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Defining the Pathway for Misfolded Protein Degradation in Retinitis Pigmentosa Caused by the Rhodopsin-P23H Mutation

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ABSTRACT

Retinitis pigmentosa (RP) is a term used to describe a wide variety of inherited degenerative diseases that affect the eye. While there are many causes of this disease, the most commonly found mutation that causes RP in North America is an autosomal dominant missense mutation in rhodopsin (Rho^{P23H}). Previous studies have shown that Rho^{P23H} is predominantly misfolded, resulting in a dramatic loss of the ability to stably bind 11-cis retinal and thus function as a photopigment. Previous work has shown that this process is conserved to some degree across many models, from pigs to mice, and even is evident when mutant mammalian rhodopsin is exogenously expressed in flies. Presently, there is limited information on the mechanism(s) that detect and degrade rhodopsin. To investigate this phenomenon, we cultured transgenic *Drosophila melanogaster* and evaluated expression of exogenous rhodopsin through western blot analysis. Our results suggest that the fly system is capable of simulating a realistic environment for murine rhodopsin. By harnessing the powerful genetic tools surrounding the fly system, future studies using RNAi techniques may be able to elucidate identification and degradation pathways important to the progression of RP.

EXECUTIVE SUMMARY

Sight is a complex sense made possible by the combination of many signaling pathways. Central to this process is an important cycle of protein modifications that convert energy from light to processed electrical signals in specialized cells called photoreceptors. The light-sensitive protein receptor responsible for this process of phototransduction in rod photoreceptors is called rhodopsin. Protein modification of rhodopsin has been observed to be a largely conserved feature of visual systems across the animal kingdom.

The complexity of the visual cycle leaves a lot of room for error. Small mutations in functional portions of DNA have been observed to have major consequences for the cycle as a whole. For example, a single missense mutation, resulting in an amino acid change from proline to histidine in the sequence that encodes the protein rhodopsin, is known to cause a disease named Retinitis pigmentosa (RP). On the biochemical level, this single missense mutation causes a misfolding of the proteins 3D structure. This aberrant conformational change in rhodopsin increases the propensity of aggregate formation in the retina. The accumulation of these aggregates has been shown to cause degeneration of first, rod photoreceptors, and then cone photoreceptors, which results in the patient level symptoms of progressive vision loss that leads to eventual blindness.

Mutations of this nature are not unique to the human system. This phenomenon has been observed in other models, including: large mammals[9], invertebrates[8], and even cultured cells[10]. While the outcome is known, the mechanism is largely unresolved.

Previous studies in mice have shown that observed Rho^{P23H} expression is much lower than wild-type controls[14]. This suggests that mutant rhodopsin is recognized in the cell before it is transported to its functional location in the outer segment of the photoreceptor. Other

experiments using transgenic frogs[17], and mice[18] have shown disruptions in the membrane integrity of the specialized outer segment of the rod cell where phototransduction occurs. Therefore, it seems reasonable to propose that the Rho^{P23H} that escapes degradation and is transported to the outer segment is contributing to rod death. By identifying and exploiting the pathways which recognize and degrade mutant rhodopsin through drug or gene therapy, it may be possible to halt or even prevent this diseases progressive fate.

The core aim of my project was to develop a protocol for screening genes of interest related to the identification and degradation of Rho^{P23H}. In our model, $mRho^{P23H}$ was exogenously expressed through the utilization of a Gal4-UAS system with an Rh1 promoter. Meaning that a mutant mouse gene for rhodopsin was inserted and expressed at a target location in the fly's eye. By characterizing the behavior of $mRho^{P23H}$ and $mRho^{wt}$ using western blot techniques, a baseline was set for future investigations using RNAi techniques. The baseline was expected to show a constant expression of $mRho^{wt}$ across several ages and a decrease across ages for $mRho^{P23H}$. The purpose of this baseline test was to confirm transgene expression and establish the correct size of gene products. However, results suggested that our controls were not behaving as previously understood. Both the $mRho^{wt}$ and $mRho^{P23H}$ fly lines displayed odd trends of degradation across different aged flies.

To further investigate this phenomenon, the fly lines were cleared of balancer chromosomes by selection and/or mating techniques. Flies were collected upon eclosion, which is when they come out of their pupal case, and reared to different ages upon which their heads were collected for protein analysis. The fly ages for the sample collections include days: 5, 7, 9, 11, and 14. Collected heads were separated by age, gender, and genotype before being homogenized and prepared for western blot analysis.

Results showed that the $mRho^{wt}$ follows a similar pattern of expression to that of a congruent system, which instead of using exogenously expressed mRho (from mice) used bRho (from cattle)[38]. Our results also confirmed that exogenously expressed $mRho^{P23H}$ is severely degraded, suggesting that the mechanism for identification and degradation of misfolded protein is conserved across mice and flies. This fortifies the usage of flies as a model system for studying this phenomenon. Flies offer many advantages in genetic studies which can be more easily exploited as compared to the mouse system. Through the use of readily available transgenic lines, identification and degradation pathways can be investigated through the use of the fly system with mouse rhodopsin.

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ADVICE TO FUTURE HONORS STUDENTS

If I had to offer two pieces of advice to future honors students, I would say this:
Start your capstone early, and make sure it is on something you enjoy because you will undoubtedly be spending an enormous amount of time on it.
And while we are speaking of time, you have much less than you may think.

