highroad Is a Carboxypeptidase Induced by Retinoids to Clear Mutant Rhodopsin-1 in Drosophila Retinitis Pigmentosa Models

Graphical Abstract

Highlights

- highroad is required for the degradation of folding-defective Rhodopsin-1

- highroad loss accelerates retinal degeneration in ninaE^{G69D} mutant flies

- highroad mRNA is induced by retinoic acids in cultured Drosophila cells

- highroad mRNA expression is induced in ninaE^{G69D} flies

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In Brief

Folding-defective mutant rhodopsins undergo degradation in photoreceptors, but the underlying mechanism was unclear. Huang et al. identify highroad as a factor required for mutant Drosophila Rhodopsin-1 degradation. Loss of highroad accelerates retinal degeneration caused by mutant Rhodopsin-1, and highroad expression is dependent on retinoids.

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**highroad** Is a Carboxypetidase Induced by Retinoids to Clear Mutant Rhodopsin-1 in *Drosophila* Retinitis Pigmentosa Models

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SUMMARY

Rhodopsins require retinoid chromophores for their function. In vertebrates, retinoids also serve as signaling molecules, but whether these molecules similarly regulate gene expression in *Drosophila* remains unclear. Here, we report the identification of a retinoid-inducible gene in *Drosophila*, **highroad**, which is required for photoreceptors to clear folding-defective mutant Rhodopsin-1 proteins. Specifically, knockdown or genetic deletion of **highroad** blocks the degradation of folding-defective Rhodopsin-1 mutant, **ninaE**

**G69D**. Moreover, loss of **highroad** accelerates the age-related retinal degeneration phenotype of **ninaE**

**G69D** mutants. Elevated **highroad** transcript levels are detected in **ninaE**

**G69D** flies, and interestingly, deprivation of retinoids in the fly diet blocks this effect. Consistently, mutations in the retinoid transporter, **santa maria**, impairs the induction of **highroad** in **ninaE**

**G69D** flies. In cultured S2 cells, **highroad** expression is induced by retinoid acid treatment. These results indicate that cellular quality-control mechanisms against misfolded Rhodopsin-1 involve regulation of gene expression by retinoids.

INTRODUCTION

As in other metazoans, *Drosophila* has several rhodopsin genes, including **ninaE**, that encode Rhodopsin-1 (Rh1) (O’Tousa et al., 1985; Zuker et al., 1985). Once synthesized, Rh1 becomes conjugated to the 11-cis-3-hydroxyretinal chromophore to detect light in the outer photoreceptors of the eye (Ahmad et al., 2006).

Certain types of mutations in human rhodopsin underlie autosomal dominant retinitis pigmentosa (ADRP), a disorder of age-related retinal degeneration (Dryja et al., 1990; Sung et al., 1991). This disease has been modeled in *Drosophila* through similar mutations in **ninaE**, including the G69D and P37H alleles, which trigger age-related retinal degeneration (Colley et al., 1995; Galy et al., 2005; Kurada and O’Tousa, 1995). The encoded mutant proteins fail to fold properly in the endoplasmic reticulum (ER) and therefore impose stress in this organelle and activate the unfolded protein response (UPR) (Ryoo et al., 2007). At the same time, healthy cells are equipped with quality-control mechanisms that act against such misfolded proteins. In the ER, a network of proteins is involved in the detection, retro-translocation, and ubiquitination of misfolded peptides for proteasomal degradation in the cytoplasm, a process referred to as ER-associated degradation (ERAD) (Brodsky, 2012; Ruggiano et al., 2014). We had previously shown that overexpression of the central ubiquitin ligase involved in ERAD, **hrd1**, strongly delayed retinal degeneration in the *Drosophila* **ninaE**

**G69D** mutant (Kang and Ryoo, 2009). In addition to ERAD, recent studies indicate that mutant and wild-type rhodopsins are partly degraded in the lysosome (Chiang et al., 2012; Chinchorne et al., 2009; Wang et al., 2014).

Without the retinal chromophore and its precursors, rhodopsins cannot function properly and fail to undergo proper maturation (Harris et al., 1977; Ozaki et al., 1993; Gu et al., 2004; Wang and Montell, 2005; Wang et al., 2007). In vertebrates, retinoids also have a second role as transcriptional regulators whose effects are mediated by the nuclear hormone receptor proteins (Mangelsdorf and Evans, 1995). Although previous studies reported that *Drosophila* that are deprived of the retinoid precursor vitamin A in the diet have altered levels of opsin and fatty-acid-binding glycoprotein transcripts (Picking et al., 1996; Shim et al., 1997), the biological role and the mechanism of retinoid-mediated gene expression control in *Drosophila* remain unclear.

