Suppression of retinal degeneration in *Drosophila* by stimulation of ER-associated degradation

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Mutations in the rhodopsin gene that disrupt the encoded protein's folding properties are a major cause of autosomal dominant retinitis pigmentosa (ADRP). This disease is faithfully modeled in *Drosophila* where similar mutations in the *ninaE* gene, encoding rhodopsin-1 (Rh-1), cause ER stress and dominantly trigger age-related retinal degeneration. In addition, mutant flies bearing certain *ninaE* alleles have dramatically reduced Rh-1 protein levels, but the underlying mechanism for this reduction and significance of its contribution to the ADRP phenotype remains unclear. To address this question, we specifically analyzed the role of *Drosophila* genes homologous to the known yeast and animal regulators of the ER-associated degradation (ERAD) pathway, a process that reduces levels of misfolded proteins in the ER through proteasomal degradation. We found that loss-of-function of these putative ERAD factors resulted in increased levels of Rh-1 in *ninaE* mutant flies. Conversely, in an ER stress assay where mutant or wild-type Rh-1 were overexpressed in developing imaginal discs beyond the ER protein folding capacity of those cells, co-expression of certain ERAD factors was sufficient to reduce Rh-1 protein levels and to completely suppress ER stress reporter activation. Significantly, those ERAD factors that specifically reduced misfolded Rh-1 in the imaginal disc assay also delayed age-related retinal degeneration caused by an endogenous *ninaE* allele, indicating that ERAD acts as a protective mechanism against retinal degeneration in the *Drosophila* model for ADRP. These results suggest that manipulation of ERAD may serve as a powerful therapeutic strategy against a number of diseases associated with ER stress.

apoptosis | endoplasmic reticulum | rhodopsin | unfolded protein response

The endoplasmic reticulum (ER) is an organelle in which membrane and secretory proteins are synthesized and folded into stable conformations. Reflecting the ER's essential role in protein folding, mutations that either cause misfolding of proteins synthesized in the ER or interfere with ER quality control mechanisms are frequent causes of degenerative diseases (1, 2).

Among the diseases associated with protein misfolding in the ER are class II autosomal dominant retinitis pigmentosa (ADRP), in which dominant rhodopsin mutations trigger age-related retinal degeneration and blindness (3, 4). The *Drosophila* genome has several rhodopsin genes, including *ninaE* that encodes the rhodopsin-1 protein (Rh-1) (5, 6). Exhibiting a striking similarity with human rhodopsin mutants, a number of *ninaE* alleles dominantly cause age-related retinal degeneration in *Drosophila* (7, 8). The amino acid substitutions that result from these *ninaE* mutations are similar to those human rhodopsin mutant proteins that fail to fold properly in cultured cells and underlie ADRP (8). In fact, *Drosophila* photoreceptors bearing these *ninaE* mutant alleles activate a specific transcriptional response that helps reduce misfolded proteins in this ER, widely referred to as the unfolded protein response (UPR) (9). Moreover, reducing the UPR through a mutation in a key component of this pathway, xbp1, aggravates the course of retinal degeneration in the *Drosophila* model (9). Similar observations have been made with the mammalian rhodopsin mutants (10), indicating that the pathology underlying ADRP is conserved between *Drosophila* and mammals.

Among a few unexplained features associated with these dominant *ninaE* alleles is a severe reduction of overall Rh-1 protein levels in the afflicted photoreceptors. While heterozygotes with a null *ninaE* allele have roughly half of the normal Rh-1 protein levels, many disease-causing rhodopsin alleles of humans and *Drosophila* lead to significantly lower rhodopsin levels under otherwise similar conditions (7, 8). However, whether such a reduction in Rh-1 levels has a functional significance in the retinal degeneration remains unclear. It is possible that the reduction of overall Rh-1 levels may have a beneficial consequence in the ADRP model, as it may protect against toxicity associated with aggregates in the ER. Alternatively, excessive Rh-1 reduction may accelerate retinal degeneration in ADRP, as insufficient levels of Rh-1 protein at the light-sensing compartment can compromise photoreceptor integrity and survival (11).

To understand the mechanism and function of Rh-1 reduction in this disease model, we investigated the role of putative regulators of *Drosophila* ER-associated degradation (ERAD). ERAD is regulated by a multiprotein complex that includes proteins involved in the recognition, retrotranslocation, and ubiquitination of misfolded proteins in the ER (12). Studies using *Saccharomyces cerevisiae* have suggested the existence of three major ERAD subpathways, defined by the subcellular location of the lesion that causes protein misfolding (13–15). A protein that misfolds in the ER lumen is thought to be degraded through the ERAD-L pathway. On the other hand, proteins with lesions in transmembrane domains are thought to be substrates of the ERAD-M pathway. Proteins with lesions on the cytoplasmic side of the ER are processed by the ERAD-C pathway. In yeast, each subpathway requires a distinct set of ERAD complex subunits. For example, the two yeast mannosidases that recognize misfolded glycoproteins, Htm1p, and membrane protein complex containing Usa1p and Derlin, appear dedicated to the ERAD-L pathway, since ERAD-M substrates bypass the requirement of those subunits (13–16). Whether ERAD regulation in metazoans follows the same rules remains unknown.

Using genetic tools of *Drosophila*, here we demonstrate that ERAD regulators help reduce stress and retinal degeneration caused by mutant Rh-1 in the *Drosophila* ADRP model and serve as a protective mechanism. Specifically, we show that disruption of the ERAD pathway leads to an increase in Rh-1 protein levels in the *Drosophila* model for ADRP. Conversely, overexpression of certain subunits of the ERAD machinery was sufficient to reduce the levels of ER stress-causing Rh-1 proteins. Individual subunits showed distinct specificity toward their substrates that were sometimes inconsistent with what is expected from the ERAD subpathways defined in yeast. Most significantly, certain factors were able to suppress late-onset retinal degeneration in a *Drosophila* model