INTRODUCTION

Retinitis pigmentosa (RP) is the umbrella term for a large set of inherited degenerative eye diseases that affect the retina. The retina is the light-sensitive tissue in the back of the inner eye that is responsible for converting what we see into electrical signals that then get sent to the brain via the optic nerve[1]. RP is the causal factor of more than 1 million cases of blindness worldwide. While the disease is well characterized and easily diagnosed, there remains no effective cure. RP dramatically degrades this process by causing the photoreceptor cells to die. Most often, the rods, which are responsible for dim light vision, are first (primary) affected and secondarily cones, which are responsible for daytime and color vision, are lost. The loss of these cells, which line the outermost layer of retina, results in the characteristic symptoms of RP. Initially, the disease presents itself as nyctalopia or night blindness, a condition in which it is difficult for the patient to see clearly in dim light conditions. Because the rods are distributed in the periphery of the retina in humans while cones are concentrated in the macular (central region), the earliest symptoms are in peripheral vision while maintaining clear vision in their central field (**Figure 1**). As the condition worsens, rod cells continue to die and central regions of the retina start to degenerate, patients begin to experience loss of their central vision[2]. Other forms of RP exist, for example cone-rod dystrophy (CRD), in which the primary cell death includes both cones and rods, thus affecting central vision more severely earlier than other forms.

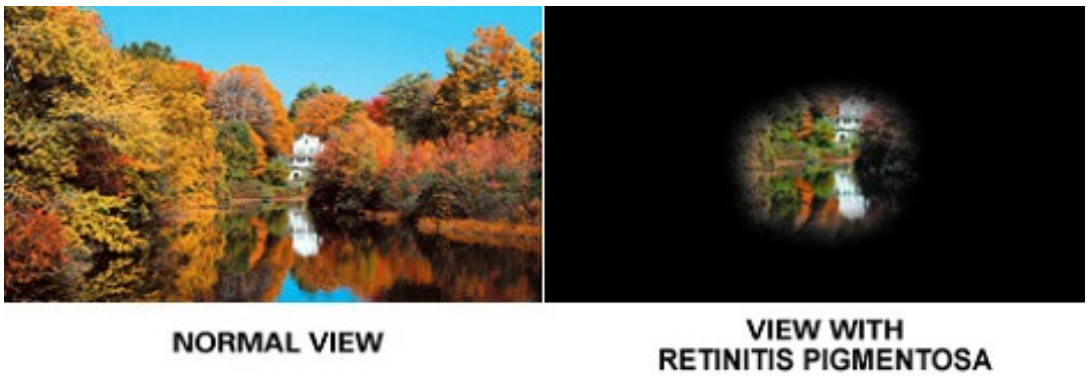


FIGURE 1. SIMULATION OF RETINITIS PIGMENTOSA (IMAGE 1)

RP is linked to many different genetic loci with about half the cases inherited[3], through one of three genetic inheritance patterns: autosomal recessive, autosomal dominant or X-linked. The most commonly found mutation that causes RP in North America is an autosomal dominant missense mutation in rhodopsin (Rho^{P23H}). Rhodopsin is the photosensitive pigment that is expressed in rod cells and absorbs light to initiate vision[4]. The mechanism by which the mutant rhodopsin causes rods to die is unknown, and the development of therapeutic strategies is therefore limited. A number of research groups have developed gene therapy approaches that have the potential to slow or reduce retinal degeneration[5]. However, these approaches are still in the early phases and the associated costs are not clear. A better understanding of the fate of Rho^{P23H} protein could identify cellular pathways that could be targeted for drug development. This project aims to uncover such pathways using a powerful model system.

Previous studies have shown that Rho^{P23H} is predominantly misfolded, resulting in a dramatic loss of the ability to stably bind 11-cis retinal and thus function as a photopigment [6,7]. Very little is known about the fate of Rho^{P23H} in human retina, so model organisms have been used extensively, from invertebrates[8] to large mammals[9], in addition to cultured cells[10]. The recent development of a knock-in mouse model[11] has provided a disease model without the complications of transgenic lines that overexpress rhodopsin[12,13]. In the knock-in heterozygote mouse, the Rho^{P23H} protein levels are much lower compared to the wild-type protein[14], suggesting most but not all of the mutant protein is targeted for degradation. However, haploinsufficiency does not explain the effects of Rho^{P23H} on rod survival, since in hemizygous rhodopsin (Rho/-) knock-out mice, the photoreceptors degenerate with a much slower time course than Rho/Rho^{P23H} [15,16]. Thus, it appears that there is a “dominant-negative” effect of the Rho^{P23H} protein, even at the low expression observed in the knock-in mice. Experiments using

transgenic Xenopus[17], and knock-in mice[18] have shown disruptions in the membrane integrity in the specialized outer segment compartment where phototransduction occurs. Therefore, it seems reasonable to propose that the Rho^{P23H} that escapes degradation and is transported to the outer segment is contributing to rod demise.

At present, there is limited information of the mechanism(s) that detect and degrade mutant rhodopsin. Although there have been extensive experiments using cultured mammalian cells[19,20], the applicability of the cell line responses to that in photoreceptor is questionable[21,22]. So, it is important that mechanistic studies be designed to investigate photoreceptors.

In most cells, there are primarily two methods of protein degradation, lysosome-mediated and proteasome-mediated. Each method of degradation has several pathways to eventual protein degradation and could be the underlying cause of photoreceptor death in Rho^{P23H}. The goal of my capstone project will be to determine if either pathway is involved in Rho^{P23H} degradation so that future studies can work towards an understanding of its process. Currently, it is thought that through an endoplasmic-reticulum-associated-degradation (a lysosome-mediated process), misfolded Rho^{P23H} proteins are recognized by an unknown protein and degraded. The proposed cause of photoreceptor death is the accumulation of residual or partially digested protein that becomes toxic for affected cells. Under this assumption, by identifying this protein we will be able to investigate in detail the mechanism of Rho^{P23H} identification and degradation. Future goals of this research will be to develop methods to aid in the degradation of misfolded rhodopsin, thus reducing cellular toxicity, which in return may save the photoreceptor cells.