In this study, we report the identification of **highroad** (**hiro**), a gene that is required for mutant Rh1 degradation in *Drosophila* that also affects the course of age-related retinal degeneration. Furthermore, our data indicate that **hiro** transcript levels increase in **ninaE**

**G69D** mutant flies, and that this is dependent on retinoid availability in vivo. These observations suggest that the degradation of mutant Rh1 is associated with retinoid-mediated gene expression control in *Drosophila*.

RESULTS AND DISCUSSION

Adult Eye Morphology-Based RNAi Screen for Genetic Interactors of **ninaE**

We previously established a facile genetic assay system to assess cellular stress caused by **ninaE**

**G69D** overexpression through the eye-specific GMR promoter (henceforth referred to as **GMR-Rh1**

**G69D**). In these flies, Rh1**

**G69D** is overexpressed in...
the early stages of eye development, resulting in adults with malformed eyes (Kang and Ryoo, 2009; Kang et al., 2012; Figure S1). This phenotype can be attributed to increased misfolded proteins in the ER, since co-overexpression of the ERAD-mediating gene \textit{hrd1} almost completely suppresses the external eye phenotype (Kang and Ryoo, 2009; Figure S1).

To identify other factors involved in misfolded Rh1 quality control, we screened for RNAi lines that impaired the protective effects of \textit{hrd1} overexpression against \textit{GMR-Rh1G69D} (Figure S1A; see also Experimental Procedures). A total of 80 RNAi lines were tested, many of which targeted \textit{Drosophila} homologs of mammalian genes with known roles in ERAD, or those that are found in protein complexes with human HRD1 and its associated proteins (Christianson et al., 2011). We also included RNAi lines that targeted annotated membrane proteases and carboxypeptidases in \textit{Drosophila} (the full list of RNAi lines is in Table S1).

RNAi knockdown of \textit{hrd1} in the developing eye did not impair eye development when expressed alone, but aggravated the eyes of flies co-expressing \textit{Rh1G69D} and \textit{hrd1} (Figure S1B). A number of other lines gave rise to phenotypes similar to \textit{hrd1} knockdown. These included not only the lines that targeted \textit{Drosophila} homologs of known ERAD genes, but also genes with no previous associations with ERAD, including CG32441, \textit{asrij}, and CG3344 (Figure S1B).

\textbf{highroad} Is a Gene Required to Reduce Mutant Rh1 Levels in Photoreceptors

As a secondary assay for validation, we turned to the classical \textit{ninaE\textsuperscript{G69D}} allele with a mutation in the endogenous \textit{ninaE} locus that dominantly reduces total Rh1 levels in newly enclosed adult flies (Colley et al., 1995; Kurada and O’Tousa, 1995). Candidate RNAi lines from the primary screen were expressed in the photoreceptors of \textit{ninaEG69D/\textsuperscript{+}} flies, and we found that one particular RNAi line (VDRC 110402) almost fully restored Rh1 levels in the \textit{ninaEG69D/\textsuperscript{+}} background to wild-type levels (Figures 1A and 1B). This line targets a previously uncharacterized carboxypeptidase, CG3344, homologous to a mammalian protein known as retinoid-inducible serine carboxypeptidase or serine carboxypeptidase 1 (SCPEP1) (Chen et al., 2001). Neither CG3344 nor its mammalian homolog has known roles in ERAD. Based on the loss-of-function phenotype, we henceforth refer to CG3344 as \textit{highroad} (\textit{hiro}).

Knockdown of known ERAD components did not restore Rh1 levels significantly in this system, which is consistent with what

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**Figure 1. \textit{hiro} Is Required for Photoreceptors to Reduce Mutant Rh1 Levels**

(A) Shown are western blots of adult head extracts with the indicated antibodies. In the \textit{ninaE} wild-type (lane 1) or in the \textit{ninaE\textsuperscript{G69D/\textsuperscript{+}}} genetic background (lanes 2–7), the indicated genes were knocked down with \textit{Rh1-Gal4} driver.

(B) Quantification of the band intensities shown in (A).

(C) A schematic diagram of the \textit{hiro} locus. The bar above indicates sequences deleted in \textit{Df(3L)5}. Below is a schematic diagram of the \textit{hiro\textsuperscript{F1}} allele.

