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

Huai-Wei Huang[1], **Brian Brown**[1], **Jaehoon Chung**[1], **Pedro M. Domingos**[2], and **Hyung Don Ryoo**[1,*,3]

[1]Department of Cell Biology, New York University School of Medicine 550 First Avenue, New York, NY 10016, USA

[2]Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa, Av. da República, Oeiras 2780-157, Portugal

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 foldingdefective 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 retinoic acid treatment. These results indicate that cellular quality-control mechanisms against misfolded Rhodopsin-1 involve regulation of gene expression by retinoids.

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.

*Correspondence: hyungdon.ryoo@nyumc.org.
[3]Lead Contact
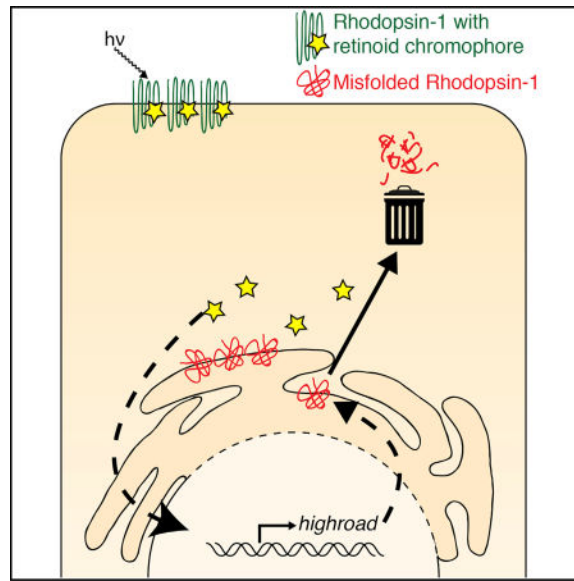
INTRODUCTION

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 agerelated 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; Chinchore 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 $ninaE^{G69D}$

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 $GMR\text{-}Rh1^{G69D}$). 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 *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 *hrd1* overexpression against $GMR\text{-}Rh1^{G69D}$ (Figure S1A; see also Experimental Procedures). A total of 80 RNAi lines were tested, many of which targeted *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 *Drosophila* (the full list of RNAi lines is in Table S1).

RNAi knockdown of *hrd1* in the developing eye did not impair eye development when expressed alone, but aggravated the eyes of flies co-expressing $Rh1^{G69D}$ and *hrd1* (Figure S1B). A number of other lines gave rise to phenotypes similar to *hrd1* knockdown. These included not only the lines that targeted *Drosophila* homologs of known ERAD genes, but also genes with no previous associations with ERAD, including CG32441, *asrij*, and CG3344 (Figure S1B).

highroad Is a Gene Required to Reduce Mutant Rh1 Levels in Photoreceptors

As a secondary assay for validation, we turned to the classical $ninaE^{G69D}$ allele with a mutation in the endogenous *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 $ninaE^{G69D/+}$ flies, and we found that one particular RNAi line (VDRC 110402) almost fully restored Rh1 levels in the $ninaE^{G69D/+}$ 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 high rhodopsin-accelerated degeneration or *highroad* (*hiro*). Knockdown of known ERAD components did not restore Rh1 levels significantly in this system, which is consistent with what 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 *ninaE$^{G69D/+}$* flies (Figure S2A). We also knocked down genes that mediate autophagy or late endosome trafficking in the *ninaE$^{G69D/+}$* 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 *hiro^{F1}*. *hiro$^{F1-/-}$* or *hiro^{F1}/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 XBP1- 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 *ninaE$^{G69D/+}$* background, however, had clear effects on Rh1 levels. Specifically, loss of *hiro* in the *Df(3L)5$^{-/-}$*, *Df(3L)/hiro^{F1}*, or *hiro$^{F1-/-}$* backgrounds resulted in the recovery of Rh1 levels in *ninaE$^{G69D/+}$* eyes to a degree that was comparable to that of *ninaE* wild-type flies (Figures 1D, lane 6, and 1E, lanes 3 and 6). This effect of *hiro^{F1}* 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 *ninaE$^{G69D/+}$* 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* (Galy 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 *ninaE^G69D* 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 antitrypsin^NHK

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 *uas-a1at^NHK* 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 Hiro protein, we inserted a GFP trangene 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 *hiro^PT* (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 this 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 *ninaE^G69D/+* eyes had most anti-Rh1 signals coming from the rhabdomeres in trapezoidal arrangements, *ninaE^G69D, 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 cytoplasm appeared to be juxtaposed or associated with sub-fractions of the ER (Figure 2B), but the resolution provided by fluorescent microscopy was insufficient to draw conclusions.