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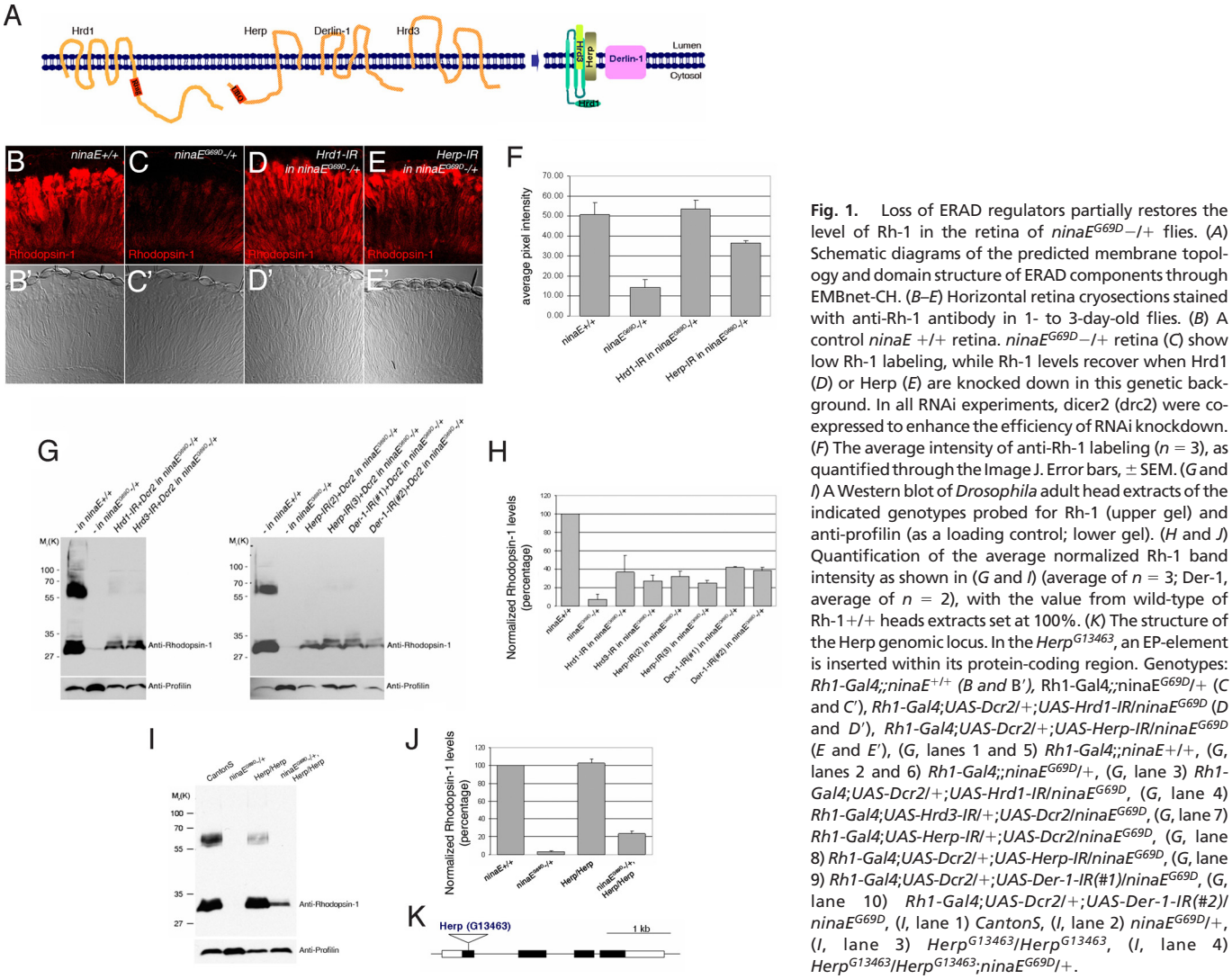

Fig. 1. Loss of ERAD regulators partially restores the level of Rh-1 in the retina of *ninaE*^{G69D}−/+ flies. (*A*) Schematic diagrams of the predicted membrane topology and domain structure of ERAD components through EMBnet-CH. (*B–E*) Horizontal retina cryosections stained with anti-Rh-1 antibody in 1- to 3-day-old flies. (*B*) A control *ninaE* +/+ retina. *ninaE*^{G69D}−/+ retina (*C*) show low Rh-1 labeling, while Rh-1 levels recover when Hrd1 (*D*) or Herp (*E*) are knocked down in this genetic background. In all RNAi experiments, dicer2 (drc2) were co-expressed to enhance the efficiency of RNAi knockdown. (*F*) The average intensity of anti-Rh-1 labeling (n = 3), as quantified through the Image J. Error bars, ± SEM. (*G* and *I*) A Western blot of *Drosophila* adult head extracts of the indicated genotypes probed for Rh-1 (upper gel) and anti-profilin (as a loading control; lower gel). (*H* and *J*) Quantification of the average normalized Rh-1 band intensity as shown in (*G* and *I*) (average of n = 3; Der-1, average of n = 2), with the value from wild-type of Rh-1+/+ heads extracts set at 100%. (*K*) The structure of the Herp genomic locus. In the *Herp*^{G13463}, an EP-element is inserted within its protein-coding region. Genotypes: *Rh1-Gal4;;ninaE*$^{+/+}$ (*B and* B′), Rh1-Gal4;;ninaE^{G69D}/+ (*C* and *C*′), *Rh1-Gal4;UAS-Dcr2/+;UAS-Hrd1-IR/ninaE*^{G69D} (*D* and *D*′), *Rh1-Gal4;UAS-Dcr2/+;UAS-Herp-IR/ninaE*^{G69D} (*E* and *E*′), (*G*, lanes 1 and 5) *Rh1-Gal4;;ninaE+/+*, (*G*, lanes 2 and 6) *Rh1-Gal4;;ninaE*^{G69D}/+, (*G*, lane 3) *Rh1-Gal4;UAS-Dcr2/+;UAS-Hrd1-IR/ninaE*^{G69D}, (*G*, lane 4) *Rh1-Gal4;UAS-Hrd3-IR/+;UAS-Dcr2/ninaE*^{G69D}, (*G*, lane 7) *Rh1-Gal4;UAS-Herp-IR/+;UAS-Dcr2/ninaE*^{G69D}, (*G*, lane 8) *Rh1-Gal4;UAS-Dcr2/+;UAS-Herp-IR/ninaE*^{G69D}, (*G*, lane 9) *Rh1-Gal4;UAS-Dcr2/+;UAS-Der-1-IR(#1)/ninaE*^{G69D}, (*G*, lane 10) *Rh1-Gal4;UAS-Dcr2/+;UAS-Der-1-IR(#2)/ninaE*^{G69D}, (*I*, lane 1) *CantonS*, (*I*, lane 2) *ninaE*^{G69D}/+, (*I*, lane 3) *Herp*^{G13463}/*Herp*^{G13463}, (*I*, lane 4) *Herp*^{G13463}/*Herp*^{G13463};*ninaE*^{G69D}/+.

for ADRP. These results suggest that specific ERAD mechanisms can be exploited as a therapeutic strategy against conformational diseases.