(D to F) Validation of the \textit{hiro} RNAi phenotype through classical alleles. Anti-Rh1 (top) and anti-\textit{\beta}-tubulin (bottom) western blot from adult fly head extracts of the indicated genotypes. Error bars represent SEM.
we reported previously (Kang and Ryoo, 2009). Although hiro was identified through a genetic interaction screen with hrd1, the precise relationship between the two genes remains unclear. Knockdown of other Drosophila carboxypeptidases, such as CG4572 or CG32821, did not result in the recovery of Rh1 levels in ninaEGD69D+ flies (Figure S2A). We also knocked down genes that mediate autophagy or late endosome trafficking in the ninaEGD69D+ photoreceptors, but overall Rh1 levels did not recover under those conditions (Figure S2B).

To further validate the RNAi result from the screen, we used two hiro mutant alleles: (1) Df(3L)5 deficiency, in which hiro and its neighboring gene, earthbound1, are deleted (Benchabane et al., 2011); and (2) an allele generated by CRISPR-Cas9-mediated deletion of the hiro locus (Figure 1C; see also Experimental Procedures), which we refer to henceforth as hiroF1-. hiro F1-- or hiroF1/Df(3L)5 flies were viable, did not exhibit any obvious external morphology defects, and did not affect total Rh1 levels in the ninaE wild-type background (Figures 1D, lanes 1 and 2, and 1E, lane 1). We also introduced the UPR reporter XBPl-EGFP (Coelho et al., 2013) into the background of adult flies that contain Df(3L)5-- mosaic clones in the eye. We found no evidence of excessive ER stress and abnormal UPR activation in these photoreceptors (Figure S3).

Loss of hiro in the ninaEGD69D+ background, however, had clear effects on Rh1 levels. Specifically, loss of hiro in the Df(3L)5--/Df(3L)5hiroF1, or hiro F1--/hiroF1/Df(3L)5 flies were viable, did not exhibit any obvious external morphology defects, and did not affect total Rh1 levels in the ninaE wild-type background (Figures 1D, lane 6, and 1E, lanes 3 and 6). This effect of hiroF1 loss of function on Rh1 was reversed by the introduction of a transgenic bacterial artificial clone (BAC), CH321-70D3, containing the hiro locus DNA (Figure 1E, lane 7). Together, these results validate that hiro is genetically required to reduce Rh1 levels in ninaEGD69D+ mutants.

hiro Affects the Levels of Other Rh1 Mutant Alleles

In humans, the most widespread rhodopsin allele associated with ADRP is the P23H mutation, which encodes a misfolding-prone rhodopsin that undergoes degradation in the ER and the lysosome (Chiang et al., 2012; Dryja et al., 1990; Liu et al., 1996). We examined a previously generated herpes simplex virus (HSV)-tagged ninaE transgenic line with the equivalent Drosophila mutation, P37H (Gal et al., 2005). Unlike the G69D mutants, we found that Rh1 levels were not noticeably lower in newly enclosed P37H flies (data not shown), but decreased significantly by 21 days post-enclosure (Figure 1F, lane 2). Similar to results with the G69D mutants, Rh1 levels in P37H mutants were almost restored to wild-type levels in the Df(3L)5--/background (Figure 1F, lane 3). Consistently, western blotting with anti-HSV to specifically detect P37H mutant protein (rather than total Rh1) showed higher P37H-HSV in the Df(3L)5--/background (Figure 1F, lanes 4, 5). These results show that the effect of hiro loss on Rh1 is not specific to the ninaEG69D allele but is applicable to other disease-relevant ninaE mutant alleles.

hiro Does Not Affect the Levels of Another ER-Stress-Causing Protein, alpha-1 antitrypsinNHK

alpha-1 antitrypsin (a1at) encodes a secreted human protein, and the NHK mutant allele underlies alpha-1 antitrypsin deficiency due to its propensity of undergoing rapid degradation through ERAD. We had previously generated a a1atNHK transgenic fly line that activates the UPR and also undergoes ERAD when expressed through a Gal4 driver in Drosophila tissues (Kang and Ryoo, 2009). We were able to detect this protein from adult head extracts when driven with the GMR-Gal4 driver, but its levels did not increase when hiro was knocked down through RNAi (Figure S2C). These results indicate that hiro does not affect all misfolding-prone proteins in the ER.

hiro Expression Pattern

To visualize the localization of the hiro protein, we inserted a GFP transgene into the hiro coding sequence within its genomic locus using minos-mediated integration cassette (MiMIC)-based protein trap technology (Nagarkar-Jaiswal et al., 2015; see Experimental Procedures). This resulted in a hiro-GFP protein trap hybrid gene with GFP in frame with hiro that we refer to as hiroPF (Figure S4A). The N-terminal signal peptide of hiro remained intact after GFP insertion. The fluorescence from the resulting GFP was readily detectable in a confined area of the larval intestine (Figures S4B and S4C). The mammalian homolog of hiro is reportedly a lysosomal protein, but the GFP signal did not overlap with LysoTracker (Figure S4D). The signal peptide within hiro indicates that the protein must be synthesized in the ER before reaching its ultimate subcellular site, and consistently, there was partial overlap of the GFP signal with the ER marker anti-Calnexin (Figure S4E). However, there were also foci with intense GFP signals that did not overlap with the ER, and we interpret that hiro is ultimately trafficked out of the ER to its final destination.