Previous approaches to large-scale screenings have been expensive, time consuming and often problematic. Using mammalian cells, and other animal models for exploratory studies have proven to be highly inefficient and time consuming. Using cell culture, an approach usually well

suited for studies such as this one, have proven to be problematic when Rho is involved because of its toxicity in cell lines when high concentration of exogenous Rho is present[24]. To screen for possible pathways affecting rhodopsin degradation, I will use a novel approach with *Drosophila* (fruit flies).

Many aspects of the *Drosophila* model are favorable for such an approach: fly photoreceptors produce rhodopsin in large amounts and elaborate light-sensitive membranes specialized for phototransduction[25], and *Drosophila* are a well-characterized model used extensively in genetic research, especially for gene discovery. *Drosophila*'s minimal maintenance and low cost, make them efficient and cost effective for larger scale studies. Since *Drosophila* are so well studied, there are many tools for genetic analysis at the cellular and molecular level. But most importantly, *Drosophila*, like mammals, possess photoreceptor neurons with the unique ability to synthesize large amounts of protein-rich membranes. New studies by the Pignoni and Knox labs have shown that *Drosophila* photoreceptors can express mouse Rho and transport the wild type protein to the appropriate membranes[26]. Moreover, in unpublished studies[31], mutant Rho^{P23H} was degraded in photoreceptors over approximately two weeks while the wild type Rho was stably expressed. This restates a key feature of the mouse knock-in model, selective degradation of Rho^{P23H}, and supports the use of *Drosophila* for dissecting the degradation pathway.

While there are many similarities between *Drosophila* and vertebrate models, it is important to note that there are many distinct differences as well. *Drosophila's* photosensitive photoreceptors are elaborated microvilli structures of stacked membranes called rhabdomeres[27,30], while rods are modified cilia with stacks of membrane disks[28]. *Dropsophila* and vertebrate phototransduction pathways follow similar conserved mechanisms of initial

events in transduction but diverge in terms of specific intermediates and interpretations of absorbed photons[27.36].

Under the direction of the Knox Laboratory and in collaboration with the Pignoni Laboratory at Upstate Medical University, a lab with specialty in *Drosophila* research, I will have the necessary mentorship and tools to effectively investigate my research goal. Using the fly model and RNA interference methods, I will search for RNAi lines that alter the degradation rate of Rho^{P23H}. The basic approach will be to select various lines from the *Drosophila* stock centers and to cross those flies with the existing *Drosophila* lines in the Pignoni lab that express either $mRho^{wt}$-eGFP or $mRho^{P23H}$-eGFP genes[30]. I will grow the flies for one to two weeks and then determine Rho protein levels using quantitative western blotting based upon luminescence detection[32]. In this way, I hope to identify candidates that may be involved in recognizing misfolded protein or directing misfolded protein to the degradation enzymes. Rho^{wt}-eGFP will serve as a control for the specificity of the RNAi line to degradation. The Specific aims of my research project are to:

1. To create Drosophila lines expressing RNAi for enzymes potentially involved in recognizing misfolded proteins, these will include initially E3 ubiquitin ligases and related genes, chaperonins, N-linked glycosylation pathway enzymes and endoplasmic reticulum-specific lectins[33,34,35], the precise number of lines will depend upon how readily the crosses can be prepared and how variable the expression level of the Rho transgene is in the various RNAi lines;
2. To confirm quantitative western blot analysis of Rho^{wt}-eGFP and Rho^{P23H}-eGFP in one to two-week old *Drosophila* is suitable for my screen. I will optimize the western blot as

needed. As controls, the VCP/ter94[8] and dPob/EMC[36] lines will be tested, as those genes have been implicated in the biosynthetic pathway for endogeneous *Drosophila* rhodopsin.

For every RNAi that induces altered degradation of Rho^{P23H}-eGFP compared to Rho^{wt}-eGFP, I will:

1. Confirm that degradation occurs at the Rho^{P23H}-eGFP protein level and not the mRNA level by showing transcript stability by qRT-PCR;
2. Confirm that the mRNA level for the targeted degradation gene has indeed decreased, using qRT-PCR and/or immunostaining if the antibodies are available commercially.

These experiments should permit an initial screen for potential genes of interest in the degradation of mutant rhodopsin.

MATERIALS AND METHODS

A. Fly Crosses

Initial fly stocks were previously created by the Pignoni Lab (SUNY Upstate Medical University, NY). They were created by transforming white plus marked flies with mRho constructs in a white minus background.

Balancers were eliminated by a brother and sister intercross, followed by a cross to a wild-type white eye fly line (w^{1118}; cs). Fly line "NT" received no modifications. For each –eGFP fly line, an -mCherry variation was made as well for future studies. A homozygous $mRho^{P23H}$-mCherry was not sufficiently viable to establish a pure stock. Balancers were eliminated from fly line "D" by crossing it to w^{1118}, followed by selection of phenotypic characteristics. The resulting genotypes of produced fly lines are listed below in **Table 1**.

Table 1. Genotypes of modified fly lines.

Fly Line Abbreviation	Genotype[1]
A	Rh1-Gal4 , UAS-$mRho^{wt}$-eGFP - // -
B	Rh1-Gal4 , UAS-$mRho^{P23H}$-eGFP - // -
NT	w ; SM5 T(2,3)ap^{xa}
D	Rh1-Gal4 , UAS-$mRho^{wt}$-eGFP - // -
F	Rh1-Gal4 , UAS-$mRho^{wt}$-eGFP - // -

B. Fly Rearing

Flies were reared and maintained at 25°C, unregulated humidity, in a 12-hour light/dark cycle. Adult flies, collected upon eclosion, were maintained with lab-made agar-based diets consisting of cornmeal, dextrose, agar, water, propionic acid, 20% tegosept and yeast (adapted

[1] "- // -" denotes the fly line is a heterozygote, "m" denotes murine

from a Perkin's lab recipe). Flies were collected twice each day, once in the morning and once at night. Each collection was reared independently until desired age was reached.

