RACK1 modulates polyglutamine-induced neurodegeneration by promoting ERK degradation in Drosophila

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Abstract

Polyglutamine diseases are neurodegenerative diseases caused by the expansion of polyglutamine (polyQ) tracts within different proteins. Although multiple pathways have been found to modulate aggregation of the expanded polyQ proteins, the mechanisms by which polyQ tracts induced neuronal cell death remain unknown. We conducted a genome-wide genetic screen to identify genes that suppress polyQ-induced neurodegeneration when mutated. Loss of the scaffold protein RACK1 alleviated cell death associated with the expression of polyQ tracts alone, as well as in models of Machado-Joseph disease (MJD) and Huntington’s disease (HD), without affecting proteostasis of polyQ proteins. A genome-wide RNAi screen for modifiers of this rack1 suppression phenotype revealed that knockdown of the E3 ubiquitin ligase, POE (Purity of essence), further suppressed polyQ-induced cell death, resulting in nearly wild-type looking eyes. Biochemical analyses demonstrated that RACK1 interacts with POE and ERK, thereby promoting ERK degradation. These results suggest that RACK1 plays a key role in polyQ pathogenesis by promoting POE-dependent degradation of ERK, and implicate RACK1/POE/ERK as potent drug targets for treatment of polyQ diseases.

Author summary

Polyglutamine (polyQ) diseases are neurodegenerative diseases caused by the expansion of glutamine repeats in specific proteins. After >100 years of effort trying to decipher the pathogenesis of polyQ diseases, the mechanisms by which these mutant proteins induced neuronal death are still unknown. Here, we performed a genome-wide genetic screen in Drosophila for mutations that alleviate polyQ cytotoxicity. We found that a mutation in the gene rack1, which encodes a scaffold protein, suppressed polyQ-induced neurodegeneration when polyQ tracts were expressed, and in models of HD and MJD. Importantly, this effect was seen without clearance of the polyQ proteins. Further, we found that RACK1 binds both the E3 ubiquitin ligase POE and ERK, thereby promoting ERK degradation.
degradation. These data suggest that the RACK1/POE/ERK pathway is involved in pathogenesis of polyQ diseases, and identify RACK1/POE/ERK as potential therapeutic targets for treating polyQ diseases.

**Introduction**

A class of neurodegenerative disorders are cause by the expansion of CAG repeats within associated genes, resulting in polyglutamine (polyQ) insertions. There are currently nine types of polyQ diseases, including spinocerebellar ataxias (SCAs) and Huntington’s disease (HD) [1, 2]. One common feature of these polyQ diseases is the formation of polyQ oligomers and aggregates in the cytosol and nucleus [1, 3, 4]. In both human patients and animal models of these diseases, polyQ structures interrupt a variety of cellular functions, including transcriptional regulation, the endoplasmic reticulum (ER), and synaptic dynamics [5–7]. A number of factors were found to reduce the severity of polyQ diseases, primarily by reducing polyQ levels [7–11], but the mechanisms by which polyQ tracts lead to neural degeneration remain unknown.

The Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway plays an important role in cellular proliferation, differentiation, and survival, as well as tumorgenesis [12, 13]. Importantly, ERK signaling is inhibited in two models of polyQ diseases, namely spinocerebellar ataxia type 17 (SCA17) and HD [14–16]. Moreover, ERK activation can protect against cellular dysfunction in cells harboring a mutant form of the gene huntingtin (htt) [14, 17]. ERK, which is also called Mitogen-activated protein kinase (MAPK), is regulated by the classic Ras/Raf/MEK/ERK signaling cascade [18, 19], but steady-state levels of ERK can also be regulated via alterations in gene expression, protein subcellular localization, and protein degradation [19–21]. It has been shown that the deubiquitinationase, USP47 (Ubiquitin carboxyl-terminal hydrolase 47) stabilizes ERK by counteracting activities of the E3 ubiquitinases POE (Purity of essence), KCMF1 (Potassium channel modulatory factor 1), and UFD4 (Ubiquitin fusion-degradation 4-like) [22]. However, it is not known whether and how steady-state levels of ERK are controlled in physiological contexts such as polyQ diseases.

Receptor for activated C kinase (RACK1) belongs to the WD repeat family of proteins [23] and functions as a scaffold protein to regulate multiple cellular processes, including translation, immunity, apoptosis, and cancer progression [24–28]. In Arabidopsis thaliana, RACK1 is involved in regulating MAPK activity [29, 30]. In mammalian cells, RACK1 serves as an adaptor to help activate MAPK JNK (c-Jun N-terminal protein kinase) [31]. RACK1 has been shown to function downstream of p38b MAPK in helping to clear aggregates of polyubiquitylated proteins from thoracic muscles of aging flies, thereby promoting proteostasis [32]. RACK1 also reduces cellular toxicity associated with protein aggregates. When overexpressed, it localizes to polyQ aggregates and protects cells from polyQ-induced neurodegeneration [33, 34]. However, in these latter studies they overexpressed human RACK1 in fly models of MJD rather than the endogenous fly RACK1, and the suppression was weak. Moreover, the mechanisms by which RACK1 regulates polyQ toxicity have not been characterized, and it remains unknown whether MAPK signaling is involved in this process.

Here we established a model of polyQ disease in the Drosophila eye, and conducted a forward genetic screen to investigate the mechanisms involved in polyQ diseases pathogenesis. Strikingly, loss of RACK1 alleviated polyQ-induced cell death without affecting the formation or clearance of protein aggregates. We then performed a genome-wide RNAi screen to identify genes that could modify the polyQ/rack1RNAi phenotype. Knockdown of the E3 ligases, POE and KCMF1, further suppressed polyQ-induced cell death, resulting in nearly wildtype looking
eyes. By contrast, overexpression of POE abolished the suppressive effects of \textit{rack1} mutation. Further, RACK1 regulated ERK levels post-translationally, as expected, and ERK was indispensable for the role of RACK1 in polyQ-induced neurodegeneration. Finally, we found that RACK1 regulated ERK level by promoting the interaction between ERK and POE. As previous studies found that the ERK pathway is inhibited in polyQ disease models, we hypothesize that the RACK1/POE/ERK pathway plays an important role in polyQ disease pathogenesis, and that this pathway should be considered a target for treating polyQ diseases.

**Results**

**Mutating the gene\textit{rack1} alleviated polyQ-induced cellular toxicity**

Several polyQ disease models have been established in flies by expressing disease-associated proteins with expanded polyQ repeat or polyQ chains alone via the GAL4/UAS system [35–38]. However, to conduct a forward genetic screen to investigate the mechanisms involved in polyQ diseases pathogenesis, we established a simplified model of polyQ disease in the \textit{Drosophila} eye. In this model, we used the \textit{GMR} promoter to express 63 CAG repeats tagged with HA (63Q) in the fly eye. The result was loss of pigment and retinal cells immediately after eclosion (S1A and S1B Fig). When 63Q was expressed in third instar eye discs, it initially localized to the cytosol (in anterior cells), but over time gradually formed aggregates (in posterior cells). By the pupal stage, most 63Q signal was detected in aggregates (S1C Fig). By contrast, 31Q localized to the cytosol in both the third instar eye disc and the pupal eye (S1C Fig). We then subjected this model to EMS mutagenesis to screen for genetic modifiers of 63Q-induced photoreceptor cell death. Because genes involved in polyQ pathogenesis could be essential, we combined the polyQ model with the “\textit{ey-flp/hid}” system to generate flies in which the EMS-induced mutation was homozygous in the eyes [39], but heterozygous in the rest of the animal. Briefly, the \textit{ey-flp/hid} system combines \textit{GMR-hid} and the \textit{FLP/FRT} system. \textit{Ey-flp} induces mitotic recombination of mutated \textit{FRT} containing chromosome arms only in the compound eyes. \textit{GMR-hid} ensures that all eye cells are killed, except for those that are homozygous for your introduced mutation. This allows one to phenotypically screen homozygous loss-of-function mutations in the eye in the F1 generation. Using this strategy, we screened chromosomes 2L, 2R, 3L, and 3R for suppressors of 63Q-induced cell death (Figs 1A, S1D and S8A).