To obtain higher resolution images of anti-Rh1 labels, we performed immunogold electron microscopy (EM) with rabbit anti-Rh1 antibody. The anti-Rh1 signal was specific based on the fact that rhabodomeres of photoreceptors R1–R6 showed immuno-labeling, whereas the

signal was lacking in R7 photoreceptors that do not normally express Rh1 (Scavarda et al., 1983; Figure 2D). In $ninaE^{G69D/+}$ flies, most anti-Rh1 signals came from the rhabdomeres (Figures 2D and 2E). In the $Df(3L)5^{-/-}$ background, $ninaE^{G69D/+}$ ommatidia still had most anti-Rh1 signals emanating from the rhabodomeres, but there were additional 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.

$ninaE^{G69D/+}$ 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 $ninaE^{G69D/+}$ flies. The integrity of the retinal photoreceptors can be determined by dissecting and labeling fly eyes with phalloidin, which marks rhabdomeres of photoreceptors (Figure 3A). In wild-type flies, phalloidin labeling showed typical trapezoidal patterns of seven photoreceptors in regular arrays (Figure 3A). In $ninaE^{G69D/+}$ flies up to 20 days old, this pattern was maintained in most dissected flies (Figures 3A). On the other hand, $ninaE^{G69D}, hiro^{F1}/hiro^{F1}$ fly eyes lost the trapezoidal rhabdomere arrangement by day 20, which is indicative of retinal degeneration (Figure 3A, lower rightmost panel). 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 $ninaE^{G69D/+}$ 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 $hiro^{F1}$ mutant background, $ninaE^{G69D/+}$ flies showed earlier signs of retinal degeneration (n = *31,* p < 0.0001, Chi-square = 178.1, when compared with $ninaE^{G69D/+}$), with more than 75% of flies showing disorganized Rh1-GFP patterns at 20 days old (Figure 3B). The control $hiro^{-/-}$ 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 epitope associated with the transgene, such conditions were not sufficient to delay the course of age-related retinal degeneration (Figure 3C). Taken together, these results indicate that *hiro* is necessary but not sufficient to protect $ninaE^{G69D/+}$ photoreceptors from age-related retinal degeneration.

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 *ninaE$^{G69D/+}$* 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 *ninaE$^{G69D/+}$* 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/b-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 *ninaE$^{G69D/+}$* 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; Wang et al., 2007). Interesting 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* hydroxylretinal (Ahmad et al., 2006), our results raise the possibility that these retinoids are released to the cytoplasm of photoreceptors with Rh1 folding mutations, thereby serving as a second messenger to instruct the expression of quality-control genes that clear misfolded Rh1. Such a speculative idea awaits more in-depth examination in future studies, along with a search for the transcription factors that directly bind retinoids for gene expression regulation in *Drosophila*.

EXPERIMENTAL PROCEDURES

Fly Genetics

The following flies used in this study were reported previously: *GMR-Rh1^{G69D}* and *uas-alpha one antitrypsin^{NHK}* (Kang and Ryoo., 2009), *ninaE^{G69D}* (Colley et al., 1995), *Rh1-GFP* (Pichaud and Desplan, 2001), *Rh1-Gal4* (Mollereau et al., 2000), *santa maria^1* (Wang et al., 2007), *Df(3L)5* (Benchabane et al., 2011), *Rh1$_p$ > ninaE^{P37H}* (Galy et al., 2005), and *uas-dicer2* (Dietzl et al., 2007). The P[acman]-derived BAC transgenic line CH321-70D3 (sequence information for this clone is available from www.flybase.org) was purchased from GenetiVision.