Results

Loss of ERAD Components Partially Restores the Level of Rhodopsin-1 in the *ninaE*^{G69D}−/+ Retina. To study the mechanism and consequences of Rh-1 reduction in the *Drosophila* model for ADRP, we examined heterozygous flies bearing a *ninaE* allele with a Gly residue in a transmembrane domain replaced by Asp (henceforth referred to as *ninaE*^{G69D}). Similar to human autosomal dominant retinitis pigmentosa (ADRP), these flies activate the UPR in their photoreceptors, which eventually undergo age-related retinal degeneration (7, 8, 11). To determine the role of ERAD in the pathogenesis of ADRP, we modulated the levels of *Drosophila* homologs of yeast and human ERAD regulator proteins, with a particular focus on those integral membrane proteins that interact with each other (12). One of these *Drosophila* gene products, known as septin interacting protein 3, was previously identified in a yeast two-hybrid assay (17). We noticed that this *Drosophila* gene is most homologous to yeast and human Hrd1, a ubiquitin ligase of the ERAD pathway (18–20), and we will henceforth refer to it as Hrd1. As in humans, *Drosophila* Hrd1 has six predicted transmembrane domains and a RING domain on the cytoplasmic side (Fig. 1*A*). Other genes we examined include a candidate retrotranslocation channel component, Derlin-1 (CG10908), and its associated proteins Hrd3 (CG10221) and Herp (CG14536) (Fig. 1*A*). When the retina of heterozygotic flies bearing the *ninaE*^{G69D} allele were immunolabeled with anti-Rh-1 antibody, we found that the average anti-Rh-1 labeling intensity was reduced to approximately 28% of wild-type levels (Fig. 1*C*), consistent with previous reports (7, 8, 21, 22) that have documented the reduction of both wild-type and mutant Rh-1 proteins under these conditions. To determine whether this reduction is due, at least in part, to enhanced ERAD, we knocked down Hrd1 and Herp in the retina of *ninaE*^{G69D}−/+ flies using inverted repeat transgenic constructs that are designed to produce double stranded (ds) RNA (see Materials and Methods). Knock down of *Drosophila* Hrd1 in this background restored the overall Rh-1 levels to an extent that was statistically indistinguishable from that of wild-type flies (Fig. 1*D*; n = 3, P = 0.6). Although to a lesser degree, Herp knock down also restored Rh-1 levels to a significant degree (Fig. 1*E*; n = 3, P = 0.0009). To complement these immunohistochemical analysis, we examined Rh-1 levels through Western blots under similar genetic conditions. The total amount of Rh-1 in *ninaE*^{G69D}−/+ flies estimated through Western blots was 7% of the wild-type levels, which was lower than that assessed through immunohistochemistry. This is most likely because insoluble membrane proteins are frequently lost during SDS/PAGE sample preparation. However, the changes of Rh-1 levels in response to ERAD inhibition was consistent with that

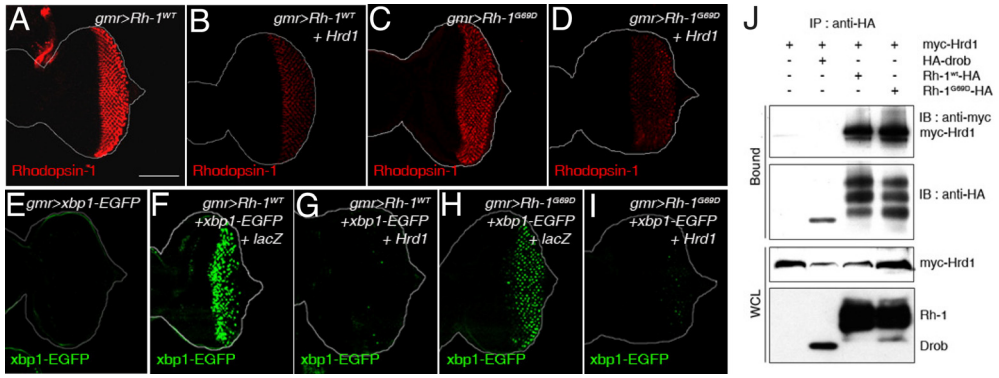

Fig. 2. ER stress caused by Rh-1 misexpression is strongly suppressed by *Drosophila* Hrd1. (*A–D*) Representative images of eye imaginal discs expressing Rh-1^{WT} (*A*) or Rh-1^{G69D} (*C*) alone, or together with Hrd1 (*B* and *D*). Anti-Rh-1 antibody labeling is in red. *E–I* Hrd1 co-expression abolished ER stress caused by wild-type or mutant Rh-1 misexpression, as determined by the ER stress marker, xbp1-EGFP (green). Shown are representative discs expressing xbp1-EGFP alone (*E*), or together with indicated genes. (*J*) Co-immunoprecipitation assays between Hrd1 and Rh-1 in 293T cells. Hrd1 was tagged with the myc epitope, while Rh-1 was tagged with HA. HA-Drob-1 is a membrane protein used as a negative control. [Scale bars, 100 μm (*A*).] Genotypes: *gmr-Gal4/+;UAS-Rh-1^{WT}/+* (*A*), *gmr-Gal4/UAS-Hrd1;UAS-Rh-1^{WT}/+* (*B*), *gmr-Gal4/+;UAS-Rh-1^{G69D}/+* (*C*), *gmr-Gal4/UAS-Hrd1;UAS-Rh-1^{G69D}/+* (*D*), *gmr-Gal4/+;UAS-xbp1-EGFP/+* (*E*), *gmr-Gal4/UAS-lacZ;UAS-Rh-1^{WT}/UAS-xbp1-EGFP* (*F*), *gmr-Gal4/UAS-Hrd1;UAS-Rh-1^{WT}/UAS-xbp1-EGFP* (*G*), *gmr-Gal4/UAS-lacZ;UAS- Rh-1^{G69D}/UAS-xbp1-EGFP* (*H*), and *gmr-Gal4/UAS-Hrd1;UAS-Rh-1^{G69D}/UAS-xbp1-EGFP* (*I*).

observed through immunohistochemistry, with the knock down of Hrd1 or other ERAD regulators partially restoring the Rh-1 levels in *ninaE^{G69D}−/+* flies (Fig. 1 *G* and *H*). The results were further validated using a Herp mutant allele, *Herp^{G13463}*, which has an EP-element inserted within its protein coding sequence (Fig. 1*K*). While the loss-of Herp did not affect the total Rh-1 protein level in the *ninaE+* background, it partially restored total Rh-1 levels in the *ninaE^{G69D}−/+* background ($p = 0.0003$) (Fig. 1 *I* and *J*). There was no statistically significant difference in the steady state Rh-1 levels between the Herp knock down and *Herp^{G13463}−/−* flies ($P = 0.45$ and 0.1 for comparisons with two independent inverted repeat lines). While these results can be most simply interpreted as evidence of misfolded Rh-1 proteins being degraded by ERAD, an alternative scenario is also possible where defective ERAD may increase Rh-1 levels by inducing ER chaperons and enhance Rh-1 folding. In fact, blocking ERAD in yeast is known to stimulate UPR signaling and induce ER chaperones (23). However, such an indirect model appears unlikely in the *Drosophila* retina, as we find that the Herp mutant flies did not induce the expression of *heat shock cognate protein 3* (*hsc3*), a major ER chaperon that is homologous to BiP and a well established target of UPR (Fig. S1). These observations support the idea that the examined *Drosophila* ERAD factors stimulate the degradation of misfolded Rh-1 in the ADRP model.

ER Stress Caused by Rh-1 Misexpression Is Strongly Suppressed by *Drosophila* Hrd1. As the retina of *ninaE^{G69D}−/+* flies contains a mixture of mutant and wild-type Rh-1 proteins that are similar in size, it is difficult to distinguish their individual fates through Western blots or immunohistochemistry. To examine the behavior of particular Rh-1 alleles, we expressed mutant or wild-type Rh-1 in the larval eye imaginal discs, a developing tissue that does not yet express Rh-1. To assess the level of ER stress caused by such Rh-1 misexpression, we used xbp1-EGFP as a reporter designed to detect the unconventional mRNA splicing of xbp1, which is a key signaling event for the initiation of UPR. This reporter allows EGFP to be expressed in frame, only when a 23-nt intron in xbp1 is spliced out by the ER stress-activated endonuclease ire-1, thereby marking with green fluorescence those cells suffering significant ER stress (9). While this marker is not activated in response to proteins that misfold in the cytoplasm (9), xbp1-EGFP fluorescence was detected in all examined eye discs in which Rh-1^{G69D} or Rh-1^{WT} were expressed (Fig. 2 *F* and *H*, $n > 10$). The observation that Rh-1^{WT} also activates the UPR in these cells was unexpected, and may be due to the limited ER capacity of the imaginal disc cells to fold and export high levels of Rh-1.

The establishment of the above assay allowed us to ask whether overexpression of any of the putative ERAD regulator genes could suppress the ER stress caused by Rh-1 misexpression. We found that this was the case, with *Drosophila* Hrd1 being one the strongest suppressors among those examined. In larval eye discs, *Drosophila* Hrd1 lowered Rh-1^{WT} and Rh-1^{G69D} protein levels when these were co-expressed through the eye-specific *gmr-Gal4* driver (Fig. 2 *A–D*). The effect of Hrd1 on the misexpressed Rh-1 proteins was further validated through an alternative gene expression system that employs *tubulin-Gal4* flip-out technology, which generates mosaic clones expressing genes of choice through the *tubulin* promoter (see Materials and Methods). When mosaic clones expressing *Drosophila* Hrd1 were generated within the population of Rh-1^{G69D}-expressing cells, those clones had distinctively lower levels of Rh-1^{G69D} protein, compared to the neighboring cells that did not express Hrd1 (Fig. S2 *B* and *B′*; $n = 8$). Hrd1 appeared to lower mutant and wild-type Rh-1 to levels that virtually eliminated ER stress itself, as evidenced by a near complete suppression of the xbp1-EGFP marker, which was otherwise activated in response to the misexpression in either wild type or mutant Rh-1 in eye discs (Fig. 2 *E–I*; $n > 10$). As an indirect measure of stress, we also examined markers for apoptosis, an active cell death program that involves, among others, proteolytic cleavage and activation of caspases. Labeling of imaginal discs with an antibody that detects such caspase cleavage event (24) showed that *Drosophila* Hrd1 co-expression suppressed the excessive apoptosis associated with Rh-1^{WT} misexpression (Fig. S2 *C* and *D*; $n = 8$). To determine whether Hrd1 interacts with either Rh-1^{WT} or Rh-1^{G69D}, we performed co-immunoprecipitation assays. Myc-tagged Hrd1 was coexpressed in 293T cells along with HA-tagged Rh-1^{WT}, Rh-1^{G69D} or a control membrane protein, Drob-1 (25). Hrd1 co-precipitated with Rh-1^{WT}and Rh-1^{G69D}, but not with Drob-1 (Fig. 2*J*), establishing that Hrd1 forms a physical complex with mutant and wild-type Rh-1 and strongly suppresses ER stress caused by these Rh-1 proteins.

Overexpression of Putative ERAD Components in *Drosophila*. The findings just described prompted us to test other *Drosophila* homologs of the ER associated degradation (ERAD) factors, including Hrd3, Herp, Derlin-1, and Derlin-2 (CG14899). In addition, we characterized the role of *Drosophila* EDEM homologs, CG3810 and CG5682, which we refer to as EDEM1 and EDEM2, respectively. Specifically, we examined their ability to suppress the rough eye phenotype generated by overexpression of Rh-1^{WT} in larval eye discs, a condition that causes ER stress (Fig. S2). Consistent with the results obtained with the xbp1-EGFP assay, Hrd1 overexpression in eye imaginal discs almost completely rescued the rough eye phenotype in adults. Herp overexpression also suppressed the eye phenotype, but to a significantly lesser extent. On the other hand,