By screening ~100,000 flies for each chromosome arm, we identified ~30 alleles that strongly suppressed polyQ protein-induced cell death. We then excluded mutants that affected transcription mediated by the GMR promoter by checking if these mutants reduced GFP levels of \textit{GMR-GFP} flies. The remaining 14 alleles belonged to 4 complementation groups. One complementation group included a single allele, which was homozygous lethal and a strong suppressor of \textit{GMR-63Q} induced cell death. Using deficiency mapping and genomic DNA sequencing, we located this mutation to the \textit{rack1} genomic locus, which encodes the scaffold protein RACK1 (Receptor for activated C kinase 1). We therefore name this allele \textit{rack1}^s12. This allele contains an RNA splicing mutation at the donor (5’) site (GT to GA) between the second and third exon (Fig 1B), which disrupts RACK1 expression (S2A Fig). A previously reported null allele of \textit{rack1}, \textit{rack1}^1, also suppressed 63Q-induced cell death (Figs 1A and S8A). Moreover, expressing wild-type \textit{rack1} via the GMR promoter restored polyQ-induced cell death in \textit{rack1}^s12 mutants (Figs 1A and S8A). Finally, knocking down \textit{rack1} by RNAi suppressed 63Q-induced cell death, although to a lesser extent, whereas expressing \textit{rack1}^{\text{RNAi}} alone did not affect eye morphology (Figs 1A and S8A). These results confirmed that \textit{rack1} mutation alleviated polyQ protein-induced cell death.

To determine whether \textit{rack1} modulates specific polyQ disease models, we examined the effect of \textit{rack1} in models of Machado-Joseph disease (MJD) and Huntington’s disease (HD).
RACK1 regulates cell death in Polyglutamine Diseases

A

wild type  rack1ts2  rack1ts  GMRe>rack1ts
GMRe-63Q;  GMRe-63Q;  GMRe-63Q;  GMRe-63Q;  GMRe-63Q;  GMRe-63Q:
rack1ts2  rack1ts  rack1ts

GMR-63Q  GMR-63Q;  GMR-63Q;  GMR-63Q;  GMR-63Q;  GMR-63Q;  GMR-63Q:
rack1ts2  rack1ts  rack1ts

1 kb

rack1ts2
splicing: GT>GA

B

rack1

TAA ➔ ATG

C

Phosphoenolpyruvate

wild type  MJD-Q78  MJD-Q78 rack1ts

D

wild type  MJD-Q78  MJD-Q78 rack1ts

E

ERG amplitude (mV)

wild type  MJD-Q78  MJD-Q78 rack1ts

F

wild type  GMR>Het96Q  GMR>Het96Q rack1ts

G

wild type  elav>MJD-Q78  elav>MJD-Q78 rack1ts

survival (%)
Fig 1. Loss of rack1 specifically suppresses polyQ toxicity. (A) Light microscope images of eyes of 1-day-old (a) wild type, (a') GMR-63Q;ey-flp GMR-63Q-HA, (b) rack1G0975 (ey-flp;GMR-GAL4;UAS-rack1FRT40A/FRT40A), and (c) GMR-63Q;tr-Q78/+/UAS-rack1FRT40A/FRT40A. (B) Rack1 protein levels in 1-day-old wild type, MJD-Q78, and MJD-Q78;rack1G0975. (C) Quantification of average number of rhabdomeres per ommatidium in 1-day-old wild type, MJD-Q78, and MJD-Q78;rack1G0975. (D-E) ERG recordings (D) and quantification of ERG amplitudes (E) from wild type, MJD-Q78, and MJD-Q78;rack1G0975 at indicated days. (F-G) Lifespan of the wild type, MJD-Q78, and MJD-Q78;rack1G0975 flies. Scale bar: 50 μm.

(A) Light microscope images of eyes of 1-day-old (a) wild type, (a') GMR-63Q;ey-flp GMR-63Q-HA, (b) rack1G0975 (ey-flp;GMR-GAL4;UAS-rack1FRT40A/FRT40A), and (c) GMR-63Q;tr-Q78/+/UAS-rack1FRT40A/FRT40A. (B) Rack1 protein levels in 1-day-old wild type, MJD-Q78, and MJD-Q78;rack1G0975. (C) Quantification of average number of rhabdomeres per ommatidium in 1-day-old wild type, MJD-Q78, and MJD-Q78;rack1G0975. (D-E) ERG recordings (D) and quantification of ERG amplitudes (E) from wild type, MJD-Q78, and MJD-Q78;rack1G0975 at indicated days. (F-G) Lifespan of the wild type, MJD-Q78, and MJD-Q78;rack1G0975 flies. Scale bar: 50 μm.

Expression of a truncated form of Ataxin-3 with 78 CAG repeats (MJD-Q78) in the fly eye caused a dramatic loss of photoreceptor cells and rhabdomere structures, which are tightly-packed microvilli required for phototransduction [41]. Knocking down rack1 in this context decreased the amount of photoreceptor cells loss [40] (Figs 1C and S2B and S1 Table). To evaluate photoreceptor function, we performed electroretinography (ERG) to measure electrical responses to light stimulation. At day 1, flies expressing MJD-Q78 exhibited decreased amplitude and loss of on- and off-transients, indicating impaired phototransduction and synaptic transmission [42]. Knocking down rack1 partially rescued ERG amplitude and restored ERG transients (Fig 1D and 1E). Similarly, knocking down rack1 alleviated the neurodegeneration associated with mutated Huntington (Htt). Expressing GFP-tagged N-terminal Htt with 96 CAG repeats (Htt96Q) in the eye progressively reduced the number of photoreceptor cells and rhabdomeres. Knocking down rack1 suppressed this retinal degeneration. In 10-day-old flies, GMR>Htt96Q ommatidia generally had only one photoreceptor cell, whereas GMR>Htt96Q ommatidia with rack1RNAi expression had ~4 intact photoreceptor cells (Figs 1F and S2C).

To exclude the possibility that loss of rack1 suppressed polyQ-induced cell death only in photoreceptor neurons, we used elav-GAL4 to express MJD-Q78 in all neurons. This greatly shortened lifespan [40]. elav>MJD-Q78 flies survived longer when rack1 was knocked down, indicating that loss of RACK1 suppressed MJD-Q78-induced neurodegeneration (Fig 1G). We next asked whether loss of rack1 could suppress cell death in models of other neurodegenerative diseases. We modeled retinitis pigmentosa by expressing mutant rhodopsin (Rh1G69D) and tauopathy by expressing TauV337M [43, 44]. Importantly, knocking down rack1 did not affect Rh1G69D- or TauV337M-mediated retinal cell death (S2D Fig). Taken together, disruption of RACK1 specifically suppressed polyQ-induced neurodegeneration.

Loss of RACK1 suppressed polyQ toxicity without clearing protein aggregates

The formation of protein aggregates is a hallmark of polyQ diseases, and most suppressors of polyQ toxicity help to clear these aggregates [9–11]. We next tested whether loss of rack1...
affected the formation of polyQ aggregates. Using the mosaic system in pupal eyes, we found no difference in the levels of 63Q aggregates or total 63Q protein between \textit{rack1}s12 cells and controls (Fig 2A–2C). We further quantified Htt96Q monomer and aggregate levels by western blot, and found that Htt96Q monomers and aggregates were also not affected by \textit{rack1} knock-down (Fig 2D–2F). Finally, we found that Htt25Q levels were not affected by knocking down \textit{rack1} (S2E and S2F Fig). These results demonstrate that loss of \textit{rack1} did not suppress polyQ toxicity by clearing polyQ aggregates.

\textit{RACK1} is a scaffold protein that helps to assemble a variety of signaling molecules involved in multiple processes, including PKC activation, protein translation, and apoptosis [31, 32, 45–47]. To reveal the mechanism by which \textit{RACK1} affects 63Q cellular toxicity, we asked whether \textit{RACK1} functions through known pathways in this context. \textit{RACK1} promotes the release of...
eukaryotic translation initiation factor 6 (eIF6) from the 60s ribosome by recruiting PKC (protein kinase C) to eIF6 [45]. However, knocking down, knocking out, or overexpressing pck53e, which encodes the major fly PKC, failed to suppress 63Q-induced cell death (S3A Fig), indicating that the classic PKC pathways were not involved in suppressing polyQ toxicity in rack1 mutants. RACK1 also regulates ribosomal quality control by interacting with ZNF598 [46]. However, knocking down znf598 did not alleviate 63Q toxicity (S3A Fig). These results indicate that the loss of rack1 did not suppress polyQ toxicity by regulating translation.

RACK1 interacts with the p38/JNK pathway to regulate apoptosis [31, 32, 47]. However, knocking down or overexpressing JNK, P38a, or P38b did not affect 63Q toxicity or the ability of rack1RNAi to suppress polyQ toxicity (S3B Fig). Considering that apoptosis is downstream of JNK, we inhibited apoptosis by knocking down apoptosis-related proteins (rpr, hid, and grim) and caspases (dronc, dcp-1, and decay) or by over-expressing Inhibitor of apoptosis (DIAP1). This did not affect 63Q-induced cell death, either with or without rack1RNAi (S3C Fig). We also assessed the hypoxia pathway and immunity-related proteins, but did not see effects on 63Q toxicity. Therefore, we hypothesized that RACK1 suppresses polyQ-induced neurodegeneration via a novel pathway.

RACK1 genetically interacted with the E3 ligases POE and KCMF1

To determine the pathway through which rack1 modulated polyQ pathology, we conducted a genome-wide RNAi screen to identify genes that modified the polyQ/rack1RNAi phenotype. We expressed ~6,000 RNAi lines individually in compound eyes that also expressed rack1RNAi and 63Q. We identified more than 300 genes that restored 63Q toxicity when knocked down, but most also induced cell death when expressed alone or combined with 63Q, suggesting effects independent of RACK1. We also identified 14 RNAi lines that further suppressed 63Q-induced cell death in combination with rack1RNAi (Table 1). Among these 14 were two E3 ligases, POE (pushover or purity of essence) and KCMF1 (zinc-finger protein Potassium Channel Modulatory Factor 1), which each further suppressed polyQ-induced cell death in combination with rack1RNAi, but only slightly suppressed 63Q toxicity when knocked down alone. In contrast, no cell death was detected when poeRNAi or kcmf1RNAi were expressed alone (Figs 3A and 8B and S1 Table).

Previous studies have shown that POE and KCMF1, together with another E3 ligase UFD4 (Ubiquitin fusion-degradation 4-like), counteract the deubiquitinase USP47 (Ubiquitin Specific Peptidase 47) to destabilize ERK [22]. However, knocking down ufd4 did not affect 63Q toxicity. This may be because UFD4 had a much weaker effect on ERK levels compared with POE or KCMF1 (Figs 3A and 8B and S1 Table). We then overexpressed USP47 in the compound eyes of GMR-63Q flies and found that USP47 suppressed polyQ-induced cell death. Moreover, the suppression of 63Q toxicity mediated by rack1 knock down was greatly enhanced by overexpressing USP47 (Figs 3A and 8B). This indicated that rack1 loss suppressed polyQ toxicity through pathways involving POE/KCMF1/USP47. Given that rack1 mutants suppressed polyQ-induced cell death independent of aggregate clearance, we next asked whether knocking down poe/kcmf1 or over-expressing USP47 reduced polyQ aggregates. We generated clones of cells expressing poeRNAi, kcmf1RNAi, or USP47 in 63Q-expressing eyes with the “flip-out” method [48]. We found that knocking down poe/kcmf1 or over-expressing USP47 did not affect 63Q aggregate formation, whereas over-expressing the chaperone, DnaJ, significantly reduced polyQ aggregates (S4A and S4B Fig) [36, 49].

We further confirmed the genetic interaction between RACK1 and POE/KCMF1/USP47 in the MJD model. As seen with 63Q, knocking down poe or kcmf1 (or overexpressing USP47) alleviated MJD-Q78-induced photoreceptor cells loss. Knocking down ufd4 had no effect (Fig
Moreover, we found that either knocking down poe or kcmf1 or overexpressing USP47 in the rack1RNAi background largely promoted MJD-Q78 photoreceptor cell survival compared with the expression of rack1RNAi, poeRNAi, kcmf1RNAi, or USP47 alone (Fig 3B and 3C). We further evaluated photoreceptor function via ERG recordings. Consistent with the degeneration results, knocking down poe or kcmf1 (or overexpressing USP47) increased ERG amplitude for MJD-Q78 flies, whereas co-expression of rack1RNAi with poeRNAi, kcmf1RNAi, or USP47 completely rescued ERG amplitude and transients for MJD-Q78 flies (Fig 4A–4C). These results suggest that rack1 alleviated polyQ toxicity through the POE/KCMF1 ubiquitin ligase systems.

rack1 mutations suppressed polyQ toxicity through ERK

Since ERK was the substrate of the POE/KCMF1 E3 ubiquitin ligase, we speculated that loss of rack1 suppresses polyQ toxicity by regulating levels of ERK. We first measured ERK levels in adult flies and found that overexpressing USP47 or knocking down rack1 increased ERK levels by two-fold (Fig 5A and 5B). We then used the “flip-out” system to express rack1RNAi in GFP-positive cells in eye imaginal discs. Comparing ERK levels between rack1RNAi cells and neighboring wild-type cells, we further confirmed that down-regulating rack1 increased ERK levels (Fig 5C and 5D). By contrast, rack1RNAi and USP47 overexpression did not impact erk mRNA levels (S6A Fig). Moreover, we found that knocking down rack1 also increased ERK levels when 63Q was expressed. Similar results were seen when poe or kcmf1 was knocked down, but

| #TH     | Gene name | 63Q>GMR>rack1RNAI | 63Q>GMR-GAL4 | Function                                                                 |
|---------|-----------|-------------------|--------------|--------------------------------------------------------------------------|
| THU0953 | kcmf1     | Yes               | Yes          | ubiquitin-dependent protein catabolic process; regulating ERK level       |
| THU1137 | poe       | Yes               | Yes          | ubiquitin-dependent protein catabolic process; regulating ERK level       |
| THU1058 | vcp       | Yes               | Yes          | AAA-ATPase; regulation of programmed cell death                          |
| TH01734.N| herc4     | Yes               | Yes          | HECT domain E3 ligase; host defense                                      |
| THU0683 | hsc70-4   | Yes               | Yes          | heat shock protein binding, protein folding                              |
| THU1190 | hsc70-2   | Yes               | Yes          | heat shock protein binding, protein folding                              |
|         | hsp70Ba   |                   |              |                                                                          |
|         | hsp70Bb   |                   |              |                                                                          |
|         | hsp70Bbb  |                   |              |                                                                          |
|         | hsp70Bc   |                   |              |                                                                          |
| THU1287 | hsp70Ba   | Yes               | Yes          | heat shock protein binding, protein folding                              |
|         | hsp70B    |                   |              |                                                                          |
|         | hsp70Bbb  |                   |              |                                                                          |
|         | hsp70Bc   |                   |              |                                                                          |
| TH03673.N| CG15739   | Yes               | Yes          | dephosphorylation                                                        |
| TH02741.N| xit       | Yes               | Yes          | germ-band extension; protein N-linked glycosylation                      |
| TH01799.N| Gnpnat    | Yes               | Yes          | UDP-N-acetylglosamine biosynthetic process                                |
| TH01611.N| lin       | Yes               | Yes          | developmental patterning and cell fate specification                     |
| TH04829.N| βTub56D   | Yes               | Yes          | microtubule-based process; muscle attachment                              |
| TH01654.N| vermilion | Yes               | Yes          | protein homotetramerization; tryptophan catabolic process to kynurenine  |
| TH01278.N2| CG15084   | Yes               | Yes          | unknown                                                                  |

a UAS-RNAi lines were crossed with 63Q>GMR>rack1RNAi to identify genes that modified the polyQ/rack1RNAi phenotype. “yes” indicates genes that when knocked-down enhanced the ability of rack1RNAi to suppress polyQ-induced cell death.

b UAS-RNAi lines were crossed with 63Q>GMR-GAL4 to identify those that suppressed polyQ-induced cell death. “yes” indicates genes that when knocked-down suppressed polyQ-induced cell death.

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not ufd4 (S5A and S5B Fig). Knocking down rack1 also did not change the rate of erk translation, as measured by polysomal loading of erk mRNA transcripts (S6B Fig). We also found that knocking down rack1 in S2 cells decreased ERK ubiquitination level (Fig 6A and 6B). Finally, knocking down rack1 using engrailed-GAL4 (en-GAL4) induced extra wing vein material within the posterior compartment, a phenotype consistent with erk activation (Fig 6C) [50]. Thus, RACK1 negatively regulated ERK protein levels post-translationally.

Consistent with the up-regulation of ERK by loss of rack1, overexpressing erk suppressed both 63Q- and MJD-Q78-associated toxicities (Figs 6D, 6E and S8C). To further prove that the suppression of polyQ toxicity by rack1 loss depended on ERK, we knocked down erk using erkRNAi and found that erkRNAi prevented rack1RNAi from suppressing both 63Q- and MJD-Q78-induced cell death (Figs 6D, 6E and S8C). Finally, we found that the knock down or overexpression of erk did not affect polyQ levels (S4A and S4B Fig). We also assessed ERK levels in polyQ disease models and found that expressing 63Q or MJD-Q87 did not affect ERK levels (S6C and S6D Fig). These results strongly indicate that the ERK pathway is involved in rack1-mediated suppression of polyQ toxicity.

**RACK1 functioned as an adaptor protein to promote the degradation of ERK by POE**

Because RACK1 is a scaffold protein and regulated ERK levels post-translationally, we hypothesized that RACK1 may recruit E3 ligases to ERK to promote its degradation. To test this hypothesis, we expressed GFP-tagged RACK1 and FLAG-tagged ERK in S2 cells, and found that RACK1 co-immunoprecipitated with ERK via multiple binding sites. The interaction between RACK1 and ERK depended on RACK1, since knocking down rack1 RNAi abolished the interaction between ERK and the E3 ligase POE, and that mutations in rack1 disrupted POE-dependent ERK degradation. These increased levels of ERK then suppressed polyQ-associated neurodegeneration.
Fig 4. POE and KCMF1 enhanced rack1RNAi restoration of photoreceptor functions in MJD model. (A) ERG recordings from 1-day-old MJD-Q78 (GMR-GAL4/+;UAS-MJD,tr-Q78/+), MJD-Q78 rack1RNAi (GMR-GAL4 UAS-rack1KK109073;UAS-MJD,tr-Q78/+), MJD-Q78 poeRNAi (GMR-GAL4/+;UAS-MJD,tr-Q78/UAS-poeHMS00739), MJD-Q78 kcmf1RNAi (GMR-GAL4/+;UAS-MJD,tr-Q78/UAS-kcmf1HMS00511), MJD-Q78 ufd4RNAi (GMR-GAL4/+;UAS-MJD,tr-Q78/UAS-ufd4HMS00693), MJD-Q78 USP47 RNAi (GMR-GAL4/+;UAS-MJD,tr-Q78/UAS-USP47HMS01557).

(B) ERG recordings from wild type, MJD-Q78 rack1RNAi, MJD-Q78 poeRNAi, MJD-Q78 kcmf1RNAi, MJD-Q78 ufd4RNAi, and MJD-Q78 USP47 RNAi.

(C) Quantification of ERG amplitude (mV) for wild type, GFP, poeRNAi, kcmf1RNAi, ufd4RNAi, and USP47 RNAi. Significant differences are indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
The fact that polyQ repeats aggregate and are toxic by themselves suggest that they are the critical driver of neurodegenerative disease pathogenesis, rather than the proteins they are.
associated with in different diseases. High-throughput screens have been performed in multiple systems to identify factors that modify polyQ aggregation [52–54], with most of these modifiers affecting the accumulation or the physical properties of polyQ aggregates [55]. In this study, we generated a simple model of polyQ disease by expressing 63 CAG repeats in the fly eye and conducted a genome-wide, loss-of-function screen to identify suppressors of polyQ-induced cell death. We found that a loss-of-function mutation in rack1 alleviated the cytotoxicity of polyQ proteins. Importantly, loss of rack1 did not affect the proteostatic control of polyQ proteins, indicating that RACK1 instead affected cytotoxicity downstream of aggregate formation. Chaperones, including HSP40/DNAJ, protect cells in the context of different polyQ disease models, again indicating that the toxicity of polyQ proteins is derived specifically from the polyQ tracts, not from the unique features of the pathogenic proteins [10, 36, 38, 56]. However, since chaperones reduce levels of both the polyQ tracts and the associated protein, this result is not definitive proof that the polyQ tracts are the primary culprit in mediated neuronal cell death. It has also been proposed that unique features of polyQ proteins also modulate pathogenesis of special polyQ diseases [57, 58]. Our results demonstrate that mutating rack1 prevented neurodegeneration in a general model of polyQ disease, as well as two models of specific polyQ diseases, namely MJD and HD. This indicates that different polyQ diseases share common pathogenic features.

RACK1 is a seven WD repeat domain protein, and provides a platform for protein-protein interactions [23]. RACK1 helps to regulate stress responses and apoptosis through scaffolding signaling proteins such as PKC, ZNF598, and JNK [31, 45, 46]. However, all these signaling pathways are not required for RACK1 function in the context of polyQ pathogenesis. Here, we demonstrate that RACK1 physically interacted with ERK and negatively regulated ERK levels post-translationally. We provide further genetic evidence that the suppression of polyQ toxicity by rack1 mutation depended on ERK stabilization. Knocking down erk abolished the suppressing effects of rack1 on polyQ-induced neurodegeneration. Therefore, we present both biochemical and genetic evidence that loss of RACK1 suppressed polyQ-induced cytotoxicity by stabilizing ERK.

POE is an N-terminal UBR box E3 ubiquitin ligase, and regulates ERK stability together with another E3 ligase, KCMF1 [22]. In a genome-wide RNAi screen for modifiers of the rack1 phenotype, we found that knockdown of POE or KCMF1 further suppressed polyQ toxicity when expressed with rack1RNAi. In contrast, overexpressing their counterpart, the deubiquitinating enzyme USP47, suppressed polyQ-induced cell death, and this suppression was improved in combination with rack1 mutation. It has been suggested that ERK is a substrate for POE, and indeed we found that ERK interacted with POE. Further, both POE and ERK bound RACK1, and interaction between POE and ERK was disrupted by knocking down rack1, suggesting that the interaction between POE and ERK depends on their ability to bind RACK1.

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Fig 7. RACK1 scaffolds POE and ERK. (A-C) RACK1 specifically interacts with ERK. FLAG-tagged ERK (A), HA-tagged KCMF1 (B) or Myc-tagged UFD4 (C) was co-expressed in S2 cells with GFP or RACK1-GFP, followed by immunoprecipitation with anti-GFP antibody. (D) Different mCherry-tagged portions
Furthermore, rack1 mutation did not suppress polyQ-induced cell death when POE was overexpressed. In contrast, KCMF1 did not interact with RACK1, and, consistent with this, overexpressing kcmf1 did not reverse the suppression effects of rack1 mutants on polyQ cytotoxicity. This suggests that KCMF1 may not be the key RACK1 cofactor. Instead, our data indicate that RACK1 plays an essential role in promoting the degradation of ERK by bringing together ERK and the E3 ligase, POE. These results further validate our finding that overexpressing rack1 does not enhance polyQ toxicity, since excessive scaffolding proteins may not enhance the interaction between POE and ERK.

The ERK pathway is involved in promoting cell survival and the clearance of protein aggregates in some neurodegenerative diseases. While ERK signaling is upregulated in HD cell lines and a mouse model of SCA-17 [14, 17], studies in a fly model of HD and patient fibroblasts indicate that polyQ proteins inhibit ERK signaling [16, 59]. Despite the fact that ERK regulation by polyQ is controversial, a number of studies agree that activating the ERK signaling pathway protects against polyQ toxicity [14, 16, 17, 59]. Further studies utilizing the SCA1 model suggest that ERK may help prevent polyQ aggregation [60]. Consistent with the role of ERK in cell survival, we found that ERK signaling protected against polyQ toxicity, both in models of general polyQ disease and models of specific polyQ diseases, namely MJD and HD. This suggests that ERK protects against polyQ disease pathogenesis. Moreover, upregulating levels of ERK protein through the genetic manipulation of POE, USP47, or RACK1 prevented polyQ-induced cell death in MJD and HD models. Although the mechanisms by which ERK signaling regulates polyQ toxicity remain unknown, our results suggest that ERK suppresses polyQ-induced cell death downstream of aggregate formation. Moreover, ERK levels was not reduced in polyQ-expressing cells, indicating that inhibition of ERK pathway polyQ is not directly involved in the pathogenesis of polyQ toxicity.

Because the abnormal expansion of polyQ repeats plays a pivotal role in pathogenesis of polyQ diseases, clearing polyQ aggregates via molecular chaperones has been extensively studied as a strategy for treating polyQ diseases. However, these types of treatment regimens could impair general cellular proteostasis, as many polyQ containing proteins play essential roles. Here we show that the RACK1/POE/ERK signaling pathway does not clear polyQ protein aggregates, but instead specifically ameliorates late pathogenic events associated with polyQ diseases. Our data thus indicate that the RACK1/POE/ERK pathway is a potential therapeutic target for treating polyQ diseases.

Methods and materials
Fly stocks
The following fly strains were obtained from the Bloomington Stock Center: (1) GMR-GAL4 [61], (2) elav-GAL4 [62], (3) UAS-MJD.tr-Q78 [40], (4) UAS-Htt9Q-eGFP [41], (5) UAS-GFP, (6) UAS-mdCD8-GFP [63], (7) UAS-p38b [64], (8) UAS-JNK [65], (9) GMR-DIAP1 [66], (10) GMR-TauV337M [44], (11) Df(3L)H99, (12) P[Trip.HMS01779]attP2 (dcp-1RNAi), (13) P[Trip. HMC06381]attP40 (znf598RNAi), (14) P[Trip. HMS01224]attP2 (p38aRNAi), (15) M(3xP3-RFP.attP)ZH-2A,M(3xP3-RFP.attP)ZH-86Fb, (16) M(3xP3-RFP.attP)ZH-2A,M(3xP3-RFP.attP)ZH-86Fb.
51C, (17) w^{118}, (18) rack1^{1.8} FRT40A/CyO. The following fly RNAi strains were obtain from the Tsinghua Fly Center: (1) P[TRiP. HMS00739]attP2 (poeRNAi), (2) P[TRiP. HMS00511] attP2 (kcmf1RNAi), (3) P[TRiP. Jf01366]attP2 (erkRNAi), (4) P[TRiP. SH05382.N]attP40 (rprRNAi), (5) P[TRiP. SH05271.N]attP2 (decayRNAi), (6) P[TRiP. Jf02641]attP2 (pkc53eRNAi), (7) P[TRiP. GL00140]attP2 (p38bRNAi), (8) P[TRiP. SH05212.N]attP2 (jnkRNAi), (9) P[TRiP. HMS00758]attP2 (droncRNAi). The P[KK109073]VIE-260B (rack1RNAi) and P[GD12135] v27858 (rack1RNAi) flies were obtained from Vienna Drosophila Resource Center [67]. The knock-down efficiency of RNAi lines were verified by qPCR (S1 Table). UAS-p38a and UAS-kcmf1 were obtained from the flyORF collection of the Zurich ORFeome Project [68]. The following flies were maintained in the laboratory of T. Wang: (1) UAS-dnal-1, (2) hs-flp; UAS-Dcr-2;actin->CD2->GAL4 UAS-GFP, (3) GMR>hs-flp;UAS-dnaJ-1, (4) hs-flp:ubi-GFP FRT40A, and (5) ey-flp ninaE-Rh1-GFP;GMR-hid CL FRT40A /CyO. For animal studies, no randomization and no blinding were used.

**Generation of plasmid constructs**

The rack1, erk, and kcmf1 cDNAs were amplified from the cDNA clones RE74715, RE08694, and LP17815, respectively, which were obtained from the Drosophila Genomic Resource Center (DGRC). The ufd4 and truncated poe cDNAs were reverse transcribed and amplified from total RNA extracted from w^{118} flies. To construct pIB-rack1-GFP, the entire coding sequence of rack1 was subcloned into the pIB-cGFP vector between the EcoRI and NotI sites. To construct pIB-erk-FLAG, the 3XFLAG tag was subcloned into the pIB vector between the BamHI and XbaI sites, followed by subcloning erk cDNA between the EcoRI and NotI sites. To construct pIB-poe.tr-mCherry, the mCherry tag was subcloned into the pIB vector between the BamHI and XbaI sites, and the specific truncated coding sequences of poe were subcloned into the pIB-mCherry vector between the EcoRI and NotI sites. To construct pIB-kcmf1-HA, the 3XHA tag was subcloned into the pIB vector between the BamHI and XbaI sites, followed by subcloning the entire coding sequence of kcmf1 between the EcoRI and NotI sites. To construct pIB-ufd4-Myc, the Myc tag was subcloned into the pIB vector between the BamHI and XbaI sites, and the entire coding sequence of ufd4 were subcloned into pIB-Myc vector between the EcoRI and NotI sites.

**Generation of transgenic flies**

To generate the pGMR-63Q-HA construct, a HA tag was added to the C-terminus of the 63Q cDNA and subsequently subcloned into the pGMR vector [69]. The construct was injected into w^{118} embryos, and transgenic insertions on the X-chromosome were maintained. To generate GMR-63Q-Myc flies, the 63Q cDNA with a C-terminal Myc tagged was subcloned into the pGMR-attB vector, and the construct was injected into M(3xP3-RFP.attP)ZH-51C embryos. To generate GMR-rack1 flies, rack1 cDNA was subcloned into the pGMR-attB vector, and the construct was injected into M(3xP3-RFP.attP)ZH-51C embryos. To generate GMR-erk flies, the cDNA was amplified from the cDNA clone LD26783 from DGRC and subcloned into the pUAST-attB vector. The construct was injected into M(3xP3-RFP.attP)ZH-86Fb embryos. To generate GMR-poe.tr-mCherry, the mCherry tag was subcloned into the pIB vector between the BamHI and XbaI sites, and the specific truncated coding sequences of poe were subcloned into the pIB-mCherry vector between the EcoRI and NotI sites. To construct pIB-kcmf1-HA, the 3XHA tag was subcloned into the pIB vector between the BamHI and XbaI sites, followed by subcloning the entire coding sequence of kcmf1 between the EcoRI and NotI sites. To construct pIB-ufd4-Myc, the Myc tag was subcloned into the pIB vector between the BamHI and XbaI sites, and the entire coding sequence of ufd4 were subcloned into pIB-Myc vector between the EcoRI and NotI sites.
The \( ufd4 \)RNAi line was generated as described [70]. Briefly, a designed two 23nt short hairpin RNA sequences (GCTGTGCTGCTAGATATTTGT) targeting the coding region of \( ufd4 \) was cloned into a VALIUM20 vector. The plasmids were then injected into \( M\{vas\cdotint.Dm\}ZH-2A;M\{3xP3-RFP.attP\}ZH-86Fb \) embryos, and transformants were identified on the basis of eye color.

\( poe \) was overexpressed using the flySAM system [51]. Briefly, one sgRNA (TGGCTCCAGCTTGACGTCGG) targeting 350 base pairs upstream of the transcription starting site of \( poe \) was cloned into the flySAM vector. Plasmids were then injected into \( M\{vas\cdotint.Dm\}ZH-2A;M\{3xP3-RFP.attP\}ZH-86Fb \) embryos, and transformants were identified on the basis of eye color, and further crossed with \( GMR\cdotGAL4 \) flies to overexpress \( poe \).

**Generation of \( pkc53e \) mutant flies**

The \( pkc53e^{ko} \) mutations were generated using the Cas9/sgRNA system [68]. Briefly, a pair of guide RNAs targeting the \( PKC53E \) locus were designed (sgRNA1: GCGGATGCGATCACACGGAG, sgRNA2: ACCATCGCAAAAGAAGGCGGA) and cloned into the \( U6b\cdotsgRNA\cdotshort \) vector. Plasmids were injected into the embryos of \( nos\cdotCas9 \) flies, and deletions were identified by PCR using the following primers: forward primer 5’-CGAGTGCTATGTTCCACTTC-3’ and reverse primer 5’-TCGTCATTGGAACGAGATGTAATT-3’.

**Objective criteria for scoring retinal phenotypes**

All genotypes presented here exhibited highly uniform retinal phenotypes. We examined eye phenotypes of \( \geq 100 \) flies per genotype, and phenotypes shown in the images (obtained via light microscopy) are present in 100% of the animals. For genetically identical flies, there was no appreciable phenotypic variability. In cases where enhancement or suppression is reported, this phenotype was present in 100% of the animals. Nevertheless, to apply quantitative analyses, we scored the severity of eye phenotypes, as previously reported [71].

**EMS screening**

The EMS screening were performed as described [72]. Briefly, the second chromosome of \( FRT40A \) or \( FRT42D \) flies and the third chromosome of \( FRT2A \) or \( FRT82B \) flies were isogenized, and young male flies were fed 25 mM EMS (Sigma, St. Louis, MO) in 2% sucrose for 8 h. Flies were then mated immediately to \( ey\cdotflp,GMR\cdot63Q\cdotHA;GMR\cdothid CL FRT40A/CyO, ey\cdotflp,GMR\cdot63Q\cdotHA;GMR\cdothid CL FRT42D/CyO, ey\cdotflp,GMR\cdot63Q\cdotHA;GMR\cdothid CL FRT2A/TM3 \) and \( ey\cdotflp,GMR\cdot63Q\cdotHA;GMR\cdothid CL FRT82B/TM3 \) flies. F1 progenies were screened by suppression of pigment loss, and approximately 100,000 F1 flies were screened for each chromosome arm (S1D Fig).

**Electroretinogram recordings**

The electroretinogram experiments were performed as described [69]. Briefly, two glass micro-electrodes were filled with Ringer’s solution; one was placed on the surface of the compound eye, and one was placed on the thorax. The light source intensity was \( \approx 2000 \text{ lux} \), and the light color was orange (source light was filtered using a FSR-OG550 filter). ERG signals were amplified with a Warner electrometer IE-210, and recorded with a MacLab/4 s A/D converter and the clampex 10.2 program (Warner Instruments, Hamden, USA).

**Transmission electron microscopy (TEM) and retinal degeneration analysis**

Transmission electron microscopy was performed as described [73]. Briefly, adult fly heads were dissected, fixed, dehydrated, and embedded in LR White resin. Thin sections (80 nm)
were prepared at a depth of 30–40 μm, then stained with uranyl acetate and lead citrate (Sigma, St. Louis, MO) and examined using a JEOL JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, JAPAN). For retinal degeneration analysis, flies were raised at 25˚C in a 12 h light/12 h dark cycle, and collected at indicated ages. More than 20 ommatidia were counted for each eye section, and more than three flies per genotype were scored.

**Lifespan assay**

Lifespan assay were performed as described [74]. Briefly, ~200 newly enclosed female flies of each genotype were collected and placed in groups of 20 individuals. Flies were kept at 25˚C with 65% humidity under 12 h light/12 h dark cycles. Flies were transferred to new vials every 2 days, and the number of dead flies was counted every day.

**Immunofluorescence analysis**

Immunofluorescence labeling was performed as described [39]. Eye imaginal discs or pupa eyes were dissected in PBS solution (pH 6.8) and fixed in 4% paraformaldehyde in PBS buffer for 30 min. Eye discs or pupa eyes were incubated with diluted antibodies targeting GFP (rabbit, 1:200, Invitrogen), HA (rat, 1:200, Roche), ERK (rabbit, 1:200, Cell Signaling Technology), and Myc (rabbit, 1:200, Sigma). Anti-rabbit and rat IgG antibodies labeled with Alexa Fluor 488, 568, or 647 (1:500, Invitrogen) were used as secondary antibodies. Images were captured with a Nikon A1-R confocal microscope (Nikon, Tokyo, Japan). Acquired images were processed using Photoshop CC 2017 and NIS-Elements AR Analysis 5.20.00.

**Western blot analysis**

Western blotting was performed as described [39]. Briefly, 20 fly eyes were dissected and homogenized in SDS sample buffer with a Pellet Pestle (Kimble/Kontes). The proteins were fractionated by SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore) in Tris-glycine buffer. The blots were probed with Mouse Tubulin primary antibodies (1:2000 dilution, Developmental Studies Hybridoma Bank), Rabbit ERK (1:1000, Cell Signaling Technology), Mouse β-Actin primary antibodies (1:1000 dilution, Santa Cruz Biotechnology) and Rabbit RACK1 antibodies (1:1000 dilution, Dr. J. Kadrmas lab) followed by IRDye 680 goat anti-Rabbit IgG (LI-COR) and IRDye 800 goat anti-Mouse IgG (LI-COR) as the secondary antibodies. The signals were detected with the Odyssey infrared imaging system (LI-COR).

**Analysis of polyQ aggregates**

For mosaic experiments, GMR-63Q-HA;rack1st12 FRT40A/CyO flies or GMR-63Q-HA;FRT40A flies were crossed with hs-flp;ubi-GFP FRT40A flies, and heat shocked at the 1st instar larva stage at 37˚C for 1 hour to induce mosaic clone. Pupa eyes were dissected at 36–40 h APF (after pupa formation), and labeled using antibodies against HA (1:200, Roche). For calculation, GFP-positive cells are control cells, and GFP negative cells are mutant cells. For flip-out experiments, flies overexpressing (UAS lines) or knocking down (RNAi lines) specific genes were crossed with hs-flp;GMR-63Q-Myc;act >> CD2 >> GALA UAS-GFP/SM-TM flies, and pupa eyes were dissected at 36–40 h APF, followed by labeling using anti-Myc antibodies (1:200, Sigma). For one pupa eye slide, GFP-positive cells are RNAi or overexpressing cells, and GFP negative cells are control cells. For each slide, five non-overlapping areas from GFP negative cells and GFP positive cells were randomly picked, and both the intensity and quantity of aggregates were counted by NIS-Elements AR Analysis 5.20.00 software.
Cell culture, dsRNA synthesis, and immunoprecipitation

S2 cells were grown at 26˚C in Schneider’s Drosophila medium (Sigma-Aldrich) with 10% fetal bovine serum (Gibco BRL), dsRNA was synthesized in vitro and transfected as reported [75]. Plasmids were transfected using Vigofect reagent (Vigorous Biotechnology), and cells were collected and lysed with 10 mM Tris-HCl lysis buffer (pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 25 mM NaF, and 1 mM Na3VO4 with 1× proteinase inhibitor cocktail [Sigma-Aldrich]). S2 cells were pre-treated with dsRNA against GFP or rack1 for four days, and then transfected with plasmids for two days. To inhibit proteasome activity, cells were treated with 5 μM MG132 for two days. Immunoprecipitations were performed with mCherry beads (Chromotek) and GFP beads (Chromotek). The bound proteins were analyzed by western blotting against Rabbit GFP antibodies (1:1000 dilution, Torrey Pines Biolabs), Mouse FLAG antibodies (1:2000 dilution, Sigma), Rat HA antibodies (1:1000 dilution, Roche), Rabbit Myc antibodies (1:1000 dilution, Biovision).

Quantitative RT-PCR

Quantitative RT-PCR was performed as described [76]. Total RNA was prepared with Trizol reagent (Invitrogen) from dissected fly eyes, and was treated with TURBO DNase (ThermoFisher). The cDNA was synthesized with RT master mix (RR036A-1; Takara). An iQ SYBR green supermix (Bio-Rad) was used for real-time PCR, and results were analyzed with a CFX96 Real-Time PCR Detection System (Bio-Rad).

The following primers were used for this study: ERK: forward, 5'-TGCACATCCCTATTTAGAGCAA-3'; reverse, 5'-AATGCGACTTCAGGCACAGCC-3'; RpI32: forward, 5'-GCCGCCTTCAAGGACAGTATCTG-3'; reverse, 5'-AAACCGGCTTCTGACATGAG-3'; RACK1: forward, 5'-TCAGGTGGCAAGGACTCCAAG-3'; reverse, 5'-GCACAGGGCTTGATGATGTCGTT-3'; POE: forward, 5'-TCAGCAAGCTACATGACTAGA-3'; reverse, 5'-TCGGGAATTGATTTGTG-3'; UFD4: forward, 5'-AATTACCAGGGACCGTAGAGG-3'; reverse, 5'-TTCCGGTCTCCATAGGAT-3'.

Polysome fractionation

Polysome fractionation was performed as described [77]. S2 cells were cultured in dsRNA for four days and then treated with cycloheximide (CHX) at 0.1 mg/ml for 30 min. For each RNAi, 10⁸ cells were pelleted and washed once with cold PBS containing 0.1 mg/ml CHX. The cells were then lysed in polysome lysis buffer (PLB; 20 mM Tris-Cl, pH 7.5, 250 mM KCl, 10 mM MgCl₂, 1% Triton X-100, 1 mM DTT, 0.1 mg/ml CHX, 1X proteinase inhibitor cocktail (Sigma-Aldrich), and 1 mM PMSF). The lysates were centrifuged at 12,000 x g for 10 min at 4˚C and 1/10 of the supernatant was removed as input sample. The remainder of each supernatant was loaded on a 20%–50% sucrose gradient prepared in PLB (without Triton X-100) and resolved by centrifugation in an SW41 rotor (Beckman) at 38,000 rpm for 3 h at 4˚C. Fractions were collected while monitoring the absorbance at 254 nm. RNA was prepared from each fraction and assayed by quantitative RT-PCR.

Quantification and statistical analysis

Statistical details for each experiment, including n numbers and the statistical test performed, can be found in the corresponding figure legend. Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism 6 software. Student’s t test
was used for datasets with a normal distribution and a single intervention. One-way ANOVA was performed with Tukey’s post hoc test for multiple comparisons, and two-way ANOVA with Sidak’s post hoc test was used to compare ribosome loading of erk mRNA. $p < 0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supporting information

S1 Fig. Genetic screen for suppressors of general polyQ model in fly eyes. (A) Light microscope images of eyes of 1-day-old wild-type and GMR-63Q (ey-flp GMR-63Q-HA) flies. Scale bar: 50 μm. (B) Transmission electron microscopy (TEM) images of 1-day-old flies from wild-type and GMR-63Q eyes. Scale bar: 2 μm. (C) Eye imaginal discs (left panels) and pupa eye (right panels) of GMR-63Q and GMR-31Q flies were stained against anti-HA antibody and anti-Myc antibody, respectively. A: anterior area, P: posterior area. Scale bar: 10 μm. (D) EMS screening strategy to identify suppressors of 63Q induced cell death. Take the screening of the second chromosome as an example. The second chromosome of flies and hs-hid63Q-HA;FRT42D GMR-hid CL/CyO hs-hidFRT2A and ey-flp;FRT2A and ey-flp;FRT82B flies were mutagenized, followed by crossing with ey-flp GMR-63Q-HA;FRT42D GMR-hid CL /CyO hs-hid, ey-flp GMR-63Q-HA;GMR-hid CL ey-flp GMR-63Q-HA;FRT82B GMR-hid CL/TM3 hs-hid flies and ey-flp GMR-63Q-HA;FRT82B GMR-hid CL/TM3 hs-hid flies, respectively. (TIF)

S2 Fig. The rack1 mutants specifically suppress polyQ induced cell death. (A) Western blot analysis of proteins extracted from wild type and rack1142 (rack1142 FRT40A/GMR-hid CL FRT40A) fly eyes with antibody against RACK1. β-Actin was used as loading control. (B) TEM images of 1-day-old flies from wild type, MJD-Q78 (GMR-GAL4/+;UAS-MJD.tr-Q78/+), and MJD-Q78 rack1RNAi (GMR-GAL4/+;UAS-MJD.tr-Q78 /UAS-rack1GD12135) eyes. Scale bar: 2 μm. (C) TEM images of wild type, GMR>Htt96Q (GMR-GAL4/+;UAS-Htt96Q-eGFP/+), and GMR>Htt96Q, rack1RNAi (GMR-GAL4/+;UAS-Htt96Q-eGFP/UAS-rack1GD12135) eye imaginal discs (left panels) and pupa eye (right panels) of GMR-63Q;GMR-GAL4/+ (middle panels), or GMR-AL4 (bottom panels). Scale bar: 50 μm. (E) Western blot analysis of Htt25Q proteins extracted from fly eyes with antibody against GFP. β-Actin was used as a loading control. (F) Fold-change of Htt25Q level in GMR>Htt25Q, rack1RNAi (GMR-GAL4/UAS-Htt25Q-eGFP/+;UAS-rack1GD12135) flies compared to GMR>Htt25Q (GMR-GAL4/UAS-Htt25Q-eGFP) flies (n = 3). ns: not significant (Student’s unpaired t test). Data are presented as mean ±SEM. (TIF)

S3 Fig. Disruption of rack1 suppresses polyQ induced cell death through a novel pathway. (A) RACK1 associated translational pathways are not involved in suppression of polyQ cytoxicity. Light microscope images of eyes of 1-day-old flies expressing GFP, pkc53eRNAi, pkc53e, and znf598RNAi or with deletion of pkc53e (pkc53eKO) in either GMR-63Q;GMR-GAL4 (top panels), GMR-63Q;GMR>(rack1RNAi (middle panels), or GMR-AL4 (bottom panels) background. (B) RACK1 associated JNK/p38 pathways are not involved in suppression of polyQ induced cell death by rack1 mutations. Light microscope images of eyes of 1-day-old
flies expressing p38bRNAi, p38b, jnkRNAi, JNK, p38aRNAi, and p38a in either GMR-63Q; GMR-GAL4 (top panels), GMR-63Q;GMR>rack1RNAi (middle panels), or GMR-GAL4 (bottom panels) background. (C) Apoptosis pathways are not involved in regulation of polyQ cytotoxicity by rack1. Light microscope images of eyes of 1-day-old flies of Df(3L)H99 (deletion of rpr, grim, and hid), UAS-rprRNAi, UAS-diap1, UAS-droncRNAi, UAS-cep1RNAi, and UAS-decayRNAi in combination with GMR-63Q;GMR-GAL4 (top panels), GMR-63Q;GMR>rack1RNAi (middle panels), or GMR-GAL4 (bottom panels). Scale bar: 50 μm. Significant differences were determined by Two-way ANOVA with Sidak’s post hoc test (n = 3).

(TIF)

S4 Fig. Aggregates of polyQ protein were unaffected by modification of poe, kcmf1, usp47 and erk. (A) Pupa eyes of indicated genotypes were stained for 63Q-Myc using anti-Myc antibodies. The “flip-out” clones expressing mCD8-GFP (hs-flp;GMR-63Q-Myc/+;UAS-mCD8-GFP/actin>>CD2>>GAL4 UAS-GFP/+, ufd4RNAi (hs-flp;GMR-63Q-Myc/+;UAS-ufd4RNAi/actin>>CD2>>GAL4 UAS-GFP), poeRNAi (hs-flp;GMR-63Q-Myc/+;UAS-poeHS00739/actin>>CD2>>GAL4 UAS-GFP), kcmf1RNAi (hs-flp;GMR-63Q-Myc/+;UAS-kcmf1HS00511/actin>>CD2>>GAL4 UAS-GFP), ufd4RNAi (hs-flp;GMR-63Q-Myc/+;UAS-ufd4RNAi/actin>>CD2>>GAL4 UAS-GFP), USP47 (hs-flp;GMR-63Q-Myc/+;UAS-USP47/actin>>CD2>>GAL4 UAS-GFP), erkRNAi (hs-flp;GMR-63Q-Myc/+;UAS-erkFJ01366/actin>>CD2>>GAL4 UAS-GFP) and poe (hs-flp;GMR-63Q-Myc/+;UAS-erk/actin>>CD2>>GAL4 UAS-GFP) were generated by heat-shock. GFP positive clones are cells expressing indicated RNAi/genes. The mCD8-GFP and dna1-1 expressing flies were served as negative and positive controls, respectively. Scale bar: 10 μm. (B) Quantification of 63Q relative fluorescence intensity (upper panel) and relative quantity of 63Q aggregates (lower panel) between mutant cells and control cells from indicated genotypes. Data are presented as mean ± standard error of the mean (SEM). Significant differences were determined by one-way ANOVA with Tukey’s post hoc test (n = 3). (C-D) Expression of ERK is unaffected by loss of rack1.

(TIF)

S5 Fig. Knocking down rack1, poe, or kcmf1 increased ERK levels in the 63Q background, whereas knocking down ufd4 did not affect ERK levels. (A) Pupa eyes of indicated genotypes were stained for ERK and 63Q-Myc using anti-ERK and anti-Myc antibodies, respectively. The “flip-out” clones expressing GFP (hs-flp;GMR-63Q-Myc/+;UAS-GFP/actin>>CD2>>GAL4 UAS-GFP/+, rack1RNAi (hs-flp;GMR-63Q-Myc/+;UAS-rack1GD12135/actin>>CD2>>GAL4 UAS-GFP), poeRNAi (hs-flp;GMR-63Q-Myc/+;UAS-poeHS00739/actin>>CD2>>GAL4 UAS-GFP), kcmf1RNAi (hs-flp;GMR-63Q-Myc/+;UAS-kcmf1HS00511/actin>>CD2>>GAL4 UAS-GFP), and ufd4RNAi (hs-flp;GMR-63Q-Myc/+;UAS-ufd4RNAi/actin>>CD2>>GAL4 UAS-GFP) were generated by heat-shock. GFP positive clones are cells expressing indicated RNAis. The GFP expressing flies were served as negative controls. Scale bar: 10 μm. (B) Quantification of ERK relative fluorescence intensity between mutant cells and control cells from indicated genotypes. Data are presented as mean ± standard error of the mean (SEM). Significant differences were determined by one-way ANOVA with Tukey’s post hoc test (n = 3).

(TIF)

S6 Fig. RACK1 regulates ERK post-translationally. (A) The erk mRNA levels are measured by qPCR in indicated fly eyes (normalized to GMR-Gal4 flies, n = 3). Data are presented as mean ± standard error error of the mean (SEM). (B) Polysomal loading of erk transcripts is not altered by rack1 depletion. S2 cells were treated with GFP or rack1 dsRNAs, and the polysomal and monosome fractions were separated on a sucrose gradient. qPCR was used to assay erk mRNA transcript levels in both fractions. Data are presented as mean ± standard error of the mean (SEM). Significant differences were determined by one-way ANOVA with Tukey’s post hoc test (n = 3).
mean (SEM). (C) Western blot analysis of proteins extracted from pupa eyes of wild-type (GMR-GAL4/+), GMR-63Q (GMR-63Q-HA), MJD-78Q (GMR-GAL4/+;UAS-MJD.tr-Q78/+), flies with antibody against ERK. β-Actin was used as an internal control. (D) Quantification of ERK levels in (C). Data are represented as mean ± SEM, and one-way ANOVA with Tukey’s post hoc test is used (n = 4).

S7 Fig. RACK1 regulates the interaction between ERK and POE. (A) Schematic representations of POE protein with the position of identifiable domains and regions. UBR: ubiquitin protein ligase E3 component n-recoggin domain; DZR: double zinc ribbon domain; UBR4: E3 ubiquitin ligase domain. Different mCherry-tagged portions of POE are presented below with their respective amino acids (a.a.) positions. (B) RACK1 interacts with 1864–2374 and 3377–4492 mCherry-tagged POE. Truncate POE-mCherry was co-expressed in S2 cells with GFP or RACK1-GFP, followed by immunoprecipitation with anti-GFP antibody. (C) S2 cells were transfected with GFP or RACK1-GFP for two days, followed by co-expression of different mCherry-tagged portions of POE and FLAG-tagged ERK. Cells were lysed and immunoprecipitated with anti-mCherry antibody. (D) Pupa eyes of indicated genotypes were stained for ERK and 63Q-Myc using anti-ERK and anti-Myc antibodies, respectively. The “flip-out” clones expressing GFP (hs-flp;GMR-63Q-Myc/+;UAS-GFP/actin>>CD2>>GAL4 UAS-GFP/+), rack1RNAi kcmf1OE (hs-flp;GMR-63Q-Myc/UAS-rack1KK109073;UAS-kcmf1/actin>>CD2>>GAL4 UAS-GFP) and rack1RNAi poeOE (hs-flp;GMR-63Q-Myc/UAS-rack1KK109073;UAS-poe/actin>>CD2>>GAL4 UAS-GFP) were generated by heat-shock. GFP positive clones are cells expressing indicated RNAi/genes. The GFP expressing flies were served as negative control. Scale bar: 10 μm. (E) Quantification of ERK relative fluorescence intensity between mutant cells and control cells from indicated genotypes. Data are presented as mean ± standard error of the mean (SEM). Significant differences were determined by one-way ANOVA with Tukey’s post hoc test (n = 3).

S8 Fig. Quantitative analysis of eye phenotypes. (A) quantification of eye degeneration of flies in Fig 1A. (B) quantification of eye degeneration of flies in Fig 3A. (C) quantification of eye degeneration of flies in Fig 6D. (D) quantification of eye degeneration of flies in Fig 7F. Data are presented as mean ± standard error of the mean (SEM). Significant differences were determined by one-way ANOVA with Tukey’s post hoc test (n = 50). Details of quantification criteria are demonstrated in Methods and Materials.

S1 Table. qPCR validation of indicated RNAi lines. UAS-RNAi lines were crossed with GMR-GAL4, and adult eyes of F1 flies were dissected for RNA extraction and subsequent qPCR. Samples were prepared in triplicate. All values were normalized to GMR-GAL4 samples. RpL32 was used as the reference gene for standardization. The p-values were obtained using a Student’s T-tests against the GMR-GAL4 samples.

S1 Data. Original data statistics.

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