Title
Mechanisms of Ubiquitylation and Endoplasmic Reticulum- Associated Degradation of P23H Mutant Rhodopsin in Retinal Degeneration

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Mechanisms of Ubiquitylation and Endoplasmic Reticulum-Associated Degradation of
P23H Mutant Rhodopsin in Retinal Degeneration

A Thesis submitted in partial satisfaction for the requirements for the degree of
Master of Science

in

Biology

by

Allen Chen

Committee in charge:

Professor Jonathan H. Lin, Chair
Professor Maho Niwa, Co-Chair
Professor Eric Bennett

2015
This Thesis of Allen Chen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2015
DEDICATION

This thesis is dedicated to the family of
Katherine, Jien, Kevin, and
Jeffrey Chen for their abundance of
love, support, and
advice.
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<td>Photoreceptor</td>
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<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
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<tr>
<td>RHO</td>
<td>The human or mouse rhodopsin gene/protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum-Associated Degradation</td>
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<tr>
<td>UPS</td>
<td>Ubiquitin-Proteasome System</td>
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<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
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<tr>
<td>RP</td>
<td>Retinitis Pigmentosa</td>
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<td>ADRP</td>
<td>Autosomal Dominant Retinitis Pigmentosa</td>
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<td>Post-Translational Modification</td>
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<td>Ub</td>
<td>Ubiquitin Protein</td>
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I would like to thank the members of my thesis committee Professor Lin, Professor Niwa, and Professor Bennett, for their counsel regarding my project as well as career advice. In addition, I would like to thank the members of the Lin Laboratory: Nobuhiko Hiramatsu, Heike Kroeger, Wei-Chieh Chiang, Priscilla Chan, and Moana Santiago for their incredible support. These scientists gave me the ability to conduct experiments and more importantly, how to think about scientific problems. Dr. Hiramatsu specifically founded the project idea and provided inspiration for experimental design.
ABSTRACT OF THESIS

Mechanisms of Ubiquitylation and Endoplasmic Reticulum-Associated Degradation of P23H Mutant Rhodopsin in Retinal Degeneration

by

Allen Chen
Master of Science in Biology

University of California, San Diego, 2015

Professor Jonathan H. Lin, Chair
Professor Maho Niwa, Co-Chair

Rhodopsin protein is the archetypal G-protein coupled receptor that is specifically and massively expressed in rod photoreceptor cells. Over 100 rhodopsin mutations lead to inheritable retinal disease. The P23H point mutation in rhodopsin leads to autosomal dominant retinitis pigmentosa, in which gradual death of rod photoreceptor cells results in night blindness and eventual total blindness. Both in vitro and in vivo systems demonstrate that P23H rhodopsin is misfolded and subjected to depletion by endoplasmic-reticulum-associated degradation (ERAD). Proper expression of mature rhodopsin is important not only for rod functionality, but also for its viability. Appropriately, we have previously shown that Rho\textsuperscript{P23H/P23H} mice demonstrate an early
degradation of rhodopsin that precedes rod photoreceptor death. However, the post-translational modifications that target rhodopsin for degradation are poorly understood. In the classical ERAD pathway, lysine residues are conjugated to poly-ubiquitin signals (ubiquitylation) for degradation by the proteasome. Here, we mutated every lysine residue (11 total) of P23H rhodopsin to arginine to test which ones are important for ubiquitylation. Using transient transfection of these rhodopsin constructs in the HEK293 human cell line, we found that a P23H rhodopsin with all lysine residues mutated (K-null P23H) is much less ubiquitylated than P23H rhodopsin with lysine residues intact. Cycloheximide blocking experiments demonstrate that the K-null P23H rhodopsin is more stable than P23H rhodopsin. Interestingly, rhodopsin constructs with singleton lysine mutations did not result in a significant change in ubiquitylation. In conclusion, ubiquitylation of multiple lysine sites on P23H rhodopsin may be involved in its ERAD processing.
**Introduction**

The eye engages visual perception through the molecular conversion of light information into electrical signals that are transmitted to the brain. This remarkable process of phototransduction begins within the eye in the retinal layer of neuronal photoreceptor (PR) cells. Akin to other neurons, the PR’s morphology is organized in a polarized fashion (Figure 1) (Kolb and Gouras, 1974). One-half of the PR cell is denoted as the inner segment. This side of the cell contains the means for gene expression and secretory transport: the nucleus, endoplasmic reticulum (ER), and the golgi apparatus. The other half of the cell, called the outer segment (OS), contains the proteins required for phototransduction. To house such reactions, the OS has membranous disks that are embedded with opsin proteins. Opsins are seven-transmembrane G-protein-coupled receptors that function through signal transduction to initiate an action potential upon photon interaction (Figure 2) (Palczewski, 2012). The importance of opsins in PR’s is highlighted by the approximation that each day, 10 million opsin molecules are recycled per PR (Palczewski, 2012; Sung and Tai, 2000). In relative terms, this opsin load is comparable to the secretory demand of pancreatic acinar cells that constantly produce and secrete digestive enzymes (Kosmaoglou et al., 2009). In light of the high flux of opsin protein and its central function in visual perception, researchers have discovered many opsin point mutations that manifest themselves in visual disease.

Heavily linked to opsin mutations, the inheritable disease Retinitis Pigmentosa (RP) is characterized by progressive PR degeneration and loss of vision. RP is found 1 in every 4,000 people, making it one of the most common forms of retinal degeneration (Hartong et al., 2006). RP is heterogeneous in nature; patients may experience loss of vision at a spectrum of ages and with varying levels of severity (Hartong et al., 2006; Sung et al., 1991a) Underlying genetic causes of RP are also variable, with over 50 chromosomal loci leading to the condition (Daiger, 2014). The overlying inheritance can be autosomal dominant (ADRP), recessive, or sex-linked.
Typical symptoms begin with the loss of night and peripheral vision. As the condition progresses through the time course of decades, patients begin to lose central vision and may suffer blindness. The pathological progression can be explained by how specific subsets of PR’s degenerate. These two subsets of PR are called rods and cones (Kolb and Gouras, 1974). The rod PR allows for sensation of less intense light such as in the scenario of night perception (Palczewski, 2012). These PR’s degenerate first, explaining the initial loss of night vision (Sharpe et al., 1999). Cone PR’s on the other hand, are responsible for colored central vision and are incapable of sensing the low intensity light sensed by rods (Palczewski, 2012). Cones, along with other layers of the retina, degenerate later during the progression of RP, potentially leading to total blindness.

Corresponding to the initial death of rods in RP pathology, the most common forms of ADRP are caused by mutations in the rod opsin protein gene (Hartong et al., 2006). First described in 1990, RP-causing rod opsin point mutations have since been found to account for nearly 25% of all ADRP cases with over 100 point mutations identified in the 348 amino acid protein (Daiger, 2014). Interestingly, different mutations can lead to different rod opsin defects. It has been researched that there are five major mutant classes that render rod opsin susceptible to problems with: endocytosis, posttranslational modification, interaction with photoregulatory elements, or proper folding (Mendes et al., 2005). While mutations in rod opsin have been studied to interfere with phototransduction function, it remains elusive how these mutations can lead to gain-of-function rod cell death and an autosomal dominant inheritance pattern.

The specific ADRP misfolding mutation that I am studying is the missense mutation occurring on the 23rd amino acid position of rod opsin changing proline to histidine (P23H). It is the most common mutation leading to ADRP in North America (Mendes et al., 2005). How the P23H mutation leads to ADRP pathology is still a matter of contention. After the discovery of the P23H mutation’s link to ADRP, researchers attempted to elucidate underlying mechanisms (Dryja
et al., 1991). First, it was found that when expressed in the HEK293 cell line, the P23H mutation caused rod opsin to misfold and localize to the endoplasmic reticulum (ER) rather than the plasma membrane (Sung et al., 1991b). Classified as an opsin misfolding mutation, P23H rod opsin was speculated to be unable to be transported to the OS in rods themselves (Olsson et al., 1992). This line of logic comes from the study that misfolded proteins are often not incorporated in regular secretory transport, do not move beyond the ER, and are subjected to endoplasmic reticulum-associated degradation (ERAD) (Dobson, 2003). In vivo modeling of P23H rhodopsin misfolding was necessary to place the mutation in the context of the rod OS.

Throughout the decade after the revealing P23H misfolding mutation, researchers sought to develop transgenic animals modeling P23H RP pathology. To this goal, researchers developed transgenic mouse, rat, and frog models expressing both human wildtype and P23H rod opsin (Olsson et al., 1992; Steinberg et al., 1996; Tam and Moritz, 2006). When studying the expression in transgenic mice, Olsson and colleagues found that expressing either WT or P23H rhodopsin at high levels lead to rod PR degeneration. This was speculated to be due to overexpression of rod opsin protein that surpassed physiological levels (Olsson et al., 1992). This made it difficult to conclude whether or not the P23H misfolding mutation specifically had a detrimental effect when expressed in rods. Additionally, researchers developed rod opsin knockout mouse models and found that a lack of rod opsin expression itself leads to PR and retinal degeneration (Humphries et al., 1997). While the use of transgenic animals did not model the P23H ADRP pathology clearly, they provide evidence that rod cell viability is sensitive to rod opsin expression.

In parallel with the investigation of transgenic rod opsin animals, researchers wanted to reveal what molecular mechanisms could be triggered by P23H rod opsin expression. As P23H misfolded opsin accumulated in the ER of HEK293 cells (Sung et al., 1991a), researchers were
interested in the potential of P23H rod opsin interaction with ER-resident molecular pathways (Illing et al., 2002; Lin et al., 2007; Saliba et al., 2002). A set of genes expressed as ER-resident or ER-associated proteins have been evolutionary developed to function in clearing misfolded proteins in the processes called the unfolded protein response (UPR) and ER associated degradation (ERAD) (Walter and Ron, 2011). These pathways function to either improve the ER folding microenvironment or to induce the proteolytic clearance of proteins, respectively. Cell death has been implicated in both of these molecular pathways. When misfolded proteins accumulate in the ER (a phenomena called “ER stress”), the ER-resident UPR signaling molecules IRE1, PERK, and ATF6 become active and transcriptionally upregulate chaperone, glycosylation, and ERAD genes (Walter and Ron, 2011). However, during elongated periods of ER stress, the activation of IRE1 or PERK can lead to caspase-mediated apoptosis (Tabas and Ron, 2011). It was thought that these UPR apoptotic programs could belie the gain-of-function rod degeneration caused by P23H rod opsin expression.

In addition to UPR-mediated cell death being a candidate for P23H rod opsin’s pathology, researchers found that expression of misfolded protein could impair the ERAD process and lead to cell death (Bence et al., 2001). ERAD is a quality control pathway that the cell uses to degrade misfolded proteins through the ubiquitin-proteasome system (UPS) (Dobson, 2003). Ubiquitin is a 76 amino acid peptide that serves as a sorting tag that can be recognized by the proteasome. The 26S proteasome itself (named for sedimentation units) is in comparison, a gargantuan 2.4 MDa complex comprised of 28 subunits that proteolytically processes ubiquitinated substrates (Adams, 2004). Ubiquitin conjugation occurs through an interaction with E3 ubiquitin ligase and accompanying E2 and E1 enzymes. Classically, this interaction results in the attachment of ubiquitin to lysine residues on the substrate. Ubiquitin molecules themselves conjugate to each other through lysine residues, forming a poly-ubiquitin chain that is recognized by the proteasome. Ubiquitin is also able to conjugate to serine, threonine, the N-terminus, and
cysteine substrate residues (Shimizu et al., 2010). In the context of P23H rod opsin-induced cell death, two mechanisms were investigated: either the activation of apoptosis through UPR signaling or the global impairment of ERAD quality control.

In 2002, two groups separately found that P23H rod opsin is a substrate for ERAD. Illing and colleagues demonstrated that P23H rod opsin protein largely accumulates within the ER of HEK293 cells and accumulates further when treated proteasomal inhibitor. Accumulation of protein upon proteasomal inhibition is evidence that the same protein is a substrate for the proteasome. WT rod opsin was not demonstrated to accumulate in the same experiments. The same research group showed that the UPS was impaired upon P23H rod opsin expression through fluorescence resonance energy transfer analysis (Illing et al., 2002). With regards to UPR activation, P23H rod opsin expression in HEK 293 cells induces the expression of Bip and Chop which is indicative of active ATF6 and PERK UPR branches, respectively (Lin et al., 2007). In addition, our lab has shown that the artificial induction of the UPR IRE1 branch in HEK293 cells potently degrades P23H, but not WT rod opsin (Chiang et al., 2012). These in vitro experiments have implicated P23H rod opsin interaction with the UPR and ERAD. However, at this point, the observation of such molecular pathways in suitable animal models became necessary for a translation to actual RP disease.

Researchers developed a P23H knock-in mouse model to analyze PR degeneration when P23H rod opsin was expressed (heterozygous and homozygous) at levels comparable to murine opsin expression (Chiang et al., 2014b; Sakami et al., 2014; Sakami et al., 2011). Sakami and colleagues found that there was a marked decrease in the number of PR nuclei in both homozygous and heterozygous P23H mouse retinas in comparison to WT mouse retinas (Sakami et al., 2011). By using the similar retina visualization methods, our lab research has demonstrated that rod nuclei degeneration can begin as early as post-natal day 8. The heterozygote knock-in
mice developed a condition most similar to that found in ADRP patients, with an initial PR degeneration accompanied with slow degeneration thereafter (Chiang et al., 2014a). Such in vivo conditions make this new P23H knock-in model ideal for analysis of the involvement of UPR and ERAD in the RP disease.

Recently, our lab has found that PR degeneration in the P23H knock-in mice do not entirely reflect the molecular implications of in vitro studies. Interestingly, unlike the P23H aggregates formed with expressed in HEK 293 or COS-7 cells (Illing et al., 2002; Saliba et al., 2002), we have found no evidence of aggregation in the rod ER of P23H knock-in mice (neither heterozygote or homozygote mice) (Chiang et al., 2014a). In fact, it appears that P23H rod opsin protein is actually transported to the base of the rod OS when visualized on the days preceding rod degeneration. Additionally, the CHOP apoptotic branch of PERK (Tabas and Ron, 2011) was not found to be responsible for PR degeneration in either homozygote or heterozygote knock-in mice. This was found because the offspring of CHOP<sup>-/-</sup> mice crossed with either heterozygous or homozygous knock-in mice were still found to undergo PR degeneration. Further investigation is necessary to understand why differences exist between the knock-in model and in vitro studies.

Although the UPR’s involvement in the rod cell death of P23H knock-in mice is unclear, our lab has demonstrated the ERAD processing of P23H rod opsin in the same mice. Through western blots of retinal lysates probing for rhodopsin, we have determined that the P23H rhodopsin is more readily depleted in the homozygous knock-in mice when compared to both the wild-type and heterozygote knock-in. Additionally, immunoprecipitation experiments demonstrate that the P23H rhodopsin protein is massively ubiquitylated and associated with ERAD factors. Also, it appears the IRE1 branch in these mice is more active, as reported by crossing the knock-in mice with a sXBP1 venus reporter mouse (Iwawaki et al., 2004). The upregulated activity of IRE1 allows for the upregulation of ERAD factors that can contribute to
the depletion of the P23H rhodopsin protein. Our current model depicts that when expressed at endogenous levels, the P23H rod opsin protein is readily triggered to undergo ERAD (shown in Figure 1).

Given the recent insights of P23H rod opsin expression in vivo, there is still a lack of a mechanistic understanding of how misfolded P23H rhodopsin protein causes rod cell death. It is questionable how a protein that undergoes depletion could still cause an irregularity within the cell. The P23H knock-in model, however, corresponds well with Humphries’ previous demonstration that rod cell viability is reduced when rod opsin no longer reaches the rod outer segment. In the knock-in mice, a lack of rod opsin protein occurs through misfolding and ERAD depletion rather than a null mutation. When rod opsin is unable to reach rod outer segments, the cells begin the degenerate. This would explain why homozygous P23H knock-in mice demonstrate a more severe phenotype in comparison to the heterozygote. However, this does not explain P23H’s autosomal dominant inheritance. A simple haploinsufficiency of wild-type rod opsin does not appear to cause PR degeneration (Humphries et al., 1997). The single copy expression of P23H rod opsin may cause an abnormality in proteostatic balance. Indeed, an overload of substrates on the proteasome has been suggested as a mechanism of retinal degeneration (Lobanova et al., 2013). Out of the context of rod opsin mutations, disruption of other UPS components, such as a mutation in a particular Kelch family E3 ligase also leads to the development of PR degeneration (Ando et al., 2014; Kigoshi et al., 2011). P23H rod opsin may irregularly disrupt the UPS activity fundamental to rod PR’s in addition to causing a lack of rod opsin protein.

While P23H rod opsin has been studied in the context of rod degeneration and ERAD, its specific interaction with E3 ligases or the proteasome itself has not been elucidated. WT rhodopsin undergoes turnover by retinal pigment epithelium phagocytosis and subsequent
metabolism (Hall et al., 1990). It is important to understand how P23H rhodopsin undergoes ERAD in comparison to properly folded WT rhodopsin. How does the cell specifically recognize, ubiquitylate, and deliver P23H rhodopsin to the cytosolic proteasome? I would like to specifically address whether or not P23H rhodopsin undergoes canonical ubiquitylation in order to be degraded by the proteasome in light of the implication that it may play a role in PR degeneration.

The classic ERAD pathway would predict that the P23H rhodopsin is poly-ubiquitinated on lysine residues in order to be recognized and degraded by the proteasome (Ciechanover and Stanhill, 2014). In order to study whether or not this indeed occurs in the case P23H rod opsin degradation, I will generate P23H rod opsin constructs with lysine residues modified to arginine residues (KXR, 11 lysine residues total in rod opsin depicted in Figure 2). These mutations should abolish the ability for ubiquitin to conjugate to those amino acid sites while still maintaining a similar biochemical structure (Boutet et al., 2007; Shimizu et al., 2010). I will make different constructs with either single, multiple or all lysines mutated to determine which lysines might be critically ubiquitylated. I will then express both normal P23H rhodopsin and KXR constructs in HEK293 cells in order to observe their UPS interaction and assumed ERAD. This can be analyzed through measuring the half-life of the protein by cycloheximide (CHX) blocking. When inhibiting translation, if P23H rhodopsin undergoes basal depletion by ERAD and ubiquitin conjugates to lysine residues, then a lysine-less (K-Null) P23H rhodopsin should have a greater half-life since it be less recognized and degraded by the proteasome. Further evidence of the importance of lysine residues for the ubiquitination of P23H rod opsin can be gathered by immunoprecipitating the KXR constructs and probing for ubiquitin signal. I would expect that the lysine-less (K-Null) P23H rhodopsin protein would be give a weaker ubiquitin signal when pulled down. Ultimately, I want to determine what amino acids on the P23H rod opsin protein are conjugated to ubiquitin during ERAD processing.
Materials and Methods

Antibodies and Plasmids

Both WT and P23H Human RHO gene was placed into a pcDNA3.1 plasmid vector downstream of a CMV promoter (Invitrogen). Site-directed mutagenesis primers were designed to modify lysine residues to arginine residues (eleven total lysine residues) by modifying in-frame 5’-AAG-3’ codons to 5’-AGG-3’ codons. Mutagenesis reactions were carried out using PrimeStar Mutagenesis (Takara Bio. Inc., Shiga, Japan). Following this method, a variety of lysine mutant pcDNA3.1-RHO (WT and P23H) plasmids were constructed to have one or more lysine modifications (see Table 1).

The following antibodies were used immunoblots and immunoprecipitation: 1D4 anti-rhodopsin (Santa Cruz Biotechnologies, Santa Cruz, CA), P4D1 anti-ubiquitin (Santa Cruz Biotechnologies), Anti-HA, horseradish peroxidase-linked anti-mouse IgG antibody (Cell Signaling Technology, Beverly, MA), and anti-actin (Millipore, Billerica, MA).

Cell Culture and Transfection

For experimental analysis, HEK293FT cell culture was maintained at 37°C and a CO₂ concentration of 5% in DMEM (Mediatech, Manassas, VA) supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 5% fetal bovine serum (Invitrogen). The cells were cultured on either 10 cm dishes, six-well, or twelve-well flat-bottom plates (Fisher Scientific). Cells were transiently transfected with the pcDNA3.1 RHO plasmids through the use of FuGENE® 6 transfection reagent (Promega, Madison, WI) and its corresponding manufacture protocol. For immunoprecipitation experiments, the cells were incubated for a post-transfection period of 24 hours before lysis. In separate experiments, cells were also incubated for a post-transfection period of 24 hours before CHX treatment and incubation. In addition, for CHX
blocking experiments cells were cultured on Poly-D Lysine coated plates according to the product’s corresponding protocol (EMD Millipore, Billerica, MA).

**Immunoblotting Analysis and Protein Quantification**

Prior to cellular lysis, cultured cell wells were washed three times with ice cold 1X PBS (Mediatech). Depending on whether or not six-well plates or 12-well plates were being analyzed, 200µl or 100µl of ice-cold NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8, supplemented with Sigma Aldrich protease inhibitor cocktail) was applied to cultured cells prior to either immunoblot or immunoprecipitation protocols. The lysates were then incubated on ice for 30 minutes and subjected to vortex bursts every five minutes. Afterwards, the lysates were sonicated for 10 seconds. Lysates were then subjected to pre-clearance by centrifugation at 15,000xg and 4°C. The supernatant was removed for measurement of protein concentration and subsequent immunoassay analysis. Cell lysate protein concentrations were normalized with the BCA Protein Assay Kit (Pierce, Rockford, IL). Normalized µg amounts of proteins were used for gel loading or immunoprecipitation substrate-antibody incubation. 4-15% Mini-PROTEAN TGX precast gels (Bio-Rad) were used for gel running in 1X SDS buffer. The protein was then subsequently transferred to PDVF membranes and blocked for 1 hour with 2% Milk in 1X TBST. For visualization, primary incubations (see antibodies above) were applied overnight at 4°C while secondary incubations generally occurred at room temperature for 1-2 hours. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce) and the ChemiDoc™XRS system (Bio-Rad). Enhanced signals of 1D4 visualization were quantified using ImageJ software by measuring total lane pixel density (from 35 kDa to approximately 200 kDa) in which rhopsin bands appeared. Similarly, P4D1 anti-Ubiquitin signals were measured by total lane analysis.
Immunoprecipitation

Dynabead Protein G magnetic beads (Life Technologies) were used for the immunoprecipitation. As noted above, cells were lysed, homogenized, and sonicated using NP-40 lysis buffer on ice. Subsequently, the lysates were pre-cleared and protein levels from the supernatant were normalized. In parallel, either 1D4 (anti-RHO) or Anti-HA antibodies were incubated with the magnetic beads for 30 minutes (20µl of antibody solution for each sample). After washing (with 1X PBS with .2% tween), the antibody-bead complex was crosslinked using dimethyl pimelimidate. Equal amounts of protein from each pre-normalized experimental sample were added to aliquots of crosslinked complexes. For substrate incubation, mixtures were incubated over night at 4°C in rotation. For elution, LDS sample buffer was added to suspend the beads to a total volume of 40 µl. Samples were then boiled at 95°C for 10 minutes before immunoblot analysis.
Results

*K-null P23H Rhodopsin is less ubiquitlated than P23H rhodopsin with intact lysine residues*

To observe whether P23H rhodopsin requires canonical ERAD pathway processing, all eleven lysine residues of rhodopsin were scrutinized. To do so, P23H Rho plasmids were subjected to site-directed mutagenesis in a sequential manner, generating multiple plasmids that contained more than one lysine mutation (Table 1). These reactions were performed until a P23H Rho plasmid was generated to have all lysines converted to arginine (Noted here as P23H K-null Rho).

To analyze the phenotypic effect of such mutations, the ubiquitylation profile of the K-null P23H rhodopsin was analyzed by immunoprecipitation (Figure 3A). The input western blot (Figure 3A, left) demonstrated similar levels of P23H and K-null P23H rhodopsin along with a similar banding pattern. The similar levels of expression given the same protocol of transfection is discomforing for stability analysis (see Figure S1 and stability analysis below). In any case, upon pull-down of P23H K-null rhodopsin, the characteristic ubiquitin smear was much less intense than that of P23H rhodopsin (Figure 3A, left). The phenotype of K-null P23H rhodopsin was consistently seen to demonstrate a nearly halved ubiquitin signal across several different experiments (Figure 3B). Quantification of the ubiquitin:rhodopsin signal demonstrates that P23H K-null rhodopsin is less than half as ubiquitinated than regular P23H rhodopsin with lysines intact. The quantification of such a ratio was performed since the basal expression of each rhodopsin construct varied across experiments. With varied amounts of rhodopsin in immunoprecipitation and loading, it was appropriate to account for greater ubiquitylation signals that could be caused simply by loading more rhodopsin. Additional experimentation using reverse immunoprecipitation of ubiquitin demonstrates a stronger P23H rhodopsin association than K-
null P23H rhodopsin (Figure S2). However, HA-epitope tagged ubiquitin was used rather than endogenous ubiquitin in the immunoprecipitation protocol.

*K-null P23H rhodopsin has a greater stability than P23H rhodopsin with intact lysine residues*

Given the lack of ubiquitylation observed when during transient transfection, it was hypothesized that the K-null P23H rhodopsin construct would be more stable within the cell. However it should be noted that the basal level of K-null P23H rhodopsin upon transient transfection was not necessarily lower than that P23H rhodopsin (intact lysines) during all experiments. The variability of basal levels would suggest that P23H rhodopsin turnover is not strongly dependent on ubiquitylation and an associated ERAD pathway. However, CHX blocking data (Figure 4A and B) suggest that P23H rhodopsin is less stable within a 12-hour time period of translational arrest. Cells were transfected with rhodopsin plasmids for a 24 hours time period before the addition of CHX (10µg/µl). Cells were sequentially harvested at time points 3, 6, and 12 hours after CHX addition. After 3 hours of translational arrest, P23H rhodopsin decreases to less than 50% of its original amount whereas K0 rhodopsin remains around 80% of its original amount. Appropriately, WT rhodopsin protein levels remained stable even after 12 hours of CHX blocking. WT rhodopsin also demonstrates a band pattern more consistent with glycosylated monomers and dimers that support such stability (Chiang et al., 2012) These data are similar to a previous *in vitro* half-life analyses performed with P23H rhodopsin (Grichiuc et al., 2010; Noorwez et al., 2009; Sung et al., 1991b) that indicated that P23H rhodopsin has a half-life in the range of 3-6 hours.

*Single lysine residues do not appear to be solely responsible for P23H rhodopsin ubiquitylation*

After demonstrating P23H rhodopsin’s dependence on lysine residues for both ubiquitylation and degradation, it was appropriate to hone in on which of the lysine residues are
responsible for such processing. For this interrogation, K-null P23H rhodopsin was subjected to
mutagenesis to recover single lysine residues. A total of ten constructs were generated to have
only a single lysine residue intact (Table 1). It should be noted that the regeneration of K311
could was not technically achieved due to a primer incompatibility. As before, each of the lysine
recovery constructs were transiently introduced into HEK293FT cells for a 24-hour period before
lysate immunoprecipitation. Upon immunoblot visualization, the Ubiquitin:Rhodopsin ratio was
quantified for each of the recovery constructs (Figure 5A and 5B). From an average of three
separate experiments, immunoprecipitates of K245, K325, and K339 appeared to reveal a
statistically greater ratio than the original K-null P23H rhodopsin. However, none of the
constructs recovered a ratio comparable to the original P23H rhodopsin with intact lysine
residues. Similar experiments with P23H rhodopsin plasmids that had single lysine-to-arginine
conversions did not demonstrate a significant ubiquitylation differential (Supplemental Figure 1).
Thus, a conclusion about the absolute contribution of single lysine residues to P23H rhodopsin’s
ubiquitylation phenotype could not be drawn from. Rather, this data potentially suggests that
P23H rhodopsin is ubiquitylated at multiple lysine residues when expressed in the cell.

*K-null WT rhodopsin also demonstrates a diminished ubiquitylation*

It has been demonstrated previously that P23H rhodopsin is more susceptible to
misfolding and degradation than WT rhodopsin. However, the nature of the misfolding event and
the elucidation of ubiquitylation is not clear. *A priori*, proteins more prone to misfolding should
be more vulnerable to ER quality control and ERAD processes. However, any protein may be
prone to some basal and physiological level of misfolding (Walter and Ron, 2011) It is interesting
to note that mass spectrometry analysis revealed more E3 ligases to associate with WT Rho<sup>+/+</sup>
mice rhodopsin than Rho<sup>P23H/P23H</sup> knock-in rhodopsin (unpublished data). In other words, any E3
ligase that associated with P23H rhodopsin, also associates with WT rhodopsin. This is
suggestive of common ubiquitylation machinery that may be involved in the ERAD processing of both WT and P23H rhodopsin. It is therefore predictable that WT rhodopsin’s are also ubiquitylated at lysine residues in a similar manner that P23H rhodopsin is. To investigate this possibility, we generated a K-null WT rhodopsin. Indeed, K-null WT rhodopsin also demonstrates a diminished ubiquitylation signal similar to that seen of K-null P23H rhodopsin (Figure 6A, B). The input immunoblot (Figure 6A, top panel) demonstrates the preferred glycosylated WT rhodopsin bands in contrast with the multiple diffuse bands visualized for P23H rhodopsin. When subjected to immunoprecipitation (Figure 6A, bottom panel), WT and K-null WT rhodopsin signals are comparable to their P23H counterparts. In contrast, the ubiquitylation signal appeared to be lower for both WT and P23H K-null rhodopsins (Figure 6A, bottom panel). It should be noted however, that WT rhodopsin’s basal ubiquitylation level appears to be to similar to that of P23H rhodopsin (Ub:Rho signal ratios). This conflicts with previous immunoprecipitation data (Illing et al., 2002) that suggests that WT rhodopsin is less ubiquitylated than P23H rhodopsin. It is indeed confounding how P23H rhodopsin’s misfolding nature would not demonstrate a higher ubiquitylation profile.
Discussion

Given the massive and highly vital expression of rod opsin in mammalian rod photoreceptors, it is crucial to understand how the protein is processed within the cell. Like other membrane proteins, rod opsin is translocated into the ER during its synthesis. Indeed, a fundamental ER utility is folding newly synthesized proteins. As such, various post-translational modifications (PTM) have been identified that may be important for the quaternary maturation of rod opsin protein (Murray et al., 2009; Murray et al., 2015). Rod opsin has been found to be palmitoylated, acetylated and N-glycosylated (Murray et al., 2009). The contribution of cysteine disulfide bond formation to tertiary and quaternary rod opsin structure has also been studied extensively (Athanasiou et al., 2014; Chen et al., 2014; McKibbin et al., 2007). Additionally, evidence demonstrates that rod opsin is phosphorylated at its C-terminal serine residues (Filipek et al., 2003; Palczewski, 2006). All of these PTM’s appear to have some importance in protein folding and trafficking. In this present study, I explore the ubiquitylation of rod opsin as a potential PTM that regulates its degradation as a mechanism of ER quality control.

The K-Null P23H rhodopsin mutant appeared to be drastically less ubiquitylated than the P23H rhodopsin with lysines intact. This finding suggests that P23H rhodopsin is ubiquitylated on at least one of its lysine residues. Such a result appears to characterize P23H rhodopsin as a classical ERAD substrate. However, the data here does not conclusively determine which of the lysine residues are crucial for ubiquitylation. There are two conceivable ways in which an E3 ligase can ubiquitylate its substrates. For some substrates such as the epidermal growth factor receptor, ubiquitylation appears to be able to occur on any lysine residue (Huang et al., 2006). Conversely, ubiquitylation of SEC31 in COPII vesicular transport has been reported to occur on only one specific lysine (Jin et al., 2012). This does not appear to be the case for P23H rod opsin. P23H rod opsins with only one lysine had generally diminished ubiquitylation profiles.
comparable to the K-null P23H rod opsin. Additionally, mutating one of each of the lysines did not appear to decrease the ubiquitylation profile as much as depleting all lysine residues. Thus, it is entirely possible that E3 ligases can ubiquitylate rod opsin on a variety of lysine residues. Perhaps generating a P23H rod opsin construct with the proper combination of intact lysine residues would allow for optimal ubiquitylation, revealing which ones are targeted by E3 ligases.

In line with its phenotypic ubiquitylation, K-Null P23H rod opsin appeared to be markedly more stable than the P23H rod opsin with lysine’s intact. CHX blocking allowed for translational arrest to observe protein amounts of rod opsin as a function of time. During these experimental time periods, several pathways may lead to rod opsin execution. P23H rod opsin has been shown to be degraded by both autophagy and ERAD when the IRE1 UPR branch is active (Chiang et al., 2012). However, CHX loosens the ER translational burden (Harding et al., 2000) and it is thus uncertain whether UPR-induced opsin clearance may play a role in this study. The induction of ER stress by P23H rod opsin transient transfection was also not studied. It has been previously shown that proteasomal inhibition and autophagic inhibition results in an accumulation of P23H rod opsin. Thus, it is understandable that some level of both degradative pathways occur when P23H rod opsin is expressed. With a lack of ubiquitylation, the increased stability of K-Null P23H rod opsin may be due to a combination of ERAD and autophagy prevention. Along with poly-ubiquitin chains targeting substrates for proteasomal degradation, it has also been shown that ubiquitin can act as a signal for selective autophagic processing (Rogov et al., 2014). Further studies using proteasomal and lysosomal inhibitors may provide clearer evidence of the role of ubiquitylation in P23H’s instability. In any case, lysine residues and subsequent ubiquitylation may be important for P23H’s degradation in its guidance to the proteasome.
The half-life studies here generally agree with reviewed literature on the topic, with however more experiments needed for support. Sung and colleagues performed an immunoprecipitation on radiolabeled samples chased with nonradioactive amino acids. They found that P23H rhodopsin had a half-life in the 2-3 hour range and was completely cleared at after six hours of chase. Similarly, Noorwez and colleagues found a drastic drop in P23H rhodopsin 12 hours after CHX addition. In contrast, however, Griciuc et al. demonstrated that P23H rhodopsin had a relatively low level of degradation within an 8-hour CHX incubation period. In addition to variability in P23H rhodopsin’s stability, throughout experiments, there was not an observation that WT rhodopsin had a drastically higher expression level than P23H rhodopsin when conducting a similar transfection protocol. An important experiment should have been carried out to quantitatively normalize transfection efficiencies in order to observe basal post-translational degradation of WT, P23H, and K-Null P23H rhodopsin. Here, the use of co-transfection controls and a measurement of transcriptional expression could be used. Indeed other labs have shown that in general WT rhodopsin has a higher expression than P23H rhodopsin (Illing et al., 2002; Saliba et al., 2002; Sung et al., 1991a). If K-null P23H rhodopsin and WT rhodopsin were both more stable than P23H rhodopsin, this should have been observed at a basal expression level. These factors affecting P23H rhodopsin aggregation nullifies the ability to simplify data analysis.

Studies of lysine ubiquitylation on rod opsin protein extend beyond the study of P23H rod opsin’s ERAD. The covalent attachment the retinal chromophore to rod opsin’s lysine is a foundational PTM. Such characterization of rhodopsin can be traced back to its crystal structure elucidation coinciding with the GPCR’s mechanistic uncovering (Schertler et al., 1993). It has previously been shown that the chief site of retinal attachment occurs on lysine residue 296. K296E and K296M are point mutations causative of ADRP (Mendes and Cheetham, 2008). Current understanding dictates that these lysine mutant rhodopsins are constitutively active,
leading to continuous transducin signaling and ultimately cell death (Yang et al., 1997). Related to the P23H mutated opsin, there is evidence that retinal binding to P23H rod opsin facilitates proper folding. As such, 11-cis retinal has been used as a pharmacological chaperone to increase amounts of folded P23H rhodopsin in vivo (Noorwez et al., 2009; Tam et al., 2010). This folding is biologically sensible since opsin’s mature functionality is a structural event dependent on retinal conformation and photon interaction. Interestingly, what could stabilize P23H rhodopsin is retinal’s competitive binding against ubiquitin in the K296 position. The covalent binding of retinal to K296 may prevent E3 ligase ubiquitylation and subsequent ERAD. In such a case, retinal’s documented folding and stabilizing properties on rhodopsin may be inherently linked to the ERAD pathway.

To more specifically address lysine residues like K296, we propose to use mass spectrometry (MS) to map ubiquitylation sites on murine-expressed rhodopsin. Being able to use endogenous rhodopsin of \( Rho^{P23H/P23H} \) knock-in mice for MS analysis will also allow for analysis of what happens to P23H rhodopsin in the more accurate rod PR context. The classical MS workflow uses trypsin to digest proteins into peptide fragments in which lysine-diglycine (KGG) sequences are remnants of covalent ubiquitin conjugation (Peng et al., 2003). In pursuing this technique for rhodopsin, there are multitudes of difficulty identifying ubiquitylation sites in any peptide, let alone ERAD substrates. In fact of the whole proteome, only several hundred peptides had been found for yeast before more recent advances. For one, ubiquitylated peptides exist at a low substochiometric amount compared to unmodified substrates (Kim et al., 2011).

Additionally, many ubiquitylation purification techniques are not able to account for every type of ubiquitin branch type and length. A more recent purification technique using an antibody that has affinity for the KGG remnant lead to the identification of many (>10,000) ubiquitylation sites (Kim et al., 2011; Udeshi et al., 2012; Xu et al., 2010). Interestingly, these techniques have been proven robust in uncovering ubiquitylation sites in rat and murine tissues (Na et al., 2012;
Wagner et al., 2012). Pertaining to our objective, Wagner and colleagues found several ubiquitylation sites on GPCR’s expressed in neural tissues. It should be noted that as discussed before, P23H rhodopsin is degraded rapidly in Rho<sup>P23H/P23H</sup> rods. With little P23H rhodopsin to begin with, the di-glycine capture may not allow for the analysis of lysine ubiquitylation. However, the technique could be potentially used to map lysine ubiquitylation sites on WT rhodopsin in normal murine retina. My data suggests that WT rhodopsin is ubiquitylated at lysine residues in the HEK293FT system. A reciprocal caveat to this experiment would be the overall low levels of ubiquitylation observed in the WT mice upon rhodopsin IP (Chiang et al., 2014b). WT rhodopsin is normally folded and transported to the outer segment, normally non-suspect to the UPS. However, the ER is not perfect at folding every protein and as such it may be possible that some basal ubiquitylation may occur. Taking these factors into consideration, MS analysis of rhodopsin lysine ubiquitylation could provide a greater insight to how rhodopsin is processed in the retinal context.

Experimentation determining the subcellular localization of K-Null P23H rhodopsin could further illustrate the importance of ubiquitylation. When expressed in HEK293 cells, P23H rod opsin was previously found to be in the ER. However, in the P23H knock-in mice (both homozygous and heterozygous), our lab and others has found that rod opsin appears to localize at the base of the rod outer segment in P23H knock-in mice. This would indicate that P23H rod opsin undergoes some form of secretory transport, perhaps additionally stabilized by retinal conjugation in PR expression (Tam et al., 2010). It was observed that only low amounts of rod opsin transported to the membrane, presumably due to the fact that it is massively degraded through ERAD. It is possible that the P23H rod opsin can reach the plasma membrane if it is no longer subjected to ERAD. As discussed above, K-null P23H rhodopsin is more stable compared to P23H rhodopsin and thus might be more likely to be transported to the plasma membrane. Such a finding would support the possibility of using therapeutics that target the ERAD pathway
in patients with the P23H mutation. Indeed, gene therapy studies in RP mouse models already attempt to restore levels of rhodopsin in order to attenuate retinal degeneration (Berger et al., 2015; Chadderton et al., 2009; Mao et al., 2012). Such strategies use adeno-associated viral vectors for gene replacement, which show promise for other retinal diseases in clinical trials (Boye et al., 2013). Evidence for the critical nature of lysine residues for ubiquitylation and rhodopsin instability could place ERAD centrally in the context of retinal degeneration.

The P23H rod opsin mutation is chief in misfolding the protein in the most common form of RP. Clinical trials using valproic acid have been successful ameliorating disease by allowing for proper rod opsin folding (Kumar et al., 2014). The exact misfolding event caused by the point mutation, however, remains elusive. P23H is unlike other rod opsin mutations that have clear causative misfolding defects in glycosylation (T17) or disulfide bridge formation (C110 and C167) (Mendes et al., 2005). Nevertheless, the pathological appearance of the P23H mutation is currently understood to predispose rod opsin to depletion (Chiang et al., 2014a; Sakami et al., 2014). In light of Rho−/− knockout mice, the production of rod opsin protein itself is important for rod outer segment formation, rod viability, and general retinal health. From this perspective, it is clear that the predisposition to depletion may be a causative factor for rod degeneration in knock-in mice. In this model, however, it is unclear how the P23H mutation has a phenotypically dominant effect. A dominant negative model is supported by the fact that expression of WT rhodopsin can titrate the disease pathology (Price et al., 2012). Previous research has suggested that expression of P23H rod opsin may lead to the activation of proapoptotic UPR branches (Lin and Lavail, 2010; Lin et al., 2007). Nevertheless, such a conclusion based on the knock-in mouse models has not been drawn.

Our current focus of P23H rod opsin pathology centers on its ERAD processing. It has previously been suggested that P23H rod opsin may subject the cell to “proteasomal overload” as
a gain-of-function toxicity (Lobanova et al., 2013). The mechanistic train follows P23H rod opsin as a substrate that is continually processed by proteasome, thus inhibiting it from degrading other substrates. Indeed, the proteasome is a cornerstone proteolytic compartment that regulates the degradation of variety of substrates out of the context of mutated misfolded proteins. Evidence has demonstrated that the UPS is involved in degrading cell cycle components (Dobson, 2003) as well as inhibitors of apoptosis (Hiramatsu et al., 2014). Thus, disruption of the proteasome by P23H rod opsin may lead to irregularities in the turnover of other crucial peptides. In line with this, proteasomal inhibitors have been found to induce apoptosis and have been explored as a therapeutic for cancer (Almond and Cohen, 2002). Proteasome regulation is perhaps important for retinal health and the P23H ADRP itself. Retinal degeneration has been linked to genetic modifications in E3 ligases and proteasomal subunits (Ando et al., 2014; Kigoshi et al., 2011). Additionally, disruption of the UPS has been implicated in neuronal function and cardiac disease (Bennett et al., 2007; Pagan et al., 2013). Aside from this mechanism, although not shown with animal models, P23H rhodopsin has been shown to accelerate the proteasomal degradation of WT rhodopsin (Rajan and Kopito, 2005). The effect of P23H rhodopsin expression on the rod’s UPS should be investigated as a potential candidate for the mutation’s dominant nature. In conclusion, we’ve characterized P23H rod opsin as a classical ERAD substrate with proteasomal recognition dependent on ubiquitylation of multiple lysine residues.
Investigating XBP-1 Splicing as a Function of Progressive ER Stress Induction in the Tauopathy PS19 Alzheimer’s Disease Mouse Model

Introduction to Study

Neurodegenerative diseases are of the greatest and ever-emerging problems confronting medicine and our aging population. Diseases such as Alzheimer’s (AD) or Huntington’s (HD), or Parkinson’s (PD) can be characterized by neuropathological protein misfolding. Unlike misfolded P23H rod opsin in the case of RP, AD mouse models and patients have shown a correlation between protein aggregation and neurological deficits (Forman et al., 2004). It still however remains unclear what the causal mechanisms of protein aggregation may be. More importantly, it is uncertain how protein processing or homeostasis may play a role in AD. Tau proteins and β-amyloid proteins comprise two major debated models regarding AD progression. APP processing and the generation of neurotoxic oligomeric Aβ peptides appear to be a current AD research focus (Chiang and Koo, 2014). The intraneuronal aggregation of the hyperphosphorylated microtubule tau protein may, however, play a greater mechanistic role due its higher correlative power with disease progression (Bierer et al., 1995). Intracellular aggregation of proteins have long been a mechanistic underpinning for neurodegenerative disease model, spanning AD, HD, and PD. The manner in which such intracellular aggregation affects neuronal function and viability is an important study to undertake.

Along with developing a robust understanding of AD mechanisms, it is important to find early markers of AD pathology. With intraneuronal defective peptide processing, the role of ER stress is a candidate for study in AD progression. A fundamental function of the ER is to process, fold and produce peptides; an accumulation of pathologically misfolded proteins may stressfully induce UPR (Walter and Ron, 2011). As such, UPR activation has been implicated in proteinopathies AD, HD, and PD (Hoozemans, 2007; Hoozemans and Scheper, 2012; Vidal et al.,
One of the first indications on ER stress in AD was the finding of *Bip* upregulation in the neocortex of diseased patients (Hamos et al., 1991). Phosphorylated PERK and eIF2α has been repeatedly been reported to be associated with AD post-mortem brains and AD tau mouse models (Abisambra, 2013; Chang et al., 2002; Nijholt et al., 2011; O’Connor et al., 2008). Appropriately, accumulation of hyperphosphorylated tau has been shown to be correlative to UPR activation (Nijholt et al., 2011). It is however curious that a cytosolic microtubule protein would lead to activation of the UPR. This preconception stems from the current understanding that UPR is dependent on the ER luminal domains of PERK, IRE1, and ATF6. Abisambra et al. have modeled a UPR mechanism in which tau accumulation blocks ERAD and disrupts overall protein homeostasis. Such affected global processes could explain the powerful pathological effect of tau accumulation. In any case, ER stress development and UPR activation stand as potential early markers and pathological inducers of AD.

*Experimental Study, Hypothesis, and Procedures*

In this study, we plan to investigate UPR activation as a function of age in a tauopathy AD mouse model. We will study the PS19 transgenic mice (Yoshiyama et al., 2007), which express a human tau protein (P301S) that is susceptible to hyperphosphorylation and aggregation. These mice have been reported to develop synaptic deficits as early as 3 months of age, synapse loss at 6 months, and have an average life span of 12 months. Our goal is to study whether P301S tau triggers ER stress as it progressively accumulates in the neuronal CNS of these mice. For an additional tool, we crossed PS19 mice with ER Stress-Activated Indicator (ERAI) transgenic mice, which contain a transgene reporter for XBP1 splicing (Iwawaki et al., 2004). The ERAI reporter entails an XBP1 partial DNA construct that contains an IRE1α RNase-dependent intron following a sequence encoding the venus fluorescent protein. Under ER stress conditions, IRE1α homodimerizes, autophosphorylates, and performs unconventional XBP1 RNA splicing (Walter
and Ron, 2011). The splicing of the ERAI construct would produce a functional venus RNA and thus a fluorescent protein. We hypothesize that tau accumulation and pathology will correlate with ER stress induction and subsequent IRE1α activation at in the PS19 mouse model.

We will cross a PS19 mouse hemizygous (PS19<sup>+/−</sup>, ERAI<sup>−/−</sup>) for the P301S tau gene with a mouse homozygous for the XBP1 venus reporter (PS19<sup>−/−</sup>, ERAI<sup>+/+</sup>). This is a simple mendelian cross that we mapped out to have 50% mice that should develop tauopathic defects (PS19<sup>+/−</sup>, ERAI<sup>−/−</sup>) and 50% control mice that express only the reporter (PS19<sup>−/−</sup>, ERAI<sup>+/−</sup>) (Figure 7). It has been reported that the PS19 mice develop tau accumulation in the hippocampus, amygdala, cortex, brain stem, and spinal cord (Yoshiyama et al., 2007). We will take these tissue subsets from the offspring cross and detect the potential appearance of venus protein. Firstly, overall brain tissues and spinal cords will be lysed and analyzed whole by immunoblot (both for human tau and venus protein). Additionally, it is important to analyze different ages of mice through the progression of their pathology (timeline shown in Figure 8A). Genotyping will be performed using primer sets for both the ERAI reporter construct and the human P301S gene (Figure 8B). Phenotypic progression of synapse loss, tau accumulation, hyperphosphorylation, and neurological defects can be observed in an aging PS19 mouse. Thus it is appropriate to detect levels of UPR activation that may correspond to a gradient of tauopathic events. We hope to identify when UPR activation will occur and whether this correlates with the neuropathology of PS19 mice.
Figure 1. An illustrative comparison: Normal Rho^{+/+} mouse rod versus Rho^{P23H/P23H} mouse rod, which exhibit pathology preceded by P23H rhodopsin ERAD (Continued on pg. 26). *(Left)* On the left is a cartoon of a rod photoreceptor cell corresponding to normal WT mouse retinal structure. Here, there is a notable polarized neuronal structure with one end called the inner segment, which contains primary organelles such as the nucleus, endoplasmic reticulum and the golgi apparatus. Below the inner segment are synaptic endings. This is the part of the cell in which communication with other neurons are made in order to send visual signals to the central nervous system. These visual signals are initiated in the part of the cell called the outer segment (OS). Here, embedded membranous discs contain rhodopsins that initiate phototransduction.

*(Right)* On the right is an illustration of what a pathologically progressed rod may appear to be in the Rho^{P23H/P23H} retina at P15. While the OS develops properly at P8, it has a stunted appearance at P15, with what seem to be misaligned membranous disks. In addition, the P23H rhodopsin to be expressed is found in low amounts and subjected to ubiquitylation in the presumable ERAD process.
Figure 2. Rod Opsin is a seven transmembrane domain protein that resides that contains 11 lysine candidates for ubiquitylation. Above is a cartoon depiction of mouse rod opsin protein as embedded in the lipid bilayer of OS disks (reproduced from (Palczewski, 2006)). The N-terminus faces the organelle lumen throughout secretory transport and finally the lumen of the membranous disks in the OS. Highlighted in red is the 23\textsuperscript{rd} amino acid proline that commonly mutated to histidine in the inherited autosomal dominant form of retinitis pigmentosa (ADRP). Highlighted in purple are all lysine residues in the rod opsin protein. These residues may be important for P23H rod opsin’s degradation in ERAD (mechanism described in text).
Figure 3. K-Null P23H Rhodopsin’s ubiquitylation profile is diminished compared to P23H rhodopsin with lysines intact. (A) These are representative western blots of rhodopsin immunoprecipitation of cells transfected with either regular P23H Rho or K-null P23H Rho, demonstrating a less ubiquitylation when K-null was pulled down. P23H Rho and K-null Rho plasmids were expressed in HEK 293FT cells. The cells were then cultured for 24 hours and lysed in 1% NP-40 for experimentation afterwards. Cell lysates were subsequently subjected to rhodopsin antibody (1D4) immunoprecipitation. (Both upper and lower left panels) Immunoprecipitates of either P23H Rho or K-null Rho transfected cells were probed with either 1D4 rhodopsin antibody or P4D1 ubiquitin antibody. (Both upper and lower right panels) These blots represent the total cell lysate input detected in a similar manner. (B) For a comparison between regular P23H and K-null ubiquitylation, the ratio of immunoprecipitate ubiquitin signal to rhodopsin signal was quantified across five different experiments. Total lane pixel density of either ubiquitin or rhodopsin signal was used for quantification. Error bars represent the standard deviation seen across each experiment.
Figure 4. K-null P23H rhodopsin demonstrates greater stability than P23H rhodopsin with lysine’s intact. (A) HEK293FT cells were transiently transfected with rhodopsin plasmids (WT, P23H, K-null P23H) simultaneously. 24 hours afterwards, the cells were treated with CHX at a concentration of 10 ng/µl. After this addition cells were sequentially harvested at time points 3, 6, and 12 hours. The extracted cells were subsequently analyzed by immunoblot and probed for rhodopsin with 1D4 antibody. Chronologically consecutive lanes were loaded from left to right. (B) Through three CHX experiments, the amount of rhodopsin was calculated through the time-course after CHX treatment by using total lane densitometry analysis. The graph plots the straight-line progression of rhodopsin amount as a fraction of rhodopsin calculated from harvesting cells at the zero time point. Error bars represent the standard deviation of these fractions across three experiments.
Figure 5. Rhodopsin constructs with single intact lysine residues do not exhibit full recovery of ubiquitylation in comparison to regular P23H rhodopsin. (A) HEK293FT cells were transfected with P23H, K-null P23H, and other rhodopsin constructs that only contain one intact lysine (denoted as K66 for a rhodopsin with K66 but all other lysines converted to arginine). The cells were then cultured for 24 hours and then lysed subsequently for analysis. The bottom third panel represents the whole cell lysate input with actin as a loading control. The lysates were subjected to rhodopsin immunoprecipitation. The concentrated elution was analyzed by western blot, probing for either rhodopsin (1D4, top panel) or ubiquitin (P4D1, bottom panel). (B) The quantification of the ubiquitin to rhodopsin signal was used for the analysis of differential ubiquitylation between constructs. Across three experiments, P23H, K-null P23H, and the 10 constructs were analyzed for the ubiquitin to rhodopsin ratio by total lane densitometry analysis. The error bars represent the standard deviation between experiments.
Figure 6. K-Null WT rhodopsin also appears to have a diminished ubiquitylation profile in comparison to a WT rhodopsin with lysine’s intact. (A) HEK293FT cells were transfected with P23H, K-Null P23H, WT, or K-Null WT rhodopsin constructs for 24 hours. Afterwards, they were subjected to immunoprecipitation analysis. The left panels represent detection of either rhodopsin (upper) or ubiquitin (lower) from elution samples whereas the upper right blot represents whole cell lysate samples used prior to immunoprecipitation. (B) The ubiquitin:rhodopsin signal ratio was calculated and graphed to compare each of the constructs across this single western blot experiment. In order to quantify the ratio, total lane densitometry analysis was applied for both rhodopsin and ubiquitin signals.
Figure 7. A simple Mendelian cross between transgenic ERAI and PS19 mice generates two cohorts for analysis. Crossing ERAI mice without the human tau PS19 transgene with a PS19 mouse will yield two different possible genotypes for analysis. All of the offspring should express the ERAI reporter (XBP-1 recombinant DNA construct with a venus fluorescent protein gene downstream of a ER stress-sensitive intron). Half of the offspring should express the PS19 transgene, which is a human tau protein with a P301S point mutation.
Figure 8. ERAI vs. PS19 cross offspring will be analyzed at timepoints following neuropathological progression. (A) A timeline was generated to observe the splicing of the ERAI construct for different ages of mice. Synapse loss was reported (Yoshiyama et al., 2007) to begin as early as 1 month. Tau accumulation, decrease in solubility, hyperphosphorylation, and neurologically defective phenotypes progress up until approximately 12 months of age. At this point, mice expressing the tau transgene have compromised viability. Throughout all ages, the mice should be sacrificed to observe XBP1 splicing, tau abnormalities, and other branches of UPR activation. (B) Genotyping of the offspring mice will be performed in order to determine which mice should be expected to have PS19 pathology. The upper DNA agarose gel represents mouse ear tissue DNA extract amplified (PCR) by primers for a control gene (~550bp) and the ERAI transgene (below 240 bp). A PS19 mouse without the ERAI transgene is used as a negative control. The lower gel represents gene amplification of the tau transgene, with a band corresponding to 600 bp. PS19 mouse extract was used as a positive control.
Table 1. Inventory of Rho plasmids produced by a variety of site-directed mutagenesis reactions. Lysine-to-Arginine (KXR) Mutations represented with middle number indicating amino acid position (e.g. K296R, Lysine at position 296 mutated to Arginine). K-null indicates lysineless constructs in which all lysines are mutated to arginine.

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Table 1. Inventory of Rho plasmids produced by a variety of site-directed mutagenesis reactions, continued.

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Supplementary Figure 1. Basal expression levels of P23H and K-null P23H rhodopsin are similar. HEK293FT cells were transfected with either P23H rhodopsin or K-Null P23H rhodopsin (six wells each). 24 hours after transfection, the cells were lysed and subjected to western blot analysis. The visualization of whole cell extract by 1D4 antibody showed that between each separate transfection execution, the amount of K-Null P23H rhodopsin was comparable to that of P23H rhodopsin. Actin was used as a loading control.
Supplementary Figure 2. Reverse immunoprecipitation of transiently expressed HA-tagged ubiquitin demonstrates K-null P23H rhodopsin’s diminished ubiquitylation. HEK293FT cells were co-transfected with HA epitope-tagged ubiquitin and either P23H rhodopsin or K-Null P23H rhodopsin. Different ratios of HA-Ub and Rhodopsin were used for transfection. 24 hours after transfection, designated wells were treated with 1 mM lactacystin for 24 hours. All wells were lysed at the same time for analysis. The cell lysates were then subject to IP using anti-HA antibody. The top two blots represent the HA IP visualization while the bottom two blots represent the whole cell lysate. Both sets were probed for either rhodopsin or HA epitope. The HA IP demonstrated that at level amounts of HA being pulled down, more P23H rhodopsin would associate than K-Null P23H rhodopsin.
Supplementary Figure 3. Single lysine mutations in P23H rhodopsin do not decrease its ubiquitylation. HEK293FT cells were transfected with either P23H rhodopsin or P23H rhodopsin with one of its lysine residues mutated to arginine. After a transfection period of 24 hours, the cells were lysed and analyzed for rhodopsin ubiquitylation. After rhodopsin 1D4 IP, the eluates were blotted and probed for either rhodopsin or ubiquitin. As shown above, single lysine residue mutations did not appear to affect the status of ubiquitylation largely in comparison to just variation in rhodopsin loading itself.
References