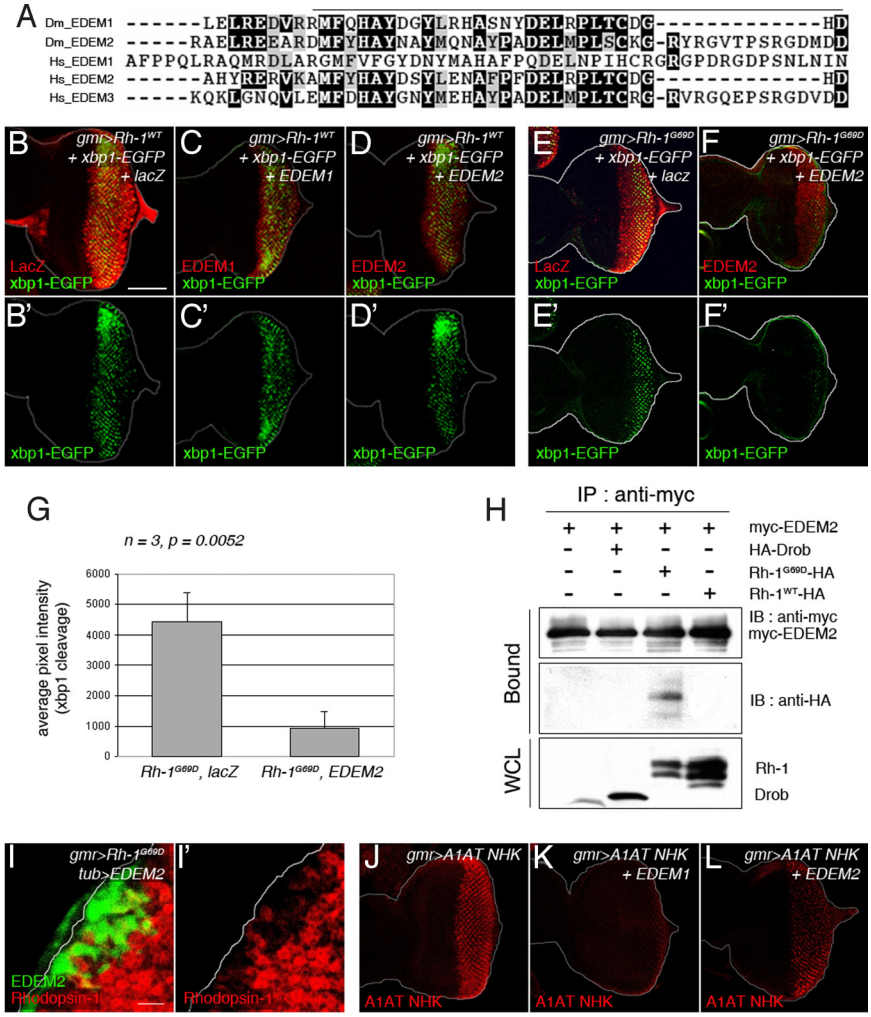

Fig. 3. *Drosophila* EDEM2 overexpression reduces mutant, but not wild-type Rh-1 levels. (*A*) The amino acid sequence alignment between EDEM family proteins of *Drosophila* and humans, generated with the ClustalW algorithm. Dark shading indicates identity, whereas light shading indicates similarity. Solid lines indicate the catalytic domain of class I mannosidase (Glycosyl hydrolase family 47). The sequences shown, with their corresponding NCBI database accession numbers, are as follows: *D. melanogaster* (*Dm*) EDEM1 (NP_726777); *D. melanogaster* (*Dm*) EDEM2 (AAF53255); human (*Hs*) EDEM1 (AAH19088); human (*Hs*) EDEM2 (NP_060687); human (*Hs*) EDEM3 (NP_079467). (*B–D*) The effect of *Drosophila* EDEM1 and EDEM2 on misexpressed Rh-1^{WT}. Shown are representative discs expressing Rh-1^{WT}, together with lacZ (*B*), EDEM1 (*C*), or EDEM2 (*D*), or expressing Rh-1^{G69D} with lacZ (*E*) or EDEM2 (*F*). The degree of ER stress is assessed through xbp1-EGFP activation (*B′–F′*, green). (*G*) Quantification of the xbp1-EGFP activation levels as shown in (*E* and *F*). Error bars, ± SEM. (*H*) EDEM2 physically interacts with Rh-1^{G69D} in S2 cells. EDEM2 was immunoprecipitated through its myc-tag, and its interaction partners were detected through their HA-epitopes. (*I* and *I′*) A disc expressing EDEM2 (green) in a flip-out mosaic clone shows reduced levels of Rh-1^{G69D} (red). (*J–L*) Both of EDEM1 and EDEM2 effectively downregulated the level of alpha 1-antitrypsin^{NHK} (A1AT NHK, in red). (Scale bars, 100 μm (*B*) and 10 μm (*I*).) Genotypes: *gmr-Gal4/UAS-lacZ;UAS-Rh-1^{WT}/UAS-xbp1-EGFP* (*B* and *B′*), *gmr-Gal4/+;UAS-Rh-1^{WT}, UAS-EDEM1/UAS-xbp1-EGFP* (*C* and *C′*), *gmr-Gal4/UAS-EDEM2;UAS-Rh-1^{WT}/UAS-xbp1-EGFP* (*D* and *D′*), *gmr-Gal4/UAS-lacZ;UAS-Rh-1^{G69D}/UAS-xbp1-EGFP* (*E* and *E′*), *gmr-Gal4/UAS-EDEM2;UAS-Rh-1^{G69D}/UAS-xbp1-EGFP* (*F* and *F′*), *hs-flp;UAS-EDEM2/+;tub>GFP>Gal4/gmr-Rh-1^{G69D}* (*I* and *I′*), *gmr-Gal4/UAS-alpha1-antitrypsin^{NHK};+/+* (*J*), *gmr-Gal4/UAS-alpha1-antitrypsin^{NHK};UAS-EDEM1/+* (*K*), and *gmr-Gal4/UAS-alpha1-antitrypsin^{NHK}, UAS-EDEM2;+/+* (*L*).

other examined genes failed to rescue the partial eye ablation caused by Rh-1^{WT} under similar conditions (Fig. S2).

***Drosophila* EDEM2 Overexpression Reduces Mutant, but Not Wild-Type Rh-1 Levels.** Although the above study suggested that the *Drosophila* EDEMs do not affect Rh-1^{WT} levels, we further tested whether EDEMs affect other ERAD substrates. The *Drosophila* EDEM1 and EDEM2 are most homologous to mammalian EDEM2 and EDEM3 respectively (Fig. 3*A*) and also to the yeast Htm1p, which are best characterized as mannosidases that extract primarily luminal misfolded glycoproteins from their chaperone cycles and deliver them to the ERAD machinery (16, 26–28). Inconsistent with the idea that Htm1p is only dedicated to ERAD-L, overexpression of EDEM2 was able to reduce the levels of a likely ERAD-M substrate, Rh-1^{G69D}, which has a mutation in a transmembrane domain (Fig. 3 *I* and *I′*; $n = 6$). More intriguingly, EDEM2 did not suppress ER stress caused by Rh-1^{WT}, as assessed through xbp1-EGFP fluorescence (Fig. 3 *B–D*). The reduction of Rh-1^{G69D} levels by EDEM2 was accompanied by a strong suppression of ER stress, as measured by xbp1-EGFP fluorescence (Fig. 3 *E′*, *F′*, and *G*; $n = 3$, $P = 0.0052$). EDEM1 overexpression neither affected Rh-1^{WT} nor Rh-1^{G69D} (Fig. 3*C* and Fig. S3*D*). We then performed immunoprecipitation assays to test whether EDEM2 forms a physical complex with Rh-1^{G69D} or Rh-1^{WT}. Consistent with EDEM2's effects on mutant and wild-type Rh-1 levels in vivo, EDEM2 co-precipitated with Rh-1^{G69D} (Fig. 3*H*, lane 3) but not with Rh-1^{WT} (Fig. 3*H*, lane 4). These results were further validated by the examination of the adult eye ablation phonotype. Similar to the case of overexpressing Rh-1^{WT}, the misexpression of Rh-1^{G69D} in larval eye discs generated partially ablated adult eyes (Fig. S3*B*). Although the degree of suppression was less than that observed with the Rh-1^{WT} misexpressing eyes, Hrd1 scored as one of the strongest suppressors the Rh-1^{G69D} overexpression phenotype, reducing the extent of eye tissue loss and de-pigmentation (Fig. S3*C*). Consistent with the results obtained with xbp1-EGFP in eye imaginal discs, EDEM2 overexpression suppressed the Rh-1^{G69D} overexpression phenotype (Fig. S3*E*).