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^{G69D}, uas-hrd1/CyO; uas-dicer2.* As a negative control, we also crossed the *UAS-RNAi* lines to *GMR-Gal4; ey-Gal4/CyO; uasdicer2* flies. To validate the hits with the *ninaE^{G69D}* endogenous allele, *UASRNAi* lines were crossed to the virgin females of the genotype: *Rh1-Gal4; uas-dicer2; ninaE^{G69D}/TM6B*. Non-TM6B progeny were collected to generate head extracts for anti-Rh1 western blot analysis.

$hiro^{PT}$ 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 $hiro^{F1}$ allele was recombined with ninaEG69D to generate $hiro^{F1}$, $nina^{EG69D/TM6B}$, which was used in subsequent crosses to make *hiro* homozygous mutants with $nina^{EG69D}$ 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 Charles Zuker's laboratory [Hsiao et al., 2013], used at 1:100), anti-β-tubulin antibody (Covance catalog no. MMS-410P), rabbit anti-ATP6V1Ba antibody (Abgent catalog no. AP11538C, used at 1:20), mouse anti-Calnexin 99 (Riedel et al., 2016; used at 1:100), guinea pig anti-Hsc3 (Ryoo et al., 2007; used at 1:25). Rhodamine-conjugated Phalloidin (Molecular Probes catalog no. R415) was used to detect rhabdomeres in whole-mount stainings.

Lysosomes were stained using LysoTracker Red DND-99 (Thermo Fisher Scientific) according to a published protocol with minor modifications (Yacobi-Sharon et al., 2013).

EM

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.

RT-PCR

qRT-PCR was performed using the Power SYBR green master mix kit (Thermo Fisher Scientific). The primer sequence can be found in the Supplemental Experimental Procedures.

Retinal Degeneration Assay

The retinal degeneration assays in Figures 3B and 3C were done in the *cn, bw*$^{-/-}$ background to eliminate eye pigments. To generate the retinal degeneration graphs in Figures 3B and 3C, green fluorescent pseudopupils appearing from *Rh1-GFP* were analyzed in live flies.

Statistics

We quantified anti-Rh1 band intensities in Figure 1 by measuring the average pixel intensities of western blot bands using ImageJ software and normalizing them to anti-α-tubulin bands. The data are representative of the average of three independent experiments

and p values were calculated through a paired Student's t test. For retinal degeneration analysis, we applied the Log-rank (Mantel-Cox) test. The graphs of Figures 3B and 3C were made by following the GraphPad Prism program using the Kaplan-Meier method and calculating the 95% confidence interval for "fractional survival." All error bars represent SEM. The n value in Figure 3 represents the number of adult flies analyzed. In other figures, n represents the number of times the experiment was repeated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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
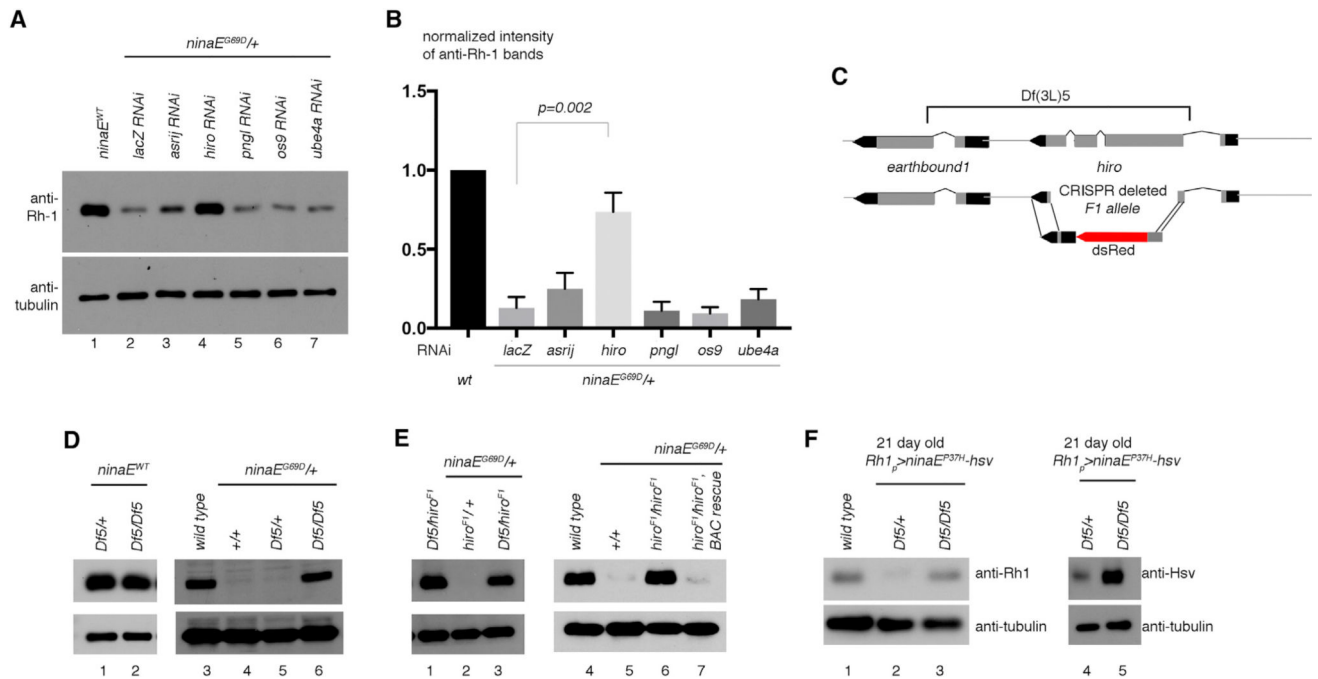
Figure 1. *hiro* Is Required for Photoreceptors to Reduce Mutant Rh1 Levels