C. Fly Head Harvesting

Heads were collected from female flies on days 5, 7, 9, 11, and 14 days after eclosion. Head collection was performed on a sanitized CO_2 flypad post CO_2 anesthesia with a sanitized scalpel. Collected heads were separated by gender, genotype, and age and immediately frozen in 1.7mL microcentrifuge tubes on dry ice after they were obtained. Collected fly heads were stored at -80°C until processed.

D. Western Blot Analysis

For each sample, 20 frozen female fly heads were homogenized in 60uL of opsin solubilization buffer (OSB) (1X PBS, pH 7.4, 1mM EDTA, pH 8.0, 1% DM (n-dodecyl-beta-D-malto (pyrano) side), and 1 tablet of Roche cOmplete™ Mini Protease Inhibitor, EDTA Free)) via tube and pestle over ice. Sample protein concentrations were calculated via Bio-RAD DC™ Protein Assay and normalized with excess OSB. See **Figure 1** for protein extraction efficiency. Lysates were diluted in 4x Laemmli sample buffer (277.8 mM Tris-HCl, pH 6.8, 44.4% glycerol, 4.4% LDS, 0.02% bromophenol blue, and 10% 2-mercaptoethanol) prior to storage at -20°C. 80ug of denatured protein was loaded into precast 10% SDS- polyacrylamide gels (Bio-Rad Mini-PROTEAN® TGX™), with a ladder mixture (3uL Thermo Scientific™ PageRuler™ Plus Prestainied Protein Ladder, and 10uL Thermo Scientific™ MagicMark™ XP Western Protein Standard) and separated by electrophoresis at 0.03 amperes for about 50 minutes. Separated samples were transferred to a polyvinylidene difluoride membrane (Immun-Blot® PVDF), blocked in blocking buffer (BB) (5% powdered milk, 0.1% Tween 20 in PBS) and incubated for at least 16 hours overnight at 4°C with a primary antibody, mouse monoclonal 1D4 (University

of British Columbia), 1: 5,000 dilution in BB. Overnight blots were then incubated with a secondary antibody, goat anti-mouse HRP (Sigma), 1: 10,000 dilution in BB, at room temperature for one hour. Membranes were visualized by Enhanced Chemiluminescence (ECL) detection with Bio-Rad Clarity™ Western ECL Blotting Substrate or Thermo Scientific™ SuperSignal™ West Femto Maximum Sensitivity Substrate. The analysis of processed blots was done on a Bio-Rad Molecular Imager ChemiDoc XRS+ using Bio-Rad Image Lab™ Software.

RESULTS

A. Homogenization Efficiency

Under the current sample preparation protocol, lysate total protein per sample did not vary significantly (**Figure 2**). When separated by group, post-homogenization lysates of 20 female fly heads displayed consistent levels of protein extraction (**Figure 2A**). When separated by age, lysates displayed uniform dispersion in all age categories with minor variation (**Figure 2B**), signifying no correlation between age and extraction efficiency. We believe that minor differences are not real differences in protein per head, but rather reflect improvements in the operator's technique.

B. Western Blot analysis

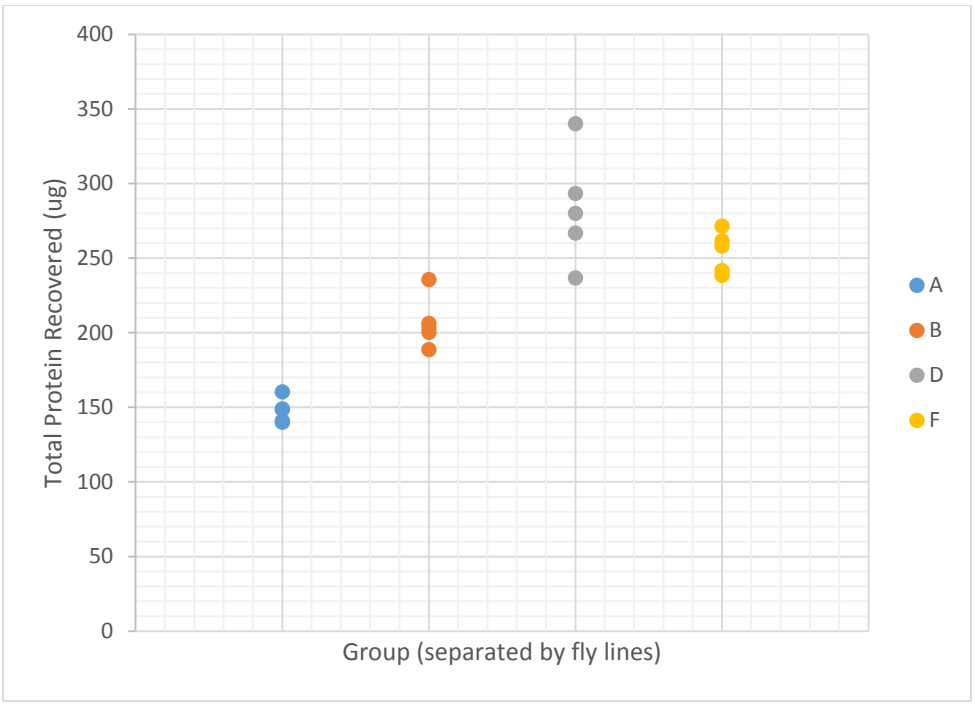
Figure 3 depicts detection of bands using primary antibody mouse monoclonal 1D4, and secondary antibody, goat anti-mouse IgG-Peroxidase, with ECL reagents. **Figure 3A** shows the state of our eGFP wild-type control (mRho^{wt}-eGFP) prior to the elimination of the balancer chromosome. Results from this line show that the expression of the transgene was variable, displaying a period of rapid increase in expression from Day 5 to Day 7, followed by a severe drop off between days 7 and 9. **Figure 3B** displays mRho^{wt}-eGFP following the removal of the balancer chromosome from the stock. The detection of mRho^{wt}-eGFP was predominately at ~55