In addition to the examination of Rh-1 levels, we also determined the effect of the EDEMs on an ER luminal ERAD substrate, alpha 1-antitrypsin^{NHK}, which in mammalian cells, has been shown to cause ER stress that can be relieved by overexpression of mammalian EDEM2 or EDEM3 (27, 29). As expected from these studies, overexpression of *Drosophila* EDEM1 or EDEM2 reduced the levels of alpha 1-antitrypsin^{NHK} (Fig. 3 *J–L*), which however was not affected by Hrd1 (Fig. S4). Collectively, these observations show that ERAD factors show specificity toward their substrates, but such specificity is not exclusively governed by the subcellular location of the lesion, as had been suggested previously (13–16).

***Drosophila* Hrd1 and EDEM2 Overexpression Suppresses Late Onset Retinal Degeneration in the *Drosophila* ADRP Model.** To determine the physiological significance of the ERAD specificity observed in the imaginal disc overexpression assay, we turned to the endogenous *ninaE^{G69D}* mutant allele that dominantly causes age-related retinal degeneration. Specifically, we overexpressed *Drosophila* Hrd1 or EDEMs in the retina of *ninaE^{G69D}*−/+ flies and examined

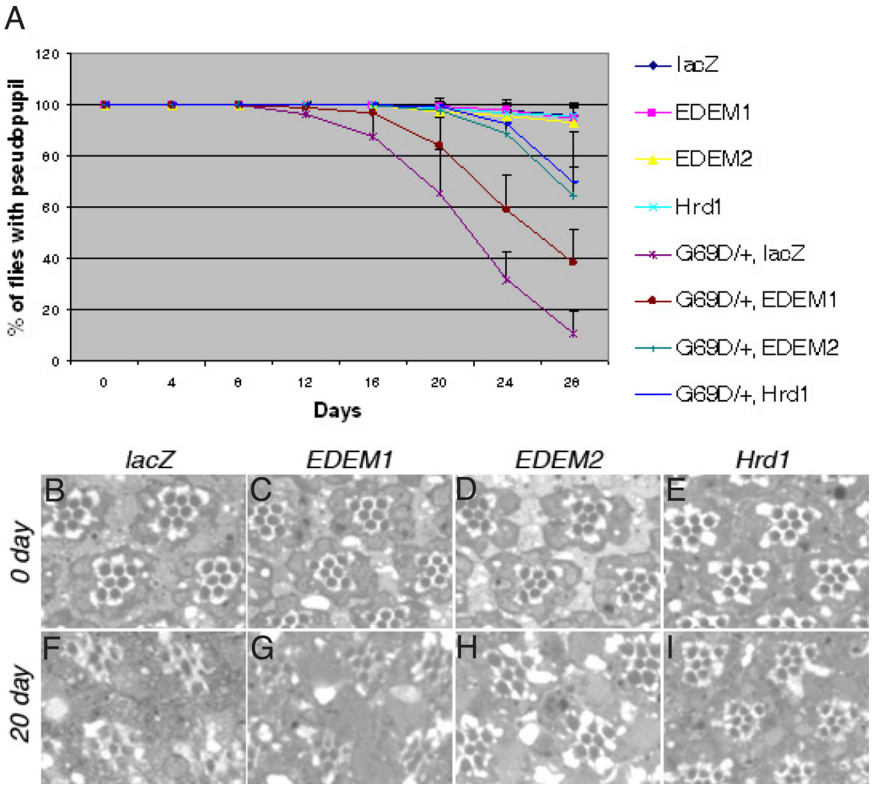

Fig. 4. *Drosophila* Hrd1 and EDEM2 overexpression suppresses late onset retinal degeneration of $ninaE^{G69D}-/+$ flies. (*A*) Quantification of the extent of retinal degeneration through the pseudopupil assay. For each genenotype, the graph shows the percentage of flies with intact pseudopupils (average of four independent crosses). Specifically, lacZ, EDEM1, EDEM2 or Hrd1 were overexpressed through the Rh1-Gal4 driver in ninaE mutant or wild type backgrounds. Overexpression of Hrd1 and EDEM2 delay the course of retinal degeneration of $ninaE^{G69D}-/+$ flies. (*B–I*) Representative images of adult retina overexpressing designated ERAD factors in the $ninaE^{G69D}-/+$ background. (*B–E*) At day 0 after eclosion, retina of all genotypes have regular array of ommatidia. (*F–I*) Retinal sections of indicated genotypes at day 20. The degree of ommatidial disarray is suppressed by overexpressing EDEM2 or Hrd1 (*H* and *I*), but not by lacZ (*F*) or EDEM1 (*G*).

their effects on the time course of retinal degeneration. To test this, we first used the pseudopupil assay, a noninvasive technique used to assess the regularity of the GFP-labeled photoreceptor array, which collectively projects to generate a single trapezoidal pseudoimage (30). While $ninaE+/+$ animals overexpressing only lacZ, EDEM1, EDEM2, or Hrd1 did not show any signs of retinal degeneration using this assay (Fig. 4*A*), $ninaE^{G69D}-/+$ flies expressing a control protein, lacZ, had their pseudopupils disappear beginning at 12 days after eclosion, in a progressive manner, with only 10% of these flies showing intact pseudopupils at 28 days after eclosion (Fig. 4*A*; 10.47 ± 8.46%, $n = 4$). Significantly, the expression of *Drosophila* Hrd1 in a $ninaE^{G69D}-/+$ background dramatically decreased the rate of deep pseudopupil loss with 70% of the examined flies showing intact pseudopupils at day 28 (Fig. 4*A*; 69.72 ± 19.58%, $n = 4$, $P = 0.0014$). The overexpression of EDEM2 in the $ninaE^{G69D}-/+$ retina also delayed the time course of retinal degeneration significantly, with 64% of flies still showing intact pseudopupils at day 28 (Fig. 4*A*; 64.43 ± 11.11%, $n = 4$, $P = 0.0002$). Consistent with what we had observed in larval imaginal disc misexpression assays, EDEM1 had only a minimal effect on delaying retinal degeneration of $ninaE^{G69D}-/+$ flies (Fig. 4*A*; 38.02 ± 13.47%, $n = 4$, $P = 0.013$).