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

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

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

(D and E) Validation of the *hiro RNAi* phenotype through classical alleles. Anti-Rh1 (top) and anti-β-tubulin (bottom) western blot from adult fly head extracts of the indicated genotypes.

(D) The effect of *Df(3L)5* deletion on wild-type (lanes 1–3) or $ninaE^{G69D/+}$ flies (lanes 4–6).

(E) $hiro^{F1-/-}$ increases Rh1 levels in $ninaE^{G69D/+}$ heads (lane 3 and 6), but introduction of the BAC rescue transgene CH321-70D3 that contains the *hiro* sequence reverses this effect (lane 7).

(F) Anti-Rh1 (lanes 1-3) and anti-Hsv (lanes 4, 5) western blots on 21-day-old $Rh1_p > ninaE^{P37H}$ fly head extracts in the indicated genotypes.

Error bars represent SEM.
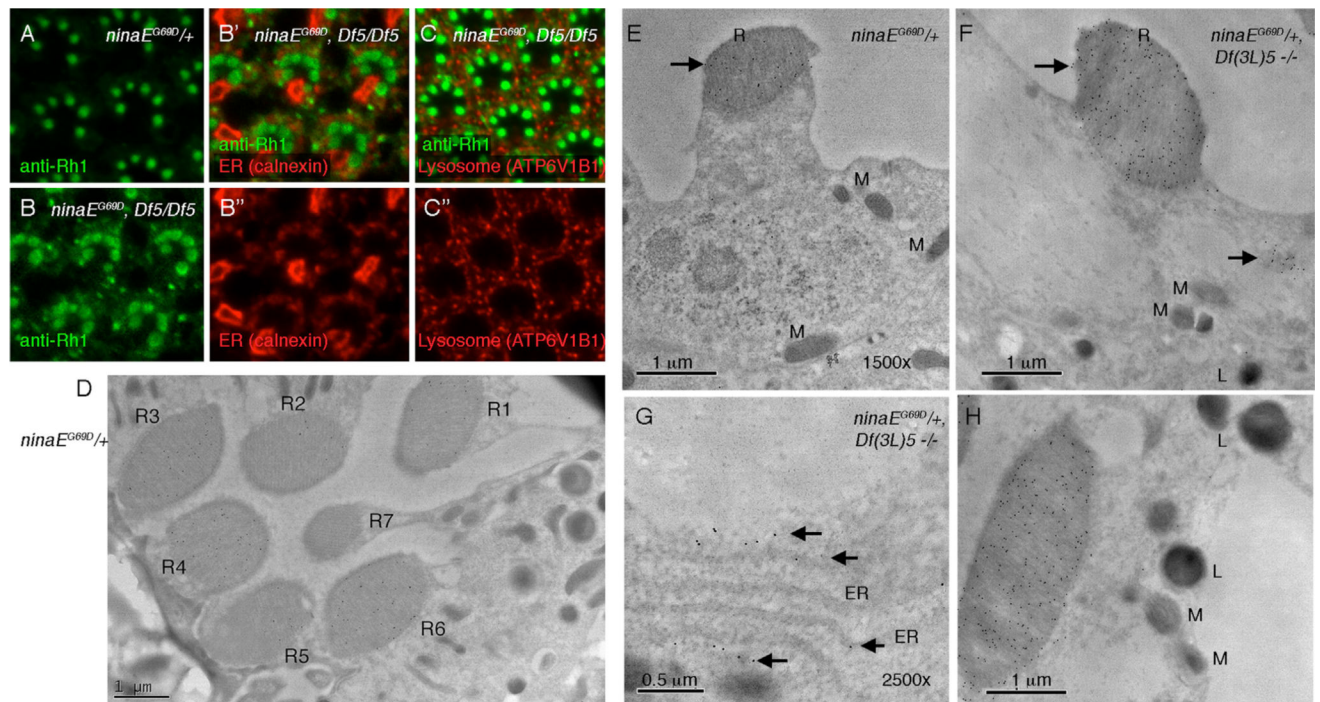
Figure 2. Subcellular Localization of Rh1 in the *hiro* Mutant Background