kda. These bands display slight smearing which may indicate some level of variable protein glycosylation in the individual rhodopsin molecules. Through volumetric and visual analysis, it is evident that protein detection is decreasing overtime. Between Day 5 and Day 9, detection decreases 17%, followed by a decrease of ~30% from Day 9 to Day 14 (**Figure 3C**).

To evaluate bands of interest from non-specific bands, we conducted a western blot with a negative control. In **Figure 3D**, non-*Rho* transgenic SM5/T(2,3)ap^{xa}, labelled "NT" was compared to mRho^{P23H}-eGFP, labelled B5, both at 5 days of age. The mRho^{P23H}-eGFP lane displays one band that is very faint around 115 kda, labelled with a red arrow, which is not present in the non-*Rho* transgenic lane. We deduce that this may represent the dimer of mRho^{P23H}-eGFP. We were also expecting to see a faint band around 55 kda in lane B5, signifying the existence of the monomer, but it appears that because of low expression or transfer efficiency this band is not visible. However, comparing lane NT to blot "E" reinforces our assumption that the band seen at ~55 kda in blot "E" is in fact our protein of interest.

The transgenic mRho^{P23H}-eGFP flies were observed to be much dimer under epiflourescence microscopy as compared to the mRho^{wt}-eGFP line. In western blot analysis, this low expression was also observed. In **Figure 3E**, the detection of the monomer for mRho^{P23H}-eGFP was observed at ~55 kda, with variety of other bands. The increase in band detection can be, in part, connected to the use of the ECL reagent Femto which is a much more sensitive substrate than Clarity which was used for **Figure 3B**. It is important to note, however, that even with Femto, exogenous rhodopsin detection was extremely low. Other notable bands: ~110 kda may represent the dimer of mRho^{P23H}-eGFP. Volumetric analysis of the monomer bands show a trend of logarithmic increase from Day 5 until Day 9, followed by negative linear regression from Day 9 to 14 resulting in a loss of expression of ~56% over five days (**Figure 3F**).

A.



B.

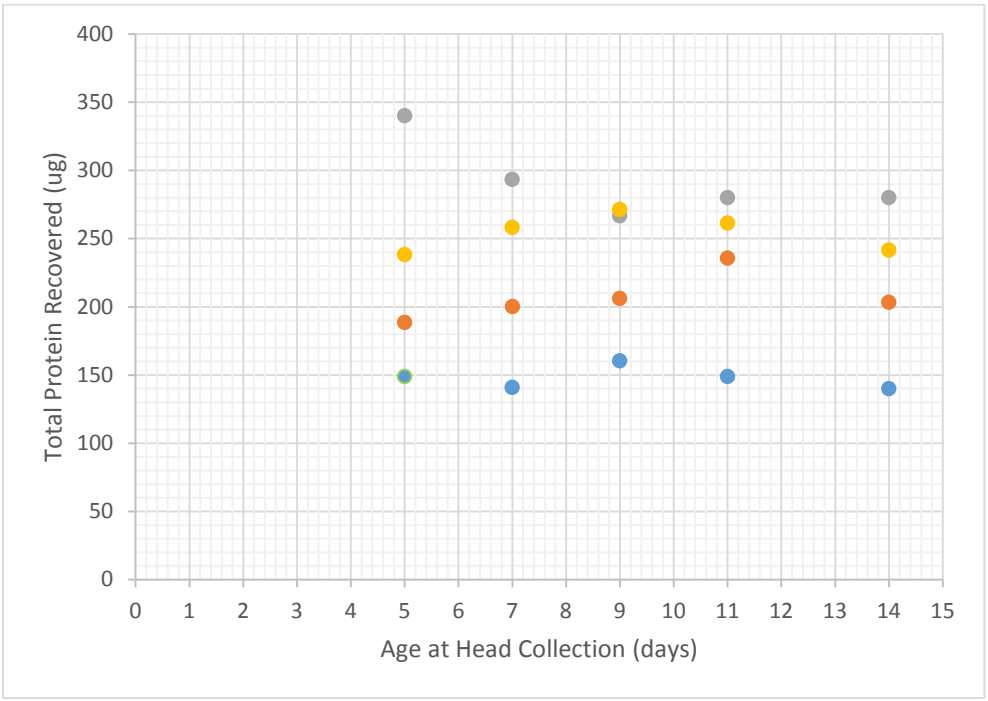


Figure 2. Post-homogenization total protein of 20 female fly heads. A. Processed samples separated into groups by fly lines. Each dot represents 20 female fly heads collected as a group on different days. **B.** Processed samples separated into groups by age of heads. Each dot represents 20 female fly heads collected as a group on different days.