hiro Mutants Increase Rh1 Protein Levels in the Photoreceptor Rhabdomeres and along the Secretory Pathway Organelles

To determine the sub-cellular localization of Rh1 protein in hiro mutants, we labeled adult ommatidia with anti-Rh1 antibody and performed initial analysis through confocal microscopy. Whereas ninaEG69D+ eyes had most anti-Rh1 signals coming from the rhabdomeres in trapezoidal arrangements, ninaEG69D Df(3L)5/Df(3L)5 eyes showed additional anti-Rh1 signals in the cell body. We performed co-localization experiments with various subcellular organelle markers, including that of the ER and the lysosome (Figures 2A–2C), but none of those markers fully overlapped with the cytoplasmic anti-Rh1 signals. Some of the anti-Rh1 signals in the cell body were also foci with intense GFP signals that did not overlap with the ER, and we interpret that hiro is ultimately trafficked out of the ER to its final destination.
subcellular sites where anti-Rh1 signals were detected (Figures 2F–2H). One of those was the ER (Figure 2G), but other subcellular sites also showed anti-Rh1 signals (Figure 2F). We did not detect any anti-Rh1 signals from the lysosome or the mitochondria (Figure 2H). Because Rh1 is ultimately trafficked to rhabdomeres in this genotype, we interpret that Rh1 levels increase in multiple subcellular organelles along the secretory pathway.

**ninaEG69D/+** Flies Show Accelerated Retinal Degeneration in the Absence of hiro

Because our data indicated that hiro mutants fail to properly regulate Rh1 levels, we decided to examine if this affected the age-related retinal degeneration of **ninaEG69D/+** flies. The integrity of the retinal photoreceptors can be determined by dissecting and labeling fly eyes with phalloidin, which marks rhabdomeres in this genotype, we interpret that Rh1 levels increase in multiple subcellular organelles along the secretory pathway. We independently validated this using the Rh1-GFP reporter that allows the examination of photoreceptor integrity in live flies over a time course of 30 days. A majority of **ninaEG69D/+** flies (n = 49) showed an organized trapezoidal pattern of Rh1-GFP for up to 20 days before showing signs of degeneration (Figure 3B). In the **hiroF1** mutant background, **ninaEG69D/+** flies showed earlier signs of retinal degeneration (n = 31, p < 0.0001, Chi-square = 178.1, when compared with **ninaEG69D/+**), with more than 75% of flies showing disorganized Rh1-GFP patterns at 20 days old (Figure 3B). The control **hiro/C0/C0** flies in the **ninaE** wild-type background showed no signs of retinal degeneration at these time points (n = 54). Although **ninaE** is only expressed in six photoreceptors (R1–R6) (Scavarda et al., 1983), all photoreceptors degenerated under these conditions, which is consistent with previous studies reporting that even R7 and R8 photoreceptor degeneration occurs in these flies due to indirect effects (Colley et al., 1995; Kurada and O’Tousa, 1995; Leonard et al., 1992).

We also performed the converse experiment of hiro overexpression using the Rh1-Gal4/ uas-hiro system (n = 50 in each genotype group). Although hiro was expressed as detected by the V5
hiro Is Inducible by Retinoids

The mammalian homolog of hiro is a gene whose expression is induced by retinoic acids (Chen et al., 2001). To test whether Drosophila hiro similarly responds to retinoic acids, we challenged Drosophila S2 cells with commercially available all-trans retinoic acid. We found that 20 min of retinoic acid treatment resulted in increased hiro transcript levels as evidenced by semiquantitative RT-PCR as well as qPCR analyses (Figures 4A and 4B).