(A–C) Confocal microscopy imaging of adult *Drosophila* ommatidia labeled with anti-Rh1 (green) together with other subcellular organelle markers (red).

(A) *ninaE*$^{G69D/+}$ ommatidia.

(B and C) The *ninaE*^{G69D}, *Df(3L)5/Df(3L)5* ommatidia, where Rh1 is not only detected in rhabdomeres, but also in the cell body.

(B' and B") A double-labeled image with the ER marker calenxin (B" is the calnexin-only channel).

(C and C") Images with the lysosome marker ATP6V1B1.

(D–H) Immunogold EM with anti-Rh1 antibody. Signals appear as black dots and are indicated with arrows.

(D) *ninaE*$^{G69D/+}$ adult fly ommatidium with seven photoreceptors labeled as R1–R7. Note that anti-Rh1 signal is not detected in the R7 rhabdomere.

(E) A *ninaE*$^{G69D/+}$ photoreceptor.

(F–H) In *ninaE*^{G69D}, *Df(3L)5/Df(3L)5* photoreceptors, Rh1 is detected in the rhabomeres (B and D) and in additional sites of the cell body. These include the ER (G) and other organelles (F). Neither the lysosome nor the mitochondria show obvious anti-Rh1 labeling (H). Subcellular structures are abbreviated as follows: R, rhabodomere; M, mitochondria; ER, endoplasmic reticulum; L, lysosome