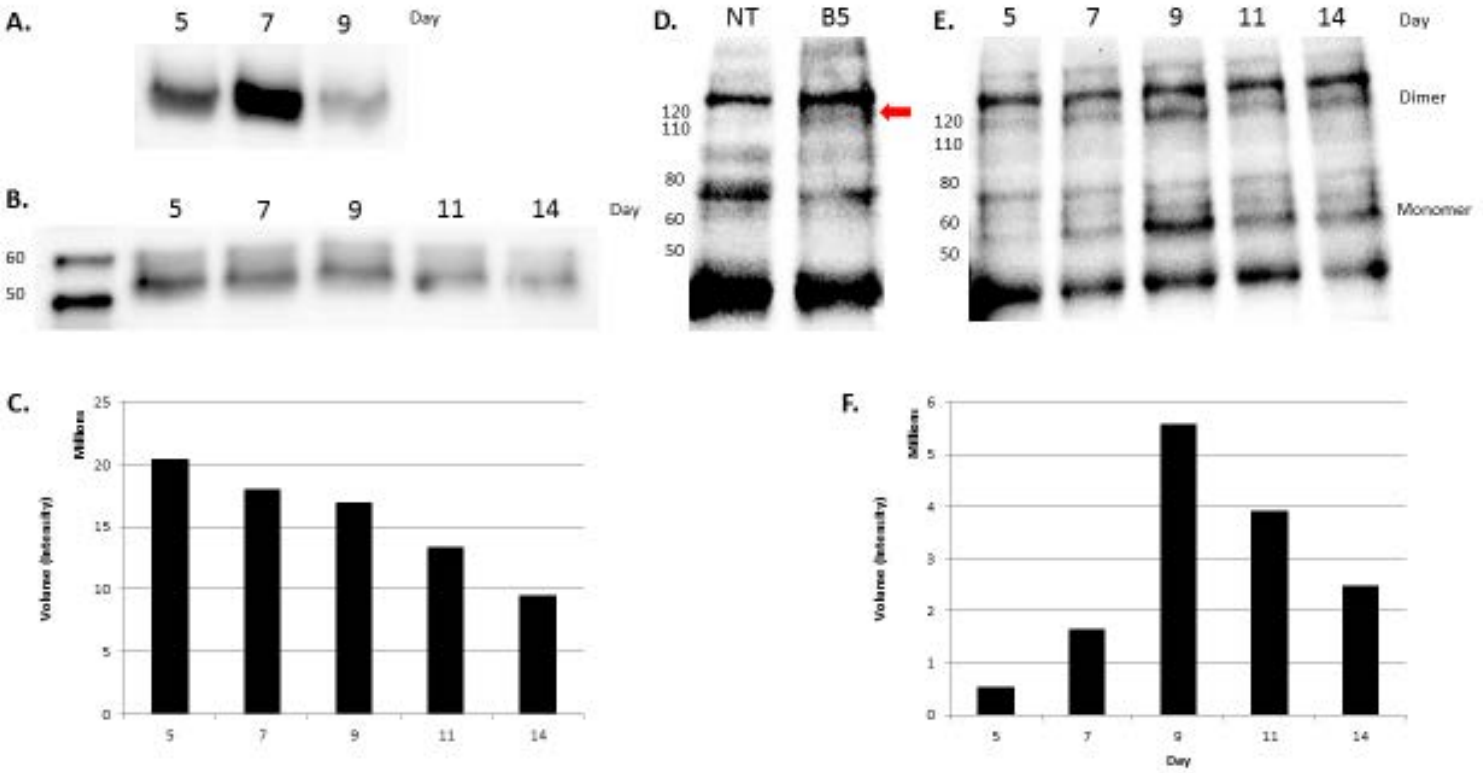


Figure 3. Western blot analysis of *Rho* variants of flies at different ages. A. Western blot analysis of fly line A (mRho^{wt}-eGFP, mixed stock with TM3 variance) for days 5, 7, and 9. 80 ug of homogenate per lane, immunodetection with mouse monoclonal 1D4, chemiluminescent with goat anti-mouse IgG-Peroxidase with Thermo Scientific™ SuperSignal™ West Femto Maximum Sensitivity Substrate, 7 second exposure. **B.** Fly line A (mRho^{wt}-eGFP with balancer chromosome TM3 eliminated) analysis of days 5, 7, 9, 11, and 14. 80 ug of homogenate per lane, immunodetection with mouse monoclonal 1D4, chemiluminescent with goat anti-mouse IgG-Peroxidase with Bio-Rad Clarity™ western ECL substrate, 112.7 second exposure. **C.** Volumetric analysis using intensity of blot "B" with Bio-Rad Image Lab™ Software. **D.** Negative Control with: NT- non-*Rho* transgenic SM5/T(2,3)ap^{xa} and B5- mRho^{P23H}-eGFP at 5 days of age. 80 ug of homogenate per lane, immunodetection with mouse monoclonal 1D4, chemiluminescent detection with goat anti-mouse IgG-Peroxidase with Thermo Scientific™ SuperSignal™ West Femto Maximum Sensitivity Substrate, 105.8 second exposure. Red arrow indicates band not present in NT. **E.** Fly line B (mRho^{P23H}-eGFP) analysis of days 5, 7, 9, 11, and 14. 80 ug of homogenate per lane, immunodetection with mouse monoclonal 1D4, chemiluminescent detection with goat anti-mouse IgG-Peroxidase with Thermo Scientific™ SuperSignal™ West Femto Maximum Sensitivity Substrate, 49.4 second exposure. **F.** Volumetric analysis of the *monomer* bands using intensity of blot "D" with Bio-Rad Image Lab™ Software.

DISCUSSION

An Rh1-mediated Gal4-UAS system was chosen, as opposed to other more general expression drivers (namely glass multiple reporter (GMR)), in an attempt to simulate the most authentic environment for our protein of interest. While it is has been shown that GMR produces much higher desired protein expression levels as compared to Rh1[37], it is difficult to evaluate cell specific protein fates, such as that of rhodopsin, when expression levels are evaluated from non-specific techniques. The use of an Rh1 promoter restricts transgene expression to cells that already express rhodopsin.