qPCR analysis of hiro from adult Drosophila heads also detected higher signals from ninaEGal4/+ samples, as compared with the wild-type controls (Figure 4C, lanes 1 and 2). To determine if such hiro induction in fly heads is due to retinoid-induced gene expression, we repeated the semiquantitative RT-PCR analysis under conditions that deprived retinoids in the fly visual system. Because metazoans require dietary vitamin A to produce retinoids and related metabolites, one way to achieve retinoid deprivation is to rear flies on vitamin-A-deficient food (Blomhoff et al., 1990; Harris et al., 1977; Ozaki et al., 1993). We found that such conditions blocked the increase in hiro transcripts in ninaEGal4/+ heads (Figure 4C, lane 3). To independently validate the role of retinoids in the regulation of hiro transcript levels, we used the santa maria mutant that has impaired vitamin A/-carotene transport to the photoreceptors (Wang et al., 2007). Similar to the results obtained with flies reared under vitamin-A-deficient food, loss of santa maria impaired hiro induction in the ninaEGal4/+ fly heads (Figure 4D). These results support an unexpected idea that retinoids regulate gene expression in Drosophila, and one such regulated gene, hiro, is involved in the clearance of mutant Rh1.

It is noteworthy that most studies on Drosophila retinoids have centered around their roles as rhodopsin chromophores (Gu et al., 2004; Harris et al., 1977; Kiefer et al., 2002; Ozaki et al., 1993; Wang and Montell, 2005). Interestingly, recent studies indicate that retinoids have additional roles in mediating an X-ray irradiation response (Halme et al., 2010), but whether those effects are due to retinoic-acid-mediated gene expression changes remain unknown. As normally folded Rh1 is bound to an 11-cis retinoid, we challenged Drosophila S2 cells with commercially available all-trans retinoic acid. We found that such conditions blocked the increase in hiro transcript levels as evidenced by semiquantitative RT-PCR as well as qPCR analyses (Figures 4A and 4B). This result is similar to the results obtained with flies reared under vitamin-A-deficient food, loss of santa maria impaired hiro induction in the ninaEGal4/+ fly heads (Figure 4D). These results suggest that retinoids regulate gene expression in Drosophila, and one such regulated gene, hiro, is involved in the clearance of mutant Rh1.

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For the RNAi screen, inverted repeat UAS (UAS-RNAi) lines from the Vienna Stock Center were crossed to the female virgins of the following genotype: GMR-Gal4; ey-Gal4, GMR-Rh1<sup>E<sub>E</sub></sup>, uas-hrd1/CyO; uas-dicer2. As a negative control, we also crossed the UAS-RNAi lines to GMR-Gal4; ey-Gal4/CyO; uas-dicer2 flies. To validate the hits with the nina<sup>E</sup> homologous endogenous allele, UAS-RNAi lines were crossed to the virgin females of the genotype: Rh1-Gal4; uas-dicer2; nina<sup>E<sub>E</sub></sup>/TM6B. Non-TM6B progeny were collected to generate head extracts for anti-Rh1 western blot analysis.

h<sup>iro</sup> <sup>h<sub>i</sub></sup> flies were generated using recombinase-mediated cassette exchange (RMCE) following a published protocol (Nagarkar-Jaiswal et al., 2015; further details in Supplemental Experimental Procedures).

To generate hiro deletion mutants, we followed the homology-directed repair CRISPR-Cas9 protocol described in www.flycrispr.molbio.wisc.edu (further details in Supplemental Experimental Procedures). The deletion of hiro was further confirmed through genomic PCR. This h<sup>iro</sup><sup>h<sub>i</sub></sup> allele was recombined with nina<sup>E<sub>E</sub></sup> to generate hiro<sup>h<sub>3</sub></sup>, nina<sup>E<sub>E</sub></sup>TM6B<sup>h<sub>3</sub></sup>, which was used in subsequent crosses to make hiro homozygous mutants with nina<sup>E<sub>E</sub></sup> mutation in the background.

**Immunofluorescence and Western Blots**

Standard protocols were followed for western blots and whole-mount immuno-labeling. The following primary antibodies were used in this study: mouse monoclonal 4C5 anti-Rh1 (Developmental Studies Hybridoma Bank, used at 1:500 for whole mount and 1:5,000 for western blots), rabbit anti-Rh1 polyclonal antibody (generated in Charley Zuker’s laboratory [Hsiao used at 1:500 for whole mount and 1:5,000 for western blots], rabbit anti-mouse monoclonal 4C5 anti-Rh1 (Developmental Studies Hybridoma Bank).

**RETINOIDS**

**Immunogold EM of Drosophila ommatidia** was done following a previously described protocol (Colley et al., 1991). Rabbit polyclonal anti-Rh1 was used as the primary antibody, and 18 nm Colloidal gold anti-rabbit antibody was used as the secondary.

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**AUTHOR CONTRIBUTIONS**

All authors were involved in the design of the experiments. H.-W.H., B.B., J.C., and P.M.D. conducted the experiments. H.D.R. wrote the manuscript based on input from all authors.
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