The pseudopupil results were independently validated by semithin sections of retinas of various genotypes. A single ommatidium of wild-type flies contains seven rhabdomeres, light-sensing organelles equivalent to the outer segments of mammalian rod cells (31). In 1-day-old $ninaE^{G69D}-/+$ flies (i.e., 0 day light incubation) expressing lacZ, EDEM1, EDEM2, or Hrd1, retinas maintained a regular repeating pattern of ommatidia, with virtually all ommatidia containing seven rhabdomeres in a regular array (Fig. 4 *B–E*). In contrast, the 21-day-old $ninaE^{G69D}-/+$ flies expressing a control lacZ gene had most ommatidia in disarray with large vacuoles and an overall reduction of rhabdomere numbers (Fig. 4*F*). Only a small percentage of the examined ommatidia retained all seven rhabdomeres within a unit (Fig. 4*J*; 2 ± 2%, $n = 3$). When EDEM2 or Hrd1 was overexpressed in the $ninaE^{G69D}-/+$ retina under otherwise similar conditions, the degree of ommatidial disarray was significantly suppressed (Fig. 4 *H* and *I*, respectively). Specifically, a majority of the ommatidia expressing EDEM2 or Hrd1 retained all seven rabdomeres associated within a unit (Fig. 4 *H* and *I*; 68.9 ± 16.1%, $n = 3$, $P = 0.002$; 68.4 ± 6.7%, $n = 3$, $P < 0.0001$, respectively). Consistent with the partial suppression of pseudopupil loss by EDEM1, ommatidial arrays in EDEM1 overexpressing $ninaE^{G69D}-/+$ flies were only mildly restored (Fig. 4*G*; 27.8 ± 4.9%, $n = 3$, $P = 0.001$).

Intriguingly, the overexpression of EDEM2 or Hrd1 in $ninaE^{G69D}-/+$ retina significantly restored overall Rh-1 levels detectable through Western blots, when compared to control $ninaE^{G69D}-/+$ retina (Fig. S5 *A* and *B*; $n = 4$, $P = 0.017$; $n = 4$, $P < 0.001$, respectively). Consistently, EDEM2 or Hrd1 overexpression enhanced the amount of Rh-1 detected in the rhabdomeres of $ninaE^{G69D}-/+$ flies. We favor the interpretation that under enhanced ERAD activity, misfolded mutant Rh-1 is quickly eliminated to allow more wild-type Rh-1 to fold and avoid degradation, for proper trafficking to rhabdomeres. Collectively, these results show that overexpression of ERAD factors that are sufficient to suppress Rh-1-induced ER stress in larval imaginal discs can delay the course of age-related retinal degeneration in the *Drosophila* model for ADRP.

Discussion

Here, we report on the mechanism and functional consequence of Rh-1 degradation through ERAD in a *ninaE* mutant allele that serves as a *Drosophila* model of ADRP. Specifically, we established a Rh-1 overexpression assay in larval imaginal discs and showed that certain ERAD factors can reduce Rh-1 levels to the extent of abolishing ER stress markers when co-expressed. The imaginal disc assay also revealed an unexpected degree of specificity between specific ERAD regulators and their substrates. Most significantly, the findings in the imaginal disc overexpression assay directly correlated with a given ERAD factor's ability to physically bind its substrates, and to suppress

retinal degeneration in a physiologically relevant disease model for ADRP, in which age-related retinal degeneration is caused by the endogenous *ninaE*^{G69D} allele.

One of the unexpected outcomes of our study is the observed specificity between given ERAD factors and their misfolded protein substrates. Previous studies conducted in yeast led to the proposal of the presence of three ERAD subpathways, defined by the subcellular locations of lesions that cause protein misfolding. In that view, the yeast homolog of EDEM, Htm1p, and membrane proteins, Derlin and Usa1p were considered specific components of the ERAD-L pathway, specializing in ER luminal protein recognition and degradation (13–16). By contrast, our study shows that *Drosophila* EDEM2, Derlin-1 and Herp are involved in reducing the levels of a mutant membrane protein, Rh-1^{G69D}. The list of ERAD genes that can reduce Rh-1^{G69D} levels were different from those involved in wild-type Rh-1 protein or alpha 1-antitrypsin^{NHK}, which is an established ER luminal substrate. We favor the interpretation that EDEM1 initiates an ERAD-L-like pathway in *Drosophila*, in part, based on the fact that EDEM1 only reduced alpha 1-antitrypsin but not the Rh-1 proteins. On the other hand, Hrd1 overexpression may initiate an ERAD-M-like pathway, as this condition only affected Rh-1 proteins, but not alpha 1-antitrypsin. The observation that Rh-1^{G69D} degradation properties are neither identical to Rh-1^{WT} nor alpha 1-antitrypsin^{NHK} degradation suggests that animals have evolved additional ERAD subpathways not reported in yeast. Such an idea has been suggested previously (32), but awaits further validation.

While the reductionist approach taken in the eye imaginal disc assay has allowed us to match specific ERAD regulators with wild-type or mutant Rh-1 alleles, we were not able to follow the fates of wild-type and mutant Rh-1 proteins that must exist as a mix in the *ninaE*^{G69D}−/+ retina. Previous studies have demonstrated that, under such a condition, the mutant Rh-1 proteins interfere with the proper maturation of the wild-type Rh-1, leading to the degradation of both species (4, 7, 8, 22). Based on this together with our imaginal disc overexpression assays, we speculate that EDEM2 or Hrd1 stimulates the degradation of misfolded Rh-1 proteins, thereby allowing more wild-type Rh-1 to undergo maturation. Supporting this idea, we found that stimulating ERAD in the *ninaE*^{G69D}−/+ retina actually enhanced overall Rh-1 levels and a more efficient Rh-1 trafficking to the rhabdomeres (Fig. S5). Elimination of misfolded Rh-1 that may otherwise cause toxicity, together with enhanced Rh-1 trafficking, most likely contribute to the suppression of retinal degeneration in this *Drosophila* model for ADRP.

Since the *ninaE*^{G69D}−/+ flies have a mutation that is molecularly similar to those mutations found in human patients, leading to a similar pattern of late-onset retinal degeneration, the role of ERAD in this disease progression is likely conserved between the two species. We exploited ERAD to delay disease in an animal disease model. As ER stress underlies a wide variety of diseases, manipulation of ERAD may be used to therapeutically intervene in a variety of ER stress-related diseases.

Materials and Methods

Plasmids and Fly Stocks. The following flies and DNA have been described previously: *ninaE*^{G69D}*/TM6B* (8), UAS-xbp1-EGFP, UAS-Rh-1^{G69D} (9), Rh1-Gal4, Rh1-GFP (30), gmr-Gal4 (33), hs-flp; tubulin>FRT>y^{+}, GFP>FRT>Gal4 flies (9), Drob-1 expression plasmid (25) alpha 1-antitrypsin^{NHK} DNA (34). The coding sequences for Hrd1, EDEM1, EDEM2, Hrd3, Herp, Der-1, and Der-2 were obtained through RT-PCR from yw larvae. Myc-tags were added to the N termini of these coding sequences and subcloned into a pUAST (35). pGMR-Rh-1^{G69D} construct was created by subcloning the corresponding cDNA into the pGMR vector (33). *Herp*^{G13463} *allele* was obtained from BMRC KAIST. For in vivo RNAi, UAS-Hrd1-IR (V6870), UAS-Hrd3-IR (V1161), UAS-Herp-IR (V11724, V11725), and UAS-Der-1-IR (V44210, V44211) were obtained from the Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at). To enhance the efficiency of RNAi knockdown, uas-dcr2 was driven for co-expression with these inverted repeat lines. Additional methods are available in *SI Text*.

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1. Marciniak SJ, Ron D (2006) Endoplasmic reticulum stress signaling in disease. *Physiol Rev* 86:1133–1149.
2. Lin JH, Walter P, Yen TS (2007) Endoplasmic reticulum stress in disease pathogenesis. *Annu Rev Pathol* 3:399–425.
3. Dryja T, et al. (1990) A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. *Nature* 343:364–366.
4. Sung CH, et al. (1991) Rhdopsin mutations in autosomal dominant retinitis pigmentosa. *Proc Natl Acad Sci USA* 88:6481–6485.
5. O'Tousa JE, *et al.* (1985) The *Drosophila* ninaE gene encodes an opsin. *Cell* 40:877–882.
6. Zuker CS, Cowman AF, Rubin GM (1985) Isolation and structure of a rhodopsin gene from *D. melanogaster*. *Cell* 40:851–858.
7. Kurada P, O'Tousa JE (1995) Retinal degeneration caused by dominant rhodopsin mutations in *Drosophila*. *Neuron* 14:571–579.
8. Colley NJ, Cassill JA, Baker EK, Zuker CS (1995) Defective intracellular transport is the molecular basis of rhodopsin-dependent dominant retinal degeneration. *Proc Natl Acad Sci USA* 92:3070–3074.
9. Ryoo HD, Domingos PM, Kang MJ, Steller H (2007) Unfolded protein response in a *Drosophila* model for retinal degeneration. *EMBO J* 26:242–252.
10. Lin JH, et al. (2007) IRE1 signaling affects cell fate during the unfolded protein response. *Science* 318:944–949.
11. Mendes HF, van der Spuy J, Chapple JP, Cheetham ME (2005) Mechanism of cell death in rhodopsin retinitis pigmentosa: Implications for therapy. *Trends Mol Med* 11:177–185.
12. Vembar SS, Brodsky JL (2008) One step at a time: Endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol* 9:944–957.
13. Vashist S, Ng DT (2004) Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J Cell Biol* 165:41–52.
14. Carvalho P, Goder V, Rapoport TA (2006) Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* 126:361–373.
15. Denic V, Quan EM, Weissman JS (2006) A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. *Cell* 126:349–359.
16. Quan EM, et al. (2008) Defining the glycan destruction signal for endoplasmic reticulum-associated degradation. *Mol Cell* 32:870–877.
17. Shih HP, Hales KG, Pringle JR, Peifer M (2002) Identification of septin-interacting proteins and characterization of the Smt3/SUMO-conjugation system in *Drosophila*. *J Cell Sci* 115:1259–1271.
18. Friedlander R, Jarosch E, Urban J, Volkwein C, Sommer T (2000) A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nat Cell Biol* 2:379–384.
19. Neuber O, Jarosch E, Volkwein C, Walter J, Sommer T (2005) Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nat Cell Biol* 7:993–998.
20. Gauss R, Jarosch E, Sommer T, Hirsch C (2006) A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. *Nat Cell Biol* 8:849–854.
21. Leonard DS, Bowman VD, Ready DF, Pak WL (1992) Degeneration of photoreceptors in rhodopsin mutants of *Drosophila*. *J Neurobiol* 23:605–626.
22. Rajan RS, Kopito RR (2005) Suppression of wild-type rhodopsin maturation by mutants linked to autosomal dominant retinitis pigmentosa. *J Biol Chem* 280:1284–1291.
23. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101:249–258.
24. Yu SY, et al. (2002) A pathway of signals regulating effector and initiator caspases in the developing *Drosophila* eye. *Development* 129:3269–3278.
25. Igaki T, et al. (2000) Drob-1, a *Drosophila* member of the Bcl-2/CED-9 family that promotes cell death. *Proc Natl Acad Sci USA* 97:662–667.
26. Molinari M, Calanca V, Galli C, Lucca P, Paganetti P (2003) Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. *Science* 299:1397–1400.
27. Oda Y, Hosokawa N, Wada I, Nagata K (2003) EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. *Science* 299:1394–1397.
28. Clerc S, et al. (2009) Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum. *J Cell Biol* 184:159–172.
29. Oda Y, Okada T, Yoshida H, Kaufman RJ, Nagata K, Mori K (2006) Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. *J Cell Biol* 172:383–393.
30. Pichaud F, Desplan C (2001) A new visualization approach for identifying mutations that affect differentiation and organization of the *Drosophila* ommatidia. *Development* 128:815–826.
31. Ready DF, Ilanson TE, Benzer S (1976) Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev Biol* 53:217–240.
32. Okuda-Shimizu Y, Hendershot LM (2007) Characterization of an ERAD pathway for nonglycosylated BiP substrates, which require Herp. *Mol Cell* 28:544–554.
33. Hay BA, Wolff T, Rubin GM (1994) Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* 120:212–219.
34. Hosokawa N, et al. (2001) A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. *EMBO Rep* 2:415–422.
35. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415.