At first, our wild-type positive control did not represent expectations (**Figure 3A**). Following intervention, which included removing balancer chromosomes, our wild-type displayed a very different, more expected, expression pattern (**Figure 3B**). There may be various reasons for the differences between **Figures 3A** and **3B**, however without further analysis it is difficult to speculate the definitive cause. Several contributing factors may be, but are not limited to: extreme variance in the genotypes of flies within a single sample, a mutation on the balancer chromosome, or a high concentration of homozygous carriers of the transgene which could result in either extreme expression or degraded expression as a result of cellular toxicity from high concentrations of rhodopsin.

The ages of flies used for our assays was determined from the results of a previous study that used flies with exogenously expressed bovine rhodopsin[38]. This study showed that in wild-type Rh1-mediated Gal4-UAS systems, exogenous bovine rhodopsin expression reached a so-called “steady state” by Day 3 which was maintained until at least Day 10. Establishing a “steady state” provides a predictable positive control to compare the results of future genetic manipulations effects on the fate of mRho. While we were unable to obtain a perfect “steady

state" of expression, our results do follow a congruent trend to Ahmad S. et al 2006. These results suggest several possibilities for the current state of our transgenic wild-type fly line (**Figure 3B**). A probable explanation is that murine rhodopsin may be inherently less stable than bovine rhodopsin when utilized this way, or there is still another factor contributing to the slow degradation of $mRho^{wt}$. A factor that may contribute to this instability is the enhanced green fluorescent protein (eGFP) tag attached to our transgene which was not used in Ahmad S. et al 2006. While there are powerful implications to keeping this tag, namely quick screening through epiflourescent microscopy, eGFP's large size or biochemical characteristics may be contributing to the transgenes instability over time. A follow up experiment, which due to time constraints we were unable to perform, involved the use of a second set of transgenes with mCherry fluorescent tags (fly lines "D" and "F"). mCherry has been shown to have a fast maturation, good pH resistance, tolerance to N-terminal fusions[39], and a low propensity for dimerization due to its monomer form.

Figure 3E confirms existing understanding that the Rho^{P23H} mutation results in extreme degradation of protein expression. This phenomenon requires highly potent ECL reagents to detect small fluctuations in band intensity leading to the subsequent detection of many non-specific bands. The trend of an exponential increase in expression followed by steady decline offers a few interpretations (**Figure 3F**). It is possible that the cells are quickly overwhelmed by an exponentially increasing amount of un-degraded or partially degraded misfolded protein. This may result in a toxic or unfavorable environment for the rod photoreceptors leading to the degradation of the cells. To assess this theory, this experiment would need to be redone with additional steps to evaluate endogenous rhodopsin expression.

For future investigations, a wider time scale, starting at Day 1 may be more appropriate. By Day 5, it appears, because of how low the detection is even with an extremely potent ECL reagent, mRho^{P23H}-eGFP may be nearing complete degradation (**Figure 3E**). It may be possible that peak mRho^{P23H} expression occurs between Day 1 and 5. Expanding the ages analyzed for future studies would also be beneficial for our Rho^{wt} line. Day 5 was observed to have the highest expression, making it difficult to infer when degradation of the protein actually began **(Figure 3B**).

Nonetheless, the fact that expression of mouse rhodopsin in fly photoreceptors mimics behavior seen in native mouse photoreceptors is both exciting and promising for future investigations of degradation pathways using the fly system. This suggests that the signaling pathways for detecting the misfolding and subsequent degradation of the mutant protein could be conserved in evolution between fly and mouse. Use of the powerful genetic tools available to the fly system, including readily available stocks with disabled genetic pathways, may prove to be a very effective method for screening candidate genes in future investigations.

References

1. Yanoff M, Cameron D. Disorders of the visual system. In: Goldman L, Schafer AI, eds. Cecil Medicine
2. Hartong DT, Berson EL, Dryja TP. "Retinitis pigmentosa" Lancet. 2006 Nov 18;368(9549):1795-809.
3. Retnet: Retinal Information Network, https://sph.uth.edu/retnet/, accessed 30 September 2015.
4. Palczewski K. Chemistry and biology of vision. J Biol Chem. 2012, 287:1612-1619.
5. Lewin AS, Rossmiller B, Mao H, Gene augmentation for adRP mutations in RHO. Cold Spring Harb Perspect Med. 2014, 4:a017400.
6. Miller LM, Gragg M, Kim TG, Park PS, Misfolded opsin mutants display elevated β-sheet structure. FEBS Lett. 2015: S0014-5793(15)00812-1;
7. Chen Y, Jastrzebska B, Cao P, Zhang J, Wang B, Sun W, Yuan Y, Feng Z, Palczewski K. Inherent instability of the retinitis pigmentosa P23H mutant opsin. J Biol Chem. 2014, 289:9288-303 and references cited therein.
8. Griciuc A, Aron L, Roux MJ, Klein R, Giangrande A, Ueffing M. Inactivation of VCP/ter94 suppresses retinal pathology caused by misfolded rhodopsin in Drosophila. PLoS Genet. 2010; 6 pii: e1001075.
9. Fernandez de Castro JP, Scott PA, Fransen JW, Demas J, DeMarco PJ, Kaplan HJ, McCall MA. Cone photoreceptors develop normally in the absence of functional rod photoreceptors in a transgenic swine model of retinitis pigmentosa. Invest Ophthalmol Vis Sci. 2014; 55(4):2460-8.
10. Kaushal S, Khorana HG. Biochemistry, 1994; 33:6121-8, Structure and function in rhodopsin. 7. Point mutations associated with autosomal dominant retinitis pigmentosa.
11. Sakami S, Maeda T, Bereta G, Okano K, Golczak M, Sumaroka A, Roman AJ, Cideciyan AV, Jacobson SG, Palczewski K. Probing mechanisms of photoreceptor degeneration in a new mouse model of the common form of autosomal dominant retinitis pigmentosa due to P23H opsin mutations. J Biol Chem. 2011; 286:10551-67.
12. Frederick JM, Krasnoperova NV, Hoffmann K, Church-Kopish J, Rüther K, Howes K,Lem J, Baehr W. Mutant rhodopsin transgene expression on a null background.Invest Ophthalmol Vis Sci. 2001; 42:826-33.
13. Naash MI, Hollyfield JG, al-Ubaidi MR, Baehr W. Simulation of human autosomal dominant retinitis pigmentosa in transgenic mice expressing a mutated murine opsin gene. Proc Natl Acad Sci U S A. 1993; 90:5499-503.
14. Sakami et al., 2011; Reks and Knox, unpublished data.
15. Lem J, Krasnoperova NV, Calvert PD, Kosaras B, Cameron DA, Nicolò M, Makino CL, Sidman RL. Morphological, physiological, and biochemical changes in rhodopsin knockout mice. Proc Natl Acad Sci U S A. 1999; 96:736-41.
16. Rakshit T, Park PS. Impact of reduced rhodopsin expression on the structure of rod outer segment disc membranes. Biochemistry. 2015; 54:2885-94.
17. Haeri M, Knox BE Rhodopsin mutant P23H destabilizes rod photoreceptor disk membranes. PLoS One. 2012;7:e30101.
18. Sakami et al., 2011; Sakami S, Kolesnikov AV, Kefalov VJ, Palczewski K. P23H opsin knock-in mice reveal a novel step in retinal rod disc morphogenesis. Hum Mol Genet. 2014 ;23:1723-4.
19. Athanasiou D, Bevilacqua D, Aguila M, McCulley C, Kanuga N, Iwawaki T, Paul Chapple J, Cheetham ME. The co-chaperone and reductase ERdj5 facilitates rod opsin biogenesis and quality control and references cited therein. Hum Mol Genet. 2014 Dec 15;23(24):6594-606.
20. Reks and Knox, unpublished results using HEK293 cell lines.
21. Chiang WC, Kroeger H, Sakami S, Messah C, Yasumura D, Matthes MT, Coppinger JA, Palczewski K, LaVail MM, Lin JH. Robust Endoplasmic Reticulum-Associated Degradation of Rhodopsin Precedes Retinal Degeneration. Mol Neurobiol. 2015; 52:679-95.
22. Adekeye, A. 2015, Retinitis Pigmentosa: Investigating the Role of the Unfolded Protein Response in Photoreceptor Death, Ph.D. dissertation, SUNY Upstate Medical University, Syracuse, NY.
23. Minikel, Eric. Basics of protein degradation. http://www.cureffi.org/2013/07/11/basics-of-protein-degradation/. Accessed 30 September 2015.
24. Reks and Knox, unpublished, Adekeye, 2015.
25. Rister J, Desplan C. The retinal mosaics of opsin expression in invertebrates and vertebrates. Dev Neurobiol. 2011 Dec;71(12):1212-26.
26. Aerni-Flessner L, Haeri M, Knox BE, Pignoni F. Can fly photoreceptors lead to treatments for rho ((P23H)) -linked retinitis pigmentosa? J Ophthalmic Vis Res. 2013; 8:86-91.

27. Xiong B, Bellen HJ. Rhodopsin homeostasis and retinal degeneration: lessons from the fly. Trends in Neurosciences, November 2013, Vol. 36, No. 11.
28. Daemen FJ. Vertebrate rod outer segment membranes. Biochim Biophys Acta. 1973; 300:255-88.
29. Fain GL, Hardie R, and Laughlin LB. Phototrnsduction and the Evolution of Photoreceptors. Current Biology 20, R114–R124, Feb. 9, 2010.
30. Pak WL. Drosophila in vision research. The Friedenwald Lecture. Invest Ophthalmol Vis Sci. 1995; 36:2340-57.
31. Reks, Aerni-Flessner, Haeri, Pignoni and Knox, in preparation
32. Peet JA, Bragin A, Calvert PD, Nikonov SS, Mani S, Zhao X, Besharse JC, Pierce EA, Knox BE, Pugh EN Jr. Quantification of the cytoplasmic spaces of living cells with EGFP reveals arrestin-EGFP to be in disequilibrium in dark adapted rod photoreceptors. J Cell Sci. 2004; 117:3049-59.
33. Nakatsukasa K, Kamura T, Brodsky JL. Recent technical developments in the study of ER-associated degradation. Curr Opin Cell Biol. 2014; 29: 82-91.
34. Shiber A, Ravid T. Chaperoning proteins for destruction: diverse roles of Hsp70 chaperones and their co-chaperones in targeting misfolded proteins to the proteasome. Biomolecules. 2014; 4:704-24.
35. Lemus L, Goder V. Regulation of Endoplasmic Reticulum-Associated Protein Degradation (ERAD) by Ubiquitin. Cells. 2014; 3:824-47.
36. Satoh T, Ohba A, Liu Z, Inagaki T, Satoh AK. Elife. EMC is essential for biosynthesis of rhodopsin and other multi-pass membrane proteins in Drosophila photoreceptors. 2015; 4.
37. Aerni-Flessner, unpublished results, 2013.
38. Ahmad S, Natochin M, Barren B, Artemyev NO, O'Tousa JE; Heterologous Expression of Bovine Rhodopsin in Drosophila Photoreceptor Cells. Invest. Ophthalmol. Vis. Sci. 2006;47(9):3722-3728.
39. Wei W S. Fluorescent proteins as tools to aid protein production. Microbial Cell Factories. 2005. 4:12.

Images

1. Retinitis Pigmentosa. Digital image. Prakash Nethralaya. Web. 30 Sept. 2015.