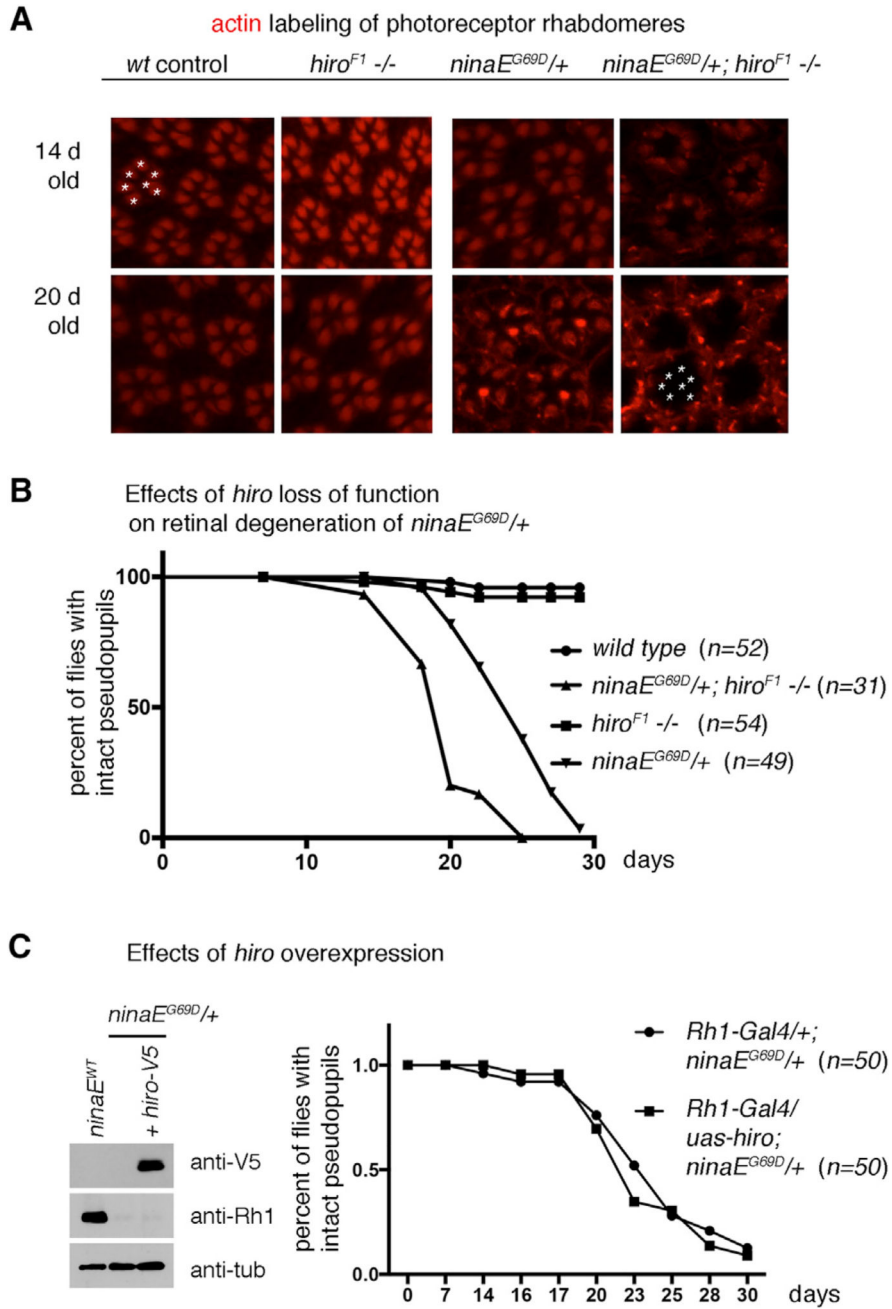
Figure 3. Loss of *hiro* Accelerates the Course of Age-Related Retinal Degeneration in *ninaE*^{G69D/+} Flies

(A) Representative images of dissected ommatidia labeled with phalloidin that labels rhabdomeres of photoreceptors (red). The genotypes are indicated on top of each panel. Intact ommatidia show a trapezoidal arrangement of seven photoreceptor rhabdomeres (one example marked with asterisks on the upper left panel). Twenty-day-old *ninaE*^{G69D}, *hiro*^{F1−/−} eyes (lower rightmost panel) lack the trapezoidal pattern of phalloidin labeling. The asterisks in that panel indicate the expected positions of phalloidin-positive rhabdomeres in an ommatidium.

(B and C) Retinal degeneration was assessed in live flies containing Rh1-GFP to visualize photoreceptors, quantified, and exhibited as a graph.

(B) The effect of *hiro* loss of function on retinal degeneration. The genotype and the numbers (n) are indicated.

