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Reduced neuronal expression of ribose-5-phosphate isomerase enhances tolerance to oxidative stress, extends lifespan, and attenuates polyglutamine toxicity in *Drosophila*

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Summary

Aging and age-related diseases can be viewed as the result of the lifelong accumulation of stress insults. The identification of mutant strains and genes that are responsive to stress and can alter longevity profiles provides new therapeutic targets for age-related diseases. Here we reported that a *Drosophila* strain with reduced expression of *ribose-5-phosphate isomerase* (*rpi*), *EP2456*, exhibits increased resistance to oxidative stress and enhanced lifespan. In addition, the strain also displays higher levels of NADPH. The knockdown of *rpi* in neurons by double-stranded RNA interference recapitulated the lifespan extension and oxidative stress resistance in *Drosophila*. This manipulation was also found to ameliorate the effects of genetic manipulations aimed at creating a model for studying Huntington's disease by overexpression of polyglutamine in the eye, suggesting that modulating *rpi* levels could serve as a treatment for normal aging as well as for polyglutamine neurotoxicity.

Key words: *ribose-5-phosphate isomerase*; pentose phosphate pathway; neuron; oxidative stress; longevity; polyglutamine toxicity; *Drosophila*.

Introduction

Aging is a universal, progressive, deleterious process and also a major cause of many diseases (Miller, 2009). The identification of mutants that can prolong lifespan can help uncover genetic mechanisms involved in regulating aging and may lead to new medical interventions to postpone aging and treat age-related diseases (Finkel, 2005; Fontana *et al.*, 2010). Several conserved mechanisms that affect aging have been identified including the following: the modulation of caloric intake by caloric restriction (CR), changing exposure to reactive oxygen species (ROS), altering the expression of target of rapamycin (TOR) and the insulin/IGF-1 pathways (Lu & Finkel, 2008; Fontana *et al.*, 2010; Kenyon, 2010).

Increased longevity is often associated with higher resistance to different environmental stress (Arking *et al.*, 1991). The manipulation of stress-responsive genes can be utilized to extend lifespan. For instance, *Drosophila* lifespan is increased by overexpression of the antioxidant Cu-Zn superoxide dismutase (SOD) (Parkes *et al.*, 1998; Sun & Tower, 1999) or the heat shock protein (HSP) genes *hsp70, hsp27, hsp26, and hsp22* (Tatar *et al.*, 1997; Morrow *et al.*, 2004; Wang *et al.*, 2004). Understanding what genes can modulate responses to stress and alter longevity can be applied to develop novel therapies. Mimicking some consequences of caloric restriction, the administration of Sir2 agonists extends lifespan in metazoans (Wood *et al.*, 2004). Increased expression of SIRT1, a human homologue of SIR2, or administration of resveratrol both promote the survival of neuronal cells, protect against Alzheimer disease (Kim *et al.*, 2007). Resveratrol improves the health and survival of mice fed high-fat diets (Baur *et al.*, 2006). Therefore, the selection for elevated tolerance to stress can be used to screen for new genes or mutants to identify targets for lifespan extension and potential disease therapies (Wang *et al.*, 2004; Liao *et al.*, 2008; Liu *et al.*, 2009).

The pentose phosphate pathway (PPP) is an important cellular defense system against oxidative stress. This pathway helps to remove excessive ROS resulting from mitochondrial oxidative phosphorylation. The major function of PPP is to generate nicotinamide adenine dinucleotide phosphate (NADPH) to provide more reduced form of glutathione to counteract the damaging effects of ROS. Long-lived flies contain higher glucose-6-phosphate dehydrogenase (G6PD) activity, a rate-limiting enzyme in PPP (Luckinbill *et al.*, 1990). In addition, Hsp27 can increase G6PD activity (Preville *et al.*, 1999), and Hsp27 overexpression transgenic flies extend lifespan and exhibit better resistance to oxidative stress (Wang *et al.*, 2004; Liao *et al.*, 2008). Modulation of G6PD expression regulates NADPH levels and protects neuronal cells against nitrosative stress-induced apoptosis (Garcia-Nogales *et al.*, 2003). Transgenic mice with neuronal expression of *G6PD* displayed neuroprotective action against oxidative stress (Mejias *et al.*, 2006). In addition, it was reported that PPP plays an important role in promoting neuronal survival upon exposure to reactive oxygen and nitrogen species (Bolanos & Almeida, 2010). Recently it was shown that *G6PD* overexpression transgenic flies with elevated NADPH levels enhance lifespan and increase tolerance to oxidative stress (Legan *et al.*, 2008). These data suggest a correlation of PPP activity with oxidative stress response and lifespan.

Neurodegenerative diseases are devastating progressive conditions that usually occur at late-age. Oxidative damage is one of the major causes of many progressive neurodegenerative diseases such as Huntington's disease (Trushina & McMurray, 2007). Huntington's disease is a neurodegenerative disease in which expanded CAG repeats cause a form of polyglutamine toxicity in neuronal cells. Several different *Drosophila* models for polyglutamine diseases were established to look for suppres-

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sor genes to alleviate polyglutamine toxicity by overexpressing toxic polyglutamine encoding constructs in the eye and the resulting rough eye used to identify enhancers or suppressors of the effect (Warrick *et al.*, 1998; Kazemi-Esfarjani & Benzer, 2000; Sang *et al.*, 2005). A number of genes able to rescue polyglutamine toxicity were identified, including *CBP*, *hsp70*, *dhdj1*, and *hsp27* (Warrick *et al.*, 1999; Kazemi-Esfarjani & Benzer, 2000; Taylor *et al.*, 2003; Liao *et al.*, 2008). Some of the genes that rescued the polyglutamine toxicity in the fly eyes were also found to promote longevity, like *hsp70*, *hsp27*, and *CBP* (Tatar *et al.*, 1997; Liao *et al.*, 2008; Zhang *et al.*, 2009). Thus, in some cases genes that enhance lifespan can also modulate polyglutamine toxicity and thus be useful for preventing neurodegeneration.

Here we reported that a *Drosophila* strain *EP2456* with reduced expression of *ribose-5-phosphate isomerase* (*rpi*) expresses higher level of NADPH and exhibits increased resistance to oxidative stress and enhanced lifespan. Furthermore, tissue-specific knockdown of *rpi* in neurons by double-stranded RNA interference recapitulated the lifespan extension and oxidative stress resistance in *Drosophila*. It also rescued the rough eye morphology resulting from polyglutamine toxicity. These data provide a new approach for postponing aging and suggest a possible treatment for neurodegenerative diseases.

Results

Reduced *ribose-5-phosphate isomerase* expression in neurons enhances lifespan and increases resistance to oxidative stress in *Drosophila*

Enhanced longevity is usually associated with better resistance to environmental stresses. We performed a screen for enhanced stress resistance, to identify genes that extend lifespan in *Drosophila* (Wang *et al.*, 2004; Liao *et al.*, 2008; Liu *et al.*, 2009). Here, we report the characterization of *EP2456,* a strain that exhibits enhanced resistance to paraquat and hyperoxia-induced oxidative stress respectively with 27 and 31% increase in mean survival time relative to the control w^{1118} (Fig. 1A,B, and Table S1 in Supporting information). *EP2456* is also long-lived with 39% increase in mean lifespan compared to w^{1118} (Fig. 1C, Table S1 in Supporting information).

To find the gene responsible for these phenotypes, we used semi-quantitative PCR to measure the level of expression of transcripts in the region near the P-element insertion, *CG30409* and *CG30410*, relative to w^{1118} (Fig. 1D). Expression of *CG30410* was reduced by 80% in the mutant, while *CG30409* remained unchanged. As *CG30410* encodes a *ribose-5-phosphate isomerase* (*rpi*), which is involved in regulating NADPH levels via the PPP and NADPH is involved in combating oxidative stress, these results are consistent with the downregulation of *rpi* affecting oxidative stress response.

To determine whether decreased *rpi* expression extends lifespan, we generated independent Gal4-driven transgenic RNA knockdown lines, *UAS-rpi*^{RNAi} with the insertion on either the second or the third chromosome. As it is known that neuronal tissue is particularly sensitive to oxidative stress, we compared the effects of reducing *rpi* expression in the neuronal tissue and eye to that of reducing the expression in the whole animal. The knockdown of *rpi* by tissue-specific drivers *Appl-Gal4* and *GMR-Gal4* resulted in increased resistance to oxidative stress. However, these effects were not seen with the ubiquitous driver *da-Gal4* (Table S2 in Supporting information). The flies with the pan-neuronal knockdown of *rpi* by *Appl-Gal4* displayed a significant increase in mean lifespan ranged from 25 to 38% compared to the controls (Fig. 2A,B, and Table S3 in Supporting information). Interestingly, using the predominantly eye-specific *GMR-Gal4* also resulted in a 35 and 38% in mean lifespan extension (Fig. 2C,D, and Table S3 in Supporting information). These effects

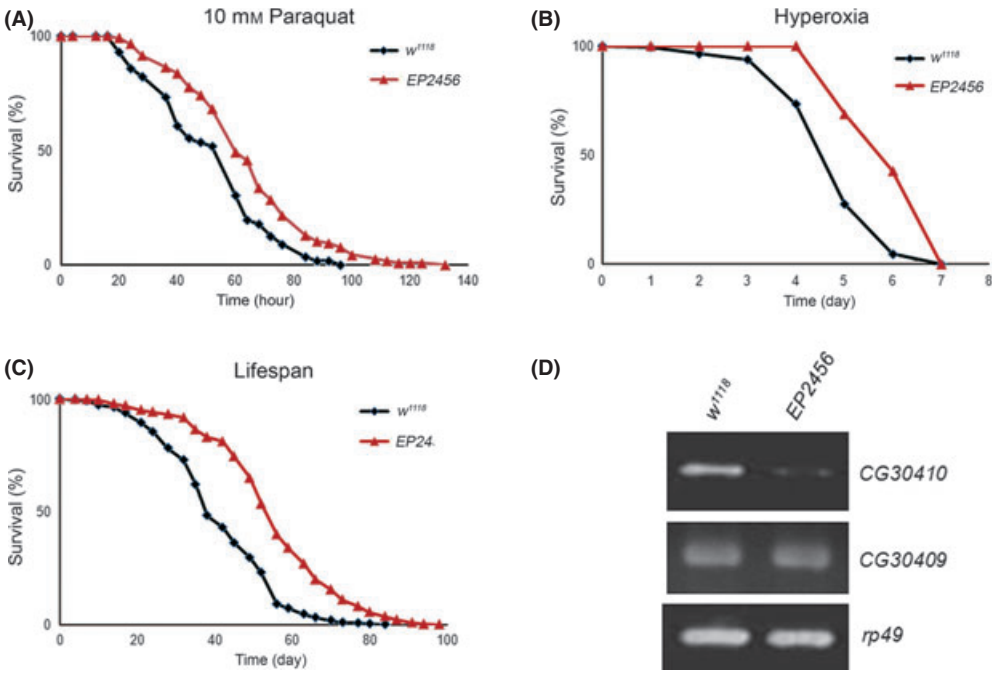

Fig. 1 Mutant fly *EP2456* with reduced expression of *rpi* displays increased resistance to oxidative stress and enhanced lifespan. *EP2456* (red triangle) exhibits elevated resistance to (A) 10 mM paraquat-induced oxidative stress ($P < 0.05$) and (B) hyperoxia (95% O_2)-induced oxidative stress ($P < 0.01$) compared to the control w^{1118} (black diamond). (C) *EP2456* displays increased mean lifespan ($P < 0.001$) compared to w^{1118} at 25°C. (D) Reduced expression of *CG30410* (*rpi*) was detected in *EP2456* compared to w^{1118}, but no differences in the expression of the neighboring *CG30409* between w^{1118} and *EP2456*. The expression of *rp49* was used as a control.

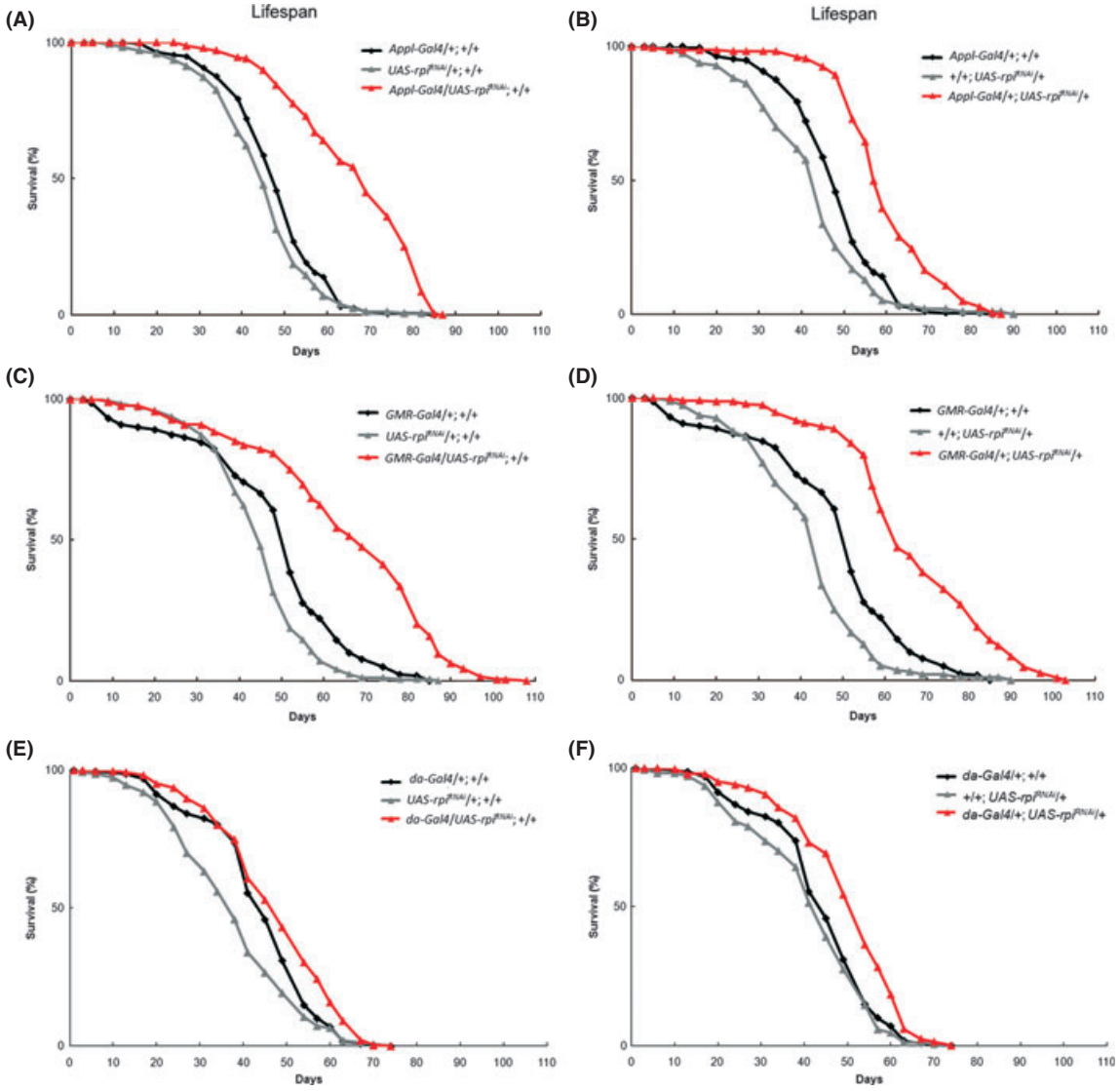

Fig. 2 The RNA interference knockdown expression of *rpi* in neurons extends lifespan in *Drosophila*. The independent transgenic fly lines with knockdown of *rpi* (A, B) by *Appl-Gal4* (red triangle) or (C, D) by *GMR-Gal4* (red triangle) exhibit dramatically increased lifespan than the control flies (black diamond and gray triangle). (E, F) The transgenic fly lines with knockdown of *rpi* ubiquitously by *da-Gal4* (red triangle) only display little increased lifespan compared to the control flies (black diamond and gray triangle).

were confirmed using five independent *UAS-rpi*^{RNAi} transgenic lines driven by *Appl-Gal4* or *GMR-Gal4* (results summarized in Table S4 in Supporting information). Ubiquitous knockdown of *rpi* by *da-Gal4* resulted in modest enhancement of longevity of only 8–14% relative to the controls (Fig. 3E,F, and Table S3 in Supporting information). It is possible that the knockdown of *rpi* by *da-Gal4* in the other tissues beside neurons nullifies the beneficial effect on lifespan and oxidative tolerance. Interestingly, adult-specific knockdown of *rpi* using the RU486-induced neuronal Gal4 driver, *elav-GS* Gal4, did not result in lifespan extension (Fig. S1 in Supporting information). This is similar to what was observed by Simonsen *et al.* where lifespan extension was detected in the *atg8a* overexpression flies driven by *Appl-Gal4* but not able by *elav-Gal4* driver (Simonsen *et al.*, 2008). Whether the age-dependent or the cell-type-specific expression differences or both of the two different neuronal Gal4 drivers contributing to the differences in the lifespan results awaits further experiments to clarify. Together, the results indicate that the tissue-specific knockdown of *rpi* in neurons increases resistance to oxidative stress and extends lifespan in *Drosophila*.

Overexpression of *rpi* does not shorten lifespan nor aggravates response to oxidative stress in *Drosophila*

As the knockdown of *rpi* in neurons extends lifespan, we examined whether overexpression of *rpi* decreases lifespan and enhances susceptibility to oxidative stress. Independent Gal4-responsive transgenic lines were generated to measure the effect of overexpressing *rpi*. Transgenic lines, containing the full-length *rpi* cDNA, *UAS-rpi*, were crossed with the same Gal4 drivers used previously, and their progeny were tested for lifespan and oxidative stress. None of the transgenic flies overexpressing *rpi* showed decreased lifespan (Figs 3A–F, S1 and Tables S5 and S7 in Supporting information), nor were they sensitive to oxidative stress (data not shown). In fact, certain specific cross-combinations resulted in marginal

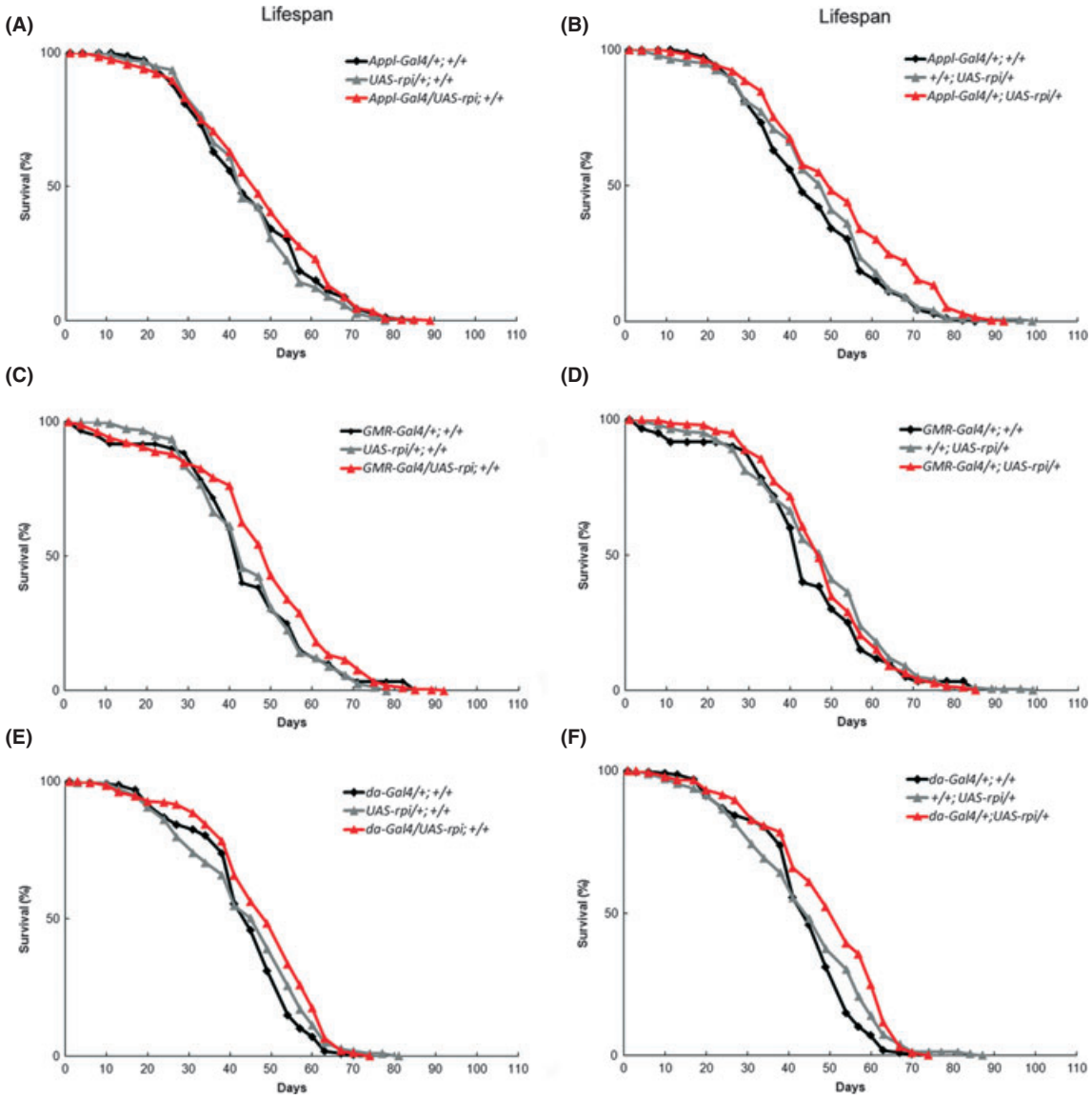

Fig. 3 The transgenic flies with overexpression of *rpi* do not have shortened lifespan. (A–F) The *rpi* overexpression transgenic flies with *UAS-rpi* either on the second or on the third chromosome driven by *Appl-Gal4*, *GMR-Gal4*, and *da-Gal4* (red triangle) do not show altered lifespan compared to their control flies (black diamond, gray triangle).

increases in mean lifespan upon Gal4 induction. The data revealed that overexpression of *rpi* does not aggravate the response to oxidative stress nor decrease lifespan in *Drosophila*.

Knockdown expression of *rpi* increases glucose-6-phosphate dehydrogenase activity and the levels of NADPH and the reduced form of glutathione

We proposed that reduced *rpi* could lead to elevated levels of NADPH, because *rpi* is involved in the PPP and thus in producing more NADPH for the generation of the reduced form of glutathione (GSH). The knockdown of *rpi* may result in excess ribulose-5-phosphate to be metabolized via nonoxidative phase of PPP and feedback to glucose-6-phosphate to generate more NADPH (Bolanos & Almeida, 2010). Therefore, we measured the NADPH levels in both the mutant and the control flies. A twofold increased level of NADPH was measured in *EP2456* compared to w^{1118} (Fig. 4A). We further examined the NADPH levels in the flies with the knockdown of *rpi* either by *Appl-Gal4* or *GMR-Gal4* driver as well as their control flies. We also detected increased levels of NADPH in the flies with the knockdown of *rpi* compared to the controls (Fig. 4B,C).

Overexpression of *G6PD* was reported to extend lifespan in *Drosophila* (Legan *et al.*, 2008), and G6PD is the rate-limiting enzyme of the PPP. To determine whether G6PD was altered in our strains, we observed both young and old *EP2456* flies and observed increased levels of *G6PD* in both (Fig. S2 in Supporting information). We also measured G6PD activity and the levels of reduced GSH in *Drosophila* S2 cells and found that *rpi* knockdown resulted in a 20% increase in G6PD activity and a 25% enhanced level of GSH compared to the untreated cells (Fig. S3 in Supporting information). Together, it suggests that reducing *rpi* results in elevated levels of NADPH as a result of changes in the PPP.

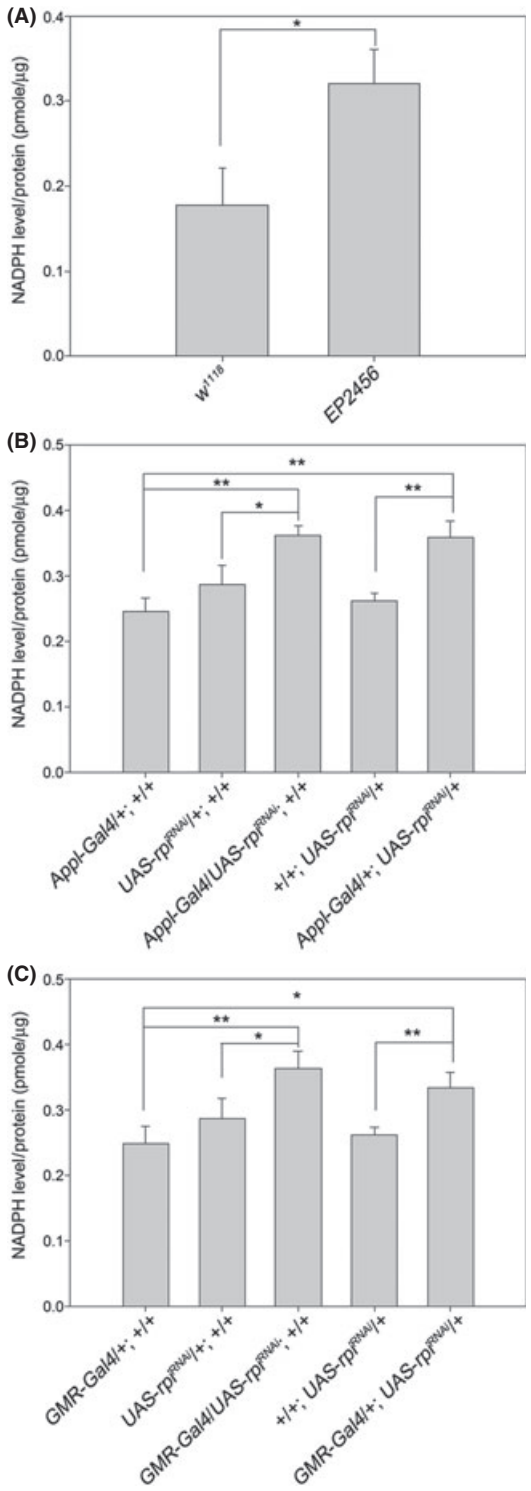

Fig. 4 Increased nicotinamide adenine dinucleotide phosphate (NADPH) levels are detected in the longevity flies with lowered *rpi* expression. The NADPH level of each fly line was determined and normalized by its protein concentration. Each bar represents mean ± SEM (n = 3) (unit: pmole/ug). *P < 0.05; **P < 0.01 (A) w^{1118} (0.18 ± 0.03) versus *EP2456* (0.32 ± 0.02). (B) *Appl-Gal4/+;+/+* (0.25 ± 0.01), *UAS-rpi^{RNAi}/+;+/+* (0.27 ± 0.02), *Appl-Gal4/UAS-rpi^{RNAi};+/+* (0.36 ± 0.01), *+/+;UAS-rpi^{RNAi}/+* (0.26 ± 0.01), *Appl-Gal4/+;UAS-rpi^{RNAi}/+* (0.36 ± 0.02). (C) *GMR-Gal4/+;+/+* (0.25 ± 0.02), *GMR-Gal4/UAS-rpi^{RNAi};+/+* (0.36 ± 0.02), *GMR-Gal4/+;UAS-rpi^{RNAi}/+* (0.33 ± 0.01).

The knockdown of *rpi* attenuates the polyglutamine toxicity-induced *Drosophila* rough eyes, which depends on *G6PD* and *transaldolase* expressions

As *rpi* knockdown flies appear to have a neuroprotective effect against oxidative damage, we tested whether this manipulation would protect against other types of neural toxicity such as polyglutamine toxicity. The *GMR-GAL4 > UAS-127Q* (Kazemi-Esfarjani & Benzer, 2000) flies and *GMR-Gal4 > 108Q* (Sang *et al.*, 2005) expressing a long tract of glutamines displayed a rough eye phenotype and loss of pigment distribution (Fig. 5B,F). We tested whether *rpi* knockdown could rescue the effect by simultaneously expressing the polyQ and the *rpi* knockdown in the eye using GMR-Gal4. The knockdown of *rpi* dramatically rescued the phenotype of the rough eye and loss of pigment in both *GMR-GAL4 > UAS-127Q* and *GMR-GAL4 > UAS-108Q* flies (Fig. 5D,E,G,H). The effect was comparable with the result by the expression of *dhdj1* (Fig. 5C). These results show that *rpi* knockdown is effective at blocking polyQ toxicity resulting from two independently generated lines in two different genetic backgrounds. In addition, the knockdown of *rpi* was shown to be dose dependent because two copies of *UAS-rpi^{RNAi}* exhibit significantly better rescue of the eye phenotype compared to only one copy of *UAS-rpi^{RNAi}* (Fig. 5G–I). To exclude the possibility that the rescue observed resulted from a titration of Gal4 proteins by the extra UAS sequences introduced, we crossed *GMR-Gal4,UAS-108/Cyo* with the fly strains harboring two copies of *UAS-GFP* (*UAS-GFP;UAS-GFP)* and none of their offspring shows any rescued eye phenotype (Fig. 5J).

As reducing *rpi* expression elevates NADPH level and increases expression of *G6PD*, we asked whether the *rpi*-mediated rescue of the polyglutamine toxicity depends on the expression of *G6PD*. We found that reducing *G6PD* levels by knockdown abolished the rescue mediated by *rpi* knockdown (Fig. 5K). However, the knockdown of *G6PD* in *GMR-Gal4 > UAS-108Q* does not further deteriorate 108Q-induced rough eyes (Fig. 5L). Overexpression of two copies of *UAS-G6PD* can partially rescue the 108Q-induced rough eyes (Fig. 5M). The results suggest that *G6PD* expression mediates the effects of *rpi* potentially by its effect on NADPH levels.

As our hypothesis is that *rpi* knockdown results in a shunt of ribulose-5-phosphate back to glucose-6-phosphate via the nonoxidative phase by a series of enzymes including transaldolase, the rescue by *rpi* knockdown of polyQ toxicity should depend on transaldolase activity. Indeed, the knockdown of *transaldolase* expression also blocks the rescue by the knockdown of *rpi* expression of 108Q toxicity (Fig. S4 in Supporting information). As seen using SEM, at higher magnification, reduced transaldolase expression suppresses the rescue of 108Q toxicity by *rpi* knockdown (Fig. S4 in Supporting information). This indicates that the rescue by the knockdown of *rpi* requires transaldolase activity. Disruption of transaldolase activity has been shown to lower NADPH level (Qian *et al.*, 2008). All these results support the conclusion that the enhancement of lifespan, resistance to oxidative stress, and alleviation of polyglutamine toxicity resulting from knockdown of *rpi* are mediated via increased G6PD activity and NADPH level.

Overexpression of *rpi* does not exacerbate the polyglutamine toxicity

Because the knockdown of *rpi* suppressed polyglutamine toxicity, we tested whether overexpression of *rpi* would enhance polyglutamine toxicity. Overexpression of *rpi* did not show any further deterioration of the rough eyes in the *GMR-GAL4 > UAS-127Q* flies (Fig. 5N) even when two copies of *UAS-rpi* were used (Fig. 5O). This result correlates with the

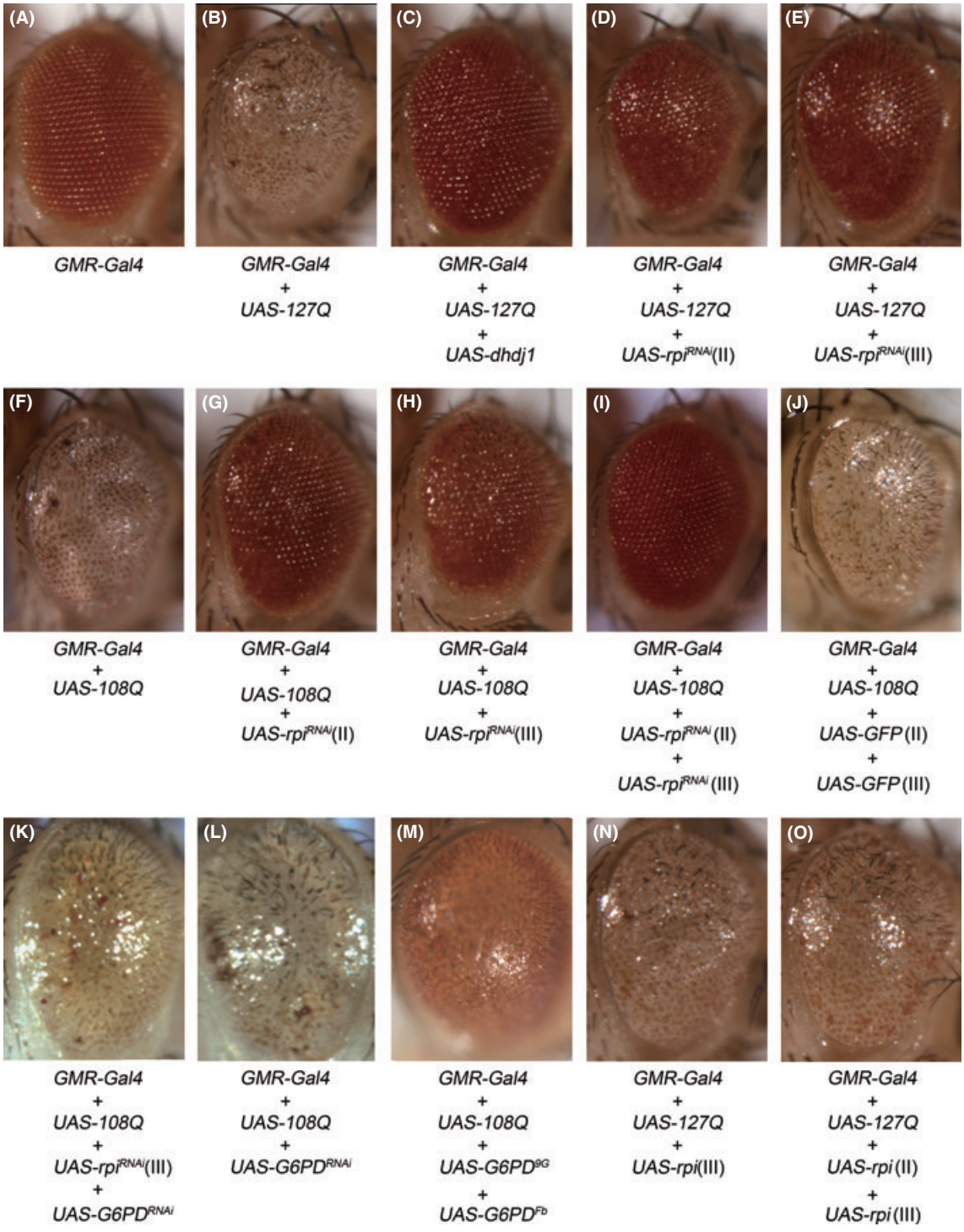

Fig. 5 The knockdown of *rpi* rescues the polyglutamine-induced rough eyes, and the overexpression of *rpi* does not further deteriorate the rough eye phenotype. The eye phenotypes resulting from different combinations of different transgenes of *127Q*, *108Q*, *rpi*, *G6PD*, *GFP* constructs are shown from A to O. (A) One copy of *GMR-Gal4* displayed normal eye appearance as a control. (B) The expression of *UAS-127Q* by *GMR-Gal4* exhibits rough eye phenotype. (C) The expression of *UAS-hdj1* rescues the rough eye phenotype (Kazemi-Esfarjani & Benzer, 2000), which was used as a positive control. (D, E) The RNAi knockdown of *rpi* by the transgene on the second (II) or the third (III) chromosome rescues the 127Q-induced rough eye. (F) The expression of *UAS-108Q* shows similar rough eye phenotype. (G, H) The RNAi knockdown of *rpi* also rescues the 108Q-induced rough eye. (I) The knockdown of *rpi* with two copies of *UAS-rpi*^{RNAi} rescues the rough eye much better than a single copy of *UAS-rpi*^{RNAi}. (J) The 108Q-induced rough eye cannot be rescued by two copies of *UAS-GFP*. (K) Knockdown of *G6PD* blocks the rescue by *rpi* knockdown on 108Q-induced rough eye. (L) Knockdown of *G6PD* does not degenerate the rough eye. (M) Overexpression of two copies of *UAS-G6PD* partially rescues the rough eye. (N) Overexpression of one copy of *UAS-rpi* does not further worsen the 127Q-induced rough eye. (O) The expression of two copies of *UAS-rpi* still does not deteriorate the rough eye.

data from lifespan and stress assays, indicating that overexpression of *rpi* does not result in any significant negative effect on the phenotypes measured.

The knockdown of *rpi* improves the abnormal retinal ultrastructure by polyglutamine toxicity

The compound eye of a fruit fly is composed of approximately 760 ommatidia. Each ommatidium contains eight photoreceptor cells (R1–R8), supporting cells, pigment cells and a cornea. Every photoreceptor cell that is responsible for phototransduction possesses the cell body and the rhabdomere. Expressing polyglutamine in the eye causes toxicity to neuronal cells, leading to dysfunction and cell death in selected neurons. Although knockdown of *rpi* effectively rescued the rough eye and pigment distribution under polyglutamine toxicity, we asked whether it can improve the integrity of neurons in fly eyes. We examined the ultrastructure of retina in the *GMR-Gal4 > UAS-108Q* with and without the knockdown of *rpi* by whole-mount staining (Sang & Ready, 2002). A longitudinal view of the retina in *GMR-Gal4 > UAS-108Q* revealed that the internal structure was disrupted and the cells had degenerated, resulting in large vacuoles not seen in the control flies (*GMR-Gal4*; Fig. 6A,B). Conversely, the retina of the flies expressing *108Q* and also having reduced *rpi* levels showed improved structural integrity and more F-actin staining by pholloidin (Fig. 6C,D), the vacuoles however still existed showing that the rescue is not complete. As expected, when two copies of *UAS-rpi^{RNAi}* were used, the rescue of the phenotype was better (Fig. 6C–E), as was seen by observing the external eye morphology (Fig. 5G–I). Thus, the knockdown of *rpi* in *GMR-Gal4 > UAS-108Q* not only rescued the external rough eye and the eye pigment but also improved the internal ultrastructural disorder of retina.

The knockdown of *rpi* attenuates adult-onset polyglutamine toxicity and ameliorates the damaged phototaxis in aged *Drosophila*

Many polyglutamine diseases are progressive and late-onset. However, *GMR-Gal4* driver expresses Gal4 in the early development throughout adult stage. To mimic the late-onset event and check whether the knockdown of *rpi* still can rescue it, we used *Rh1-Gal4* driver, which expresses Gal4 in the late pupa stage, to express *UAS-108Q* alone or in combination with *UAS-rpi^{RNAi}*. At 1 week of age, no photoreceptor degeneration was observed either in *Rh1-Gal4 > UAS-108Q* flies (Fig. 7B,F) or in the knockdown of *rpi*, *Rh1-Gal4 > UAS-108Q,UAS-rpi^{RNAi}* (Fig. 7C,D,G,H), relative to the control *Rh1-Gal4* driver alone (Fig. 7A,E). This reveals that 108Q toxicity is not observable in the 1-week-old flies. Four-week-old control fly *Rh1-Gal4* showed normal ommatidium morphology, an ordered array of R1-R6 photoreceptor neurons surrounding the central R7 cell and the intact continuous structure of rhabdomeres as those in the 1-week-old *Rh1-Gal4* (Fig. 7A,E,I,M). In age-matched *Rh1-Gal4 > UAS-108Q* flies, the photoreceptor neurons degenerated and the rhabdomeres were fragmented (Fig. 7J,N). Knockdown of *rpi* significantly improved rhabdomere structure and rescued the photoreceptor neurons of each ommatidium (Fig. 7 K,L,O,P). The percentage of the intact ommatidia containing seven photoreceptors were significantly improved from 38% to 72–90% in the flies with the knockdown of *rpi* in *Rh1-Gal4 > UAS108Q;UAS-rpi^{RNAi}* compared to *Rh1-Gal4 > UAS108Q* alone (Fig. 7Q). These data demonstrate that *rpi* knockdown also relieves adult-onset polyglutamine toxicity.

To determine whether the rescued eyes also function better, we measured phototaxis of the young and old *Rh1-Gal4 > UAS108Q* flies with or without the knockdown of *rpi*. As the *Rh1*-mediated Gal4 expression starts in the late pupa stage, there is no difference of the phototaxis activity among the young flies under 108Q with or without the knockdown of *rpi* (Fig. 7R, the upper panel). However, as flies aged polyglutamine toxicity reduced the phototaxis activity from 48 to 20%. The effect of polyglutamine toxicity on phototaxis was largely rescued by the *rpi* knockdown back to 39 and 42%, respectively (Fig. 7R, the lower panel). Together, knockdown of *rpi* not only rescued the abnormal structure of ommatidia but also functionally amended the damaged phototaxis activity by polyglutamine toxicity.

Discussion

In this study, we report that reduction in the expression of *rpi* can lead to enhanced longevity, resistance to oxidative damage and protect against polyglutamine-induced neurodegeneration. The reