rhodopsin) and K62-82 (recognizes total rhodopsin) primary antibodies, washed in PBS, and subsequently probed with a goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (K62-82) with DAPI in 10% goat serum in PBS for 1 h at room temperature. Sections were imaged on Olympus IX81 spinning disc confocal microscope. To examine retinas for apoptosis, TUNEL labeling was performed using the ApopTag Red kit (Millipore) following the manufacturer’s protocol with rhodopsins labeled as stated previously. Labeled nuclei were counted per section, and a two-tailed t test was used to statistically analyze the data. Transmission electron microscopy on 4-week old +/+ heterozygote rhodopsin KO (+/−) Ter349Glu/+, and Ter349Glu/Ter349Glu mouse eyes was performed by the Electron Microscopy Core in the Department of Ophthalmology at University of Alabama at Birmingham.

**RESULTS**

Effects of the Ter349Glu Addition on Receptor Folding and Ligand Interaction—Because improper folding of rhodopsin is a key component of endoplasmic reticulum stress-induced cell death in photoreceptors of many RhoADRP Class II cases, we assayed for the ability of Ter349Glu opsin to properly fold and bind 11-cis-retinal using UV/visible spectrophotometry. Ter349Glu-1D4 rhodopsin also appeared similar to WT (data not shown). Perinuclear localization of Ter349Glu opsin in COS cells suggests
some mislocalization or endoplasmic reticulum retention of the mutant protein (Fig. 1D); however, normal membrane localization was observed more frequently than perinuclear localization. This is similar to WT and Gln344Ter rhodopsin, a known Class I (mislocalizing) mutant, indicating the mutant may aggregate or misfold slightly, but not to the extent of the known Class II (misfolding) mutant P23H.

**Effects of the Ter349Glu Addition on G Protein Activation—** The activation of the G protein transducin by rhodopsin is undetectable in the dark and increases dramatically after exposure to light (21). To test the ability of Ter349Glu rhodopsin to catalyze the exchange of radiolabeled GTPγS for bound GDP in transducin, a radioactive filter binding assay was used. Rhodopsin concentrations were estimated by Western blot analysis and equivalent molar concentrations used in the assays (data not shown). Similarly to WT, Ter349Glu rhodopsin exhibits minimal G protein activation in the dark and a subsequent light-dependent activation with similar rate constants (Fig. 1C).

**Localization of Ter349Glu Opsin in Cultured Cells—** To assess localization in polarized, monociliated cells, IMCD cells were transfected with high expressing WT, Ter349Glu, Ter349Glu-1D4, and Gln344Ter rhodopsin plasmid constructs and subjected to immunocytochemistry for opsin, the ciliary protein Arl13b, and the tight junction protein ZO-1 (Fig. 2A) followed by quantification of both apical and basolateral localizations (Fig. 2B). WT opsin localized apically to the cilium and plasma membrane of the IMCDs. Similarly, Ter349Glu and Gln344Ter rhodopsins localized apically; however, fluorescence was also observed basolaterally, indicating that some of the protein was mislocalized. Fluorescence did not appear to colocalize to the cilium. Interestingly, adding the 1D4 epitope (ETSQVAPA) onto the C terminus restored localization in a manner slightly more similar to that of WT, although ciliary entry was not observed and the Ter349Glu-1D4 protein mislocalized basolaterally.

As localization in the cilia of IMCDs could be a consequence of high apical expression, we used a lower expression method to transfect IMCDs with cDNA coding for the photoreceptors mutants to test this hypothesis. The pRevTRE vector contains a CMV promoter that governs heterologous expression levels by tetracycline. This construct provides low basal levels of expression in the absence of tetracycline (22). Transfection with the derivatized pRevTRE vector yielded low rhodopsin expression allowing for observation of ciliary colocalization (Fig. 2C). In striking contrast to the high expression vector, the localization patterns were altered with some of the mutants. WT, Ter349Glu, and Ter349Glu-1D4 at this lowered level of expression appeared to colocalize with the cilium labeling, indicating ciliary localization and entry. Gln344Ter rhodopsin appeared to localize proximally to the cilium and basal body, but was unable to enter, possibly due to the loss of its C-terminal sorting motif. None of the mutants was found to localize to the basolateral membrane. These data indicate that at low levels of expression, Ter349Glu rhodopsin is capable of entering the cilium. Contrarily, high expression of the receptor causes the protein to localize throughout the cellular membrane, regardless of the presence of a free C-terminal motif (i.e. Ter349Glu-1D4), unlike WT rhodopsin.

**Localization of Ter349Glu Rhodopsin in X. laevis Photoreceptors—** To monitor the mutants in the native photoreceptor environment, transgenic *X. laevis* expressing Ter349Glu rhodopsin under the XOP were generated and the retinas of tadpoles examined for mutant rhodopsin localization using immunohistochemistry (Fig. 3). Ter349Glu rhodopsin labeling using antibody A5–3–12 (which does not recognize endogenous frog rhodopsin) revealed that Ter349Glu rhodopsin is able to localize to the ROS, likely due to the copious amounts of WT rhodopsin being expressed in these transgenic animals. Interestingly, some mislocalized Ter349Glu rhodopsin, as well as malformed ROS, can be observed in the transgenic animals, indicating that even with WT rhodopsin expression, expression of the mutant protein results in a deleterious phenotype.

**Localization and Degeneration of Ter349Glu Rhodopsin in the Knock-in Mouse Retina—** Due to the C-terminal site of the Ter349Glu rhodopsin mutation, along with the high expressing IMCD cells and transgenic *X. laevis* findings, it is logical to think that Ter349Glu rhodopsin may mislocalize in intact mouse rod cells as well. To examine for possible mislocalization, we immunohistochemically labeled retinal sections from both Ter349Glu+/ and Ter349Glu/Ter349Glu mice up to 12 weeks of age. Using an antibody specific for WT rhodopsin
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FIGURE 3. Localization of Ter349Glu rhodopsin in transgenic X. laevis. Using a modified Amaya and Kroll method of transgenesis, WT and Ter349Glu rhodopsin-expressing tadpoles were generated and euthanized at 2 weeks of age, processed and cryosectioned, and labeled using immunohistochemistry. WT rhodopsin was labeled using B6-30N primary antibody. Ter349Glu rhodopsin was labeled using mammalian rhodopsin-specific primary antibody A5-3-12. Both were labeled with anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (green). Nuclei were labeled with DAPI (blue). OPL, outer plexiform layer. Arrows, normally developed outer segments; arrowheads (†), inner segment mislocalization. Asterisks, synaptic mislocalization. Scale bar, 20 μm.

(1D4) and an antibody which recognizes the N terminus of all rhodopsins (K62-82) on Ter349Glu/+ animals and using an N-terminal antibody (B6-30N) on Ter349Glu/Ter349Glu and +/+ retinas, we observed localization to the distal RIS in Ter349Glu/Ter349Glu mice, which do not form proper outer segments, and predominantly outer segment localization in Ter349Glu/+ mice. We frequently observed WT and Ter349Glu rhodopsins localized to the RIS, outer nuclear layer (ONL), and synapse of Ter349Glu/+ mice (Fig. 4A). Despite its disruption of outer segment formation, when expressed in the absence of WT rhodopsin, Ter349Glu rhodopsin is transported from the Golgi to the proper region of the cell for transport through the connecting cilium to the outer segment. These results correlate with the phenotype observed in transgenic X. laevis and support a dominant negative mechanism of Ter349Glu rhodopsin aggregating with and mislocalizing WT rhodopsin under coexpressed conditions (i.e. Ter349Glu/+).

ONL counts were performed and averages taken over the course of 12 weeks of age (Fig. 4B). The Ter349Glu knock-in mouse degenerates with faster kinetics than the RhoGFP mutant nor Ter349Glu-1D4 was able to enter the primary cilium, behaving more similarly to Gln344Ter rhodopsin. At low levels of expression, Ter349Glu rhodopsin behaved more similarly to WT by exhibiting ciliary trafficking and entry.

A major difference exists between normal primary cilia and ROS, however, in that the ROS contains hundreds to thousands of stacked membranous discs. Previous work done involving the RhoGFP mouse showed that in homozygote mice lacking any WT rhodopsin, proper outer segment morphology was unable to occur (15). This phenotype was attributed to occlusion of the C terminus by fusion of the EGFP protein,
prohibiting proper disc formation. Therefore, it is logical to hypothesize the Ter349Glu rhodopsin read-through mutant would act in a similar manner. Expression of Ter349Glu rhodopsin in X. laevis suggests that the mutant protein mislocalizes when expressed in conjunction with WT rhodopsin and alters ROS morphology but may not result in degeneration, at least not by 2 weeks of age. In transgenic X. laevis, however, it is possible that because the transgene coded for bovine rhodopsin, it may delay degeneration to a time point beyond 2 weeks (28). Therefore, we generated the knock-in mouse model for Ter349Glu rhodopsin to better monitor the in vivo effects of the mutant in mammalian retina in the presence and absence of endogenous rhodopsin.

Prior knock-in mouse models for rhodopsin mutations exhibit progressively slow or little to no degenerative phenotype in heterozygotes including mice expressing P23H, Gln344Ter, D190N, or the RhoGFP fusion with degeneration often halting once ~8 nuclei remain in the ONL, even when carried out to 7 months of age (15, 29, 30). The Ter349Glu/+ mouse also degenerates to 8 ONL nuclei by 12 weeks of age; however, aberrant morphological phenotypes were not observed in either Gln344Ter/+ or RhoGFP/+ mice whereas the Ter349Glu/+ mice exhibited morphogenic abnormalities in the ROS discs including discs oriented parallel to the axoneme and disc membrane whirls similar to those observed in the retinas of both the Ter349Glu/Ter349Glu and RhoGFP/
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Rhodopsin homozygote mice. Tubby-like protein-1 (Tulp1) is a vesicular trafficking protein that, when mutated, causes the blinding disease Leber congenital amaurosis, an early onset congenital blindness (31). In Tulp1 KO mice, extracellular vesicles accumulate at the connecting cilium when the retina is observed by transmission electron microscopy (32). Interestingly, the Ter349Glu/Ter349Glu mouse exhibits extracellular vesicles near the tip of the cilium where the ROS forms, linking severe degenerative phenotypes of two different blinding diseases. Diseases causing syndromic RP-like Usher syndrome, Bardet-Biedl syndrome, and Meckel syndrome also result in phenotypes similar to the Ter349Glu rhodopsin mutant including mislocalization of rhodopsin and a loss of ciliary formation or function, resulting in aberrant ROS formation (33–42). Because the rhodopsin C terminus is occluded in both the RhoGFP mouse and the Ter349Glu mouse and both result in improper disc formation, perhaps some of the proteins found in syndromic RP and Leber congenital amaurosis patients are responsible for interacting with a free rhodopsin C terminus for either localization from Golgi to connecting cilium or proper disc and ROS morphogenesis.

Considering the copious abundance of GPCRs encoded by the human genome, the likelihood of a read-through mutation occurring in a GPCR-encoding gene is probable, even with the rarity of read-through mutations as a whole. Studies of rhodopsin as the prototypical GPCR have been ongoing for decades and have laid the foundation for understanding characteristics common to not only Class A (rhodopsin-like) GPCRs, but all GPCRs. By performing this in-depth analysis of the Ter349Glu rhodopsin mutant, perhaps a better understanding of GPCR read-through mutations and insights into the molecular mechanisms governing the phenotypes associated with them will be gained.

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