(C) The effect of *hiro* overexpression. *UAS-hiro-V5* was expressed in *ninaE$^{G69D/+}$* photoreceptors using *Rh1-Gal4.* (Left) Western blot showing V5 epitope detection in the adult fly head extracts (top gel) and *hiro-V5*'s effect on Rh1 levels (middle gel). Anti-β-tubulin was used as a loading control. (Right) Comparison of the course of age-related retinal degeneration in control *ninaE$^{G69D/+}$* flies with those that express *hiro-V5* in an otherwise identical genetic background.
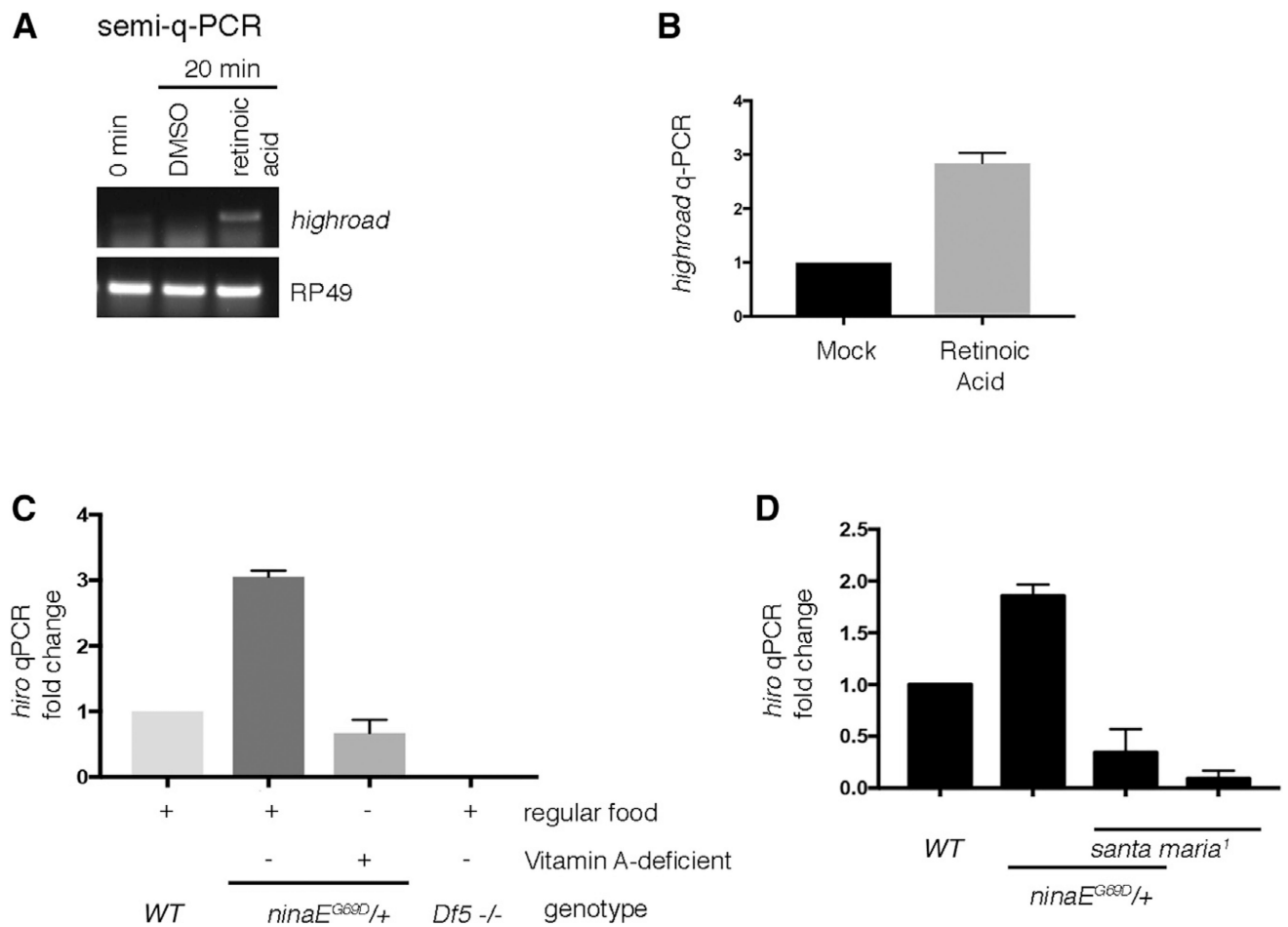
Figure 4. *hiro* Transcript Levels Are Regulated by Retinoids

(A) Semiquantitative RT-PCR of *hiro* (top) and the control RP49 (bottom) from cultured *Drosophila* S2 cells with or without retinoic acid treatment.

(B) qPCR of *hiro* from S2 cells treated with DMSO (left) as control or with retinoic acids (right).

(C) qPCR of *hiro* from fly head extracts of indicated genotypes. Flies were either raised in regular cornmeal medium or in vitamin-A-deficient food.

(D) qPCR analysis of *hiro* induction in wild-type (WT) and in *santa maria[1]* mutants that impair retinoid transport to the nervous system (right).

Error bars represent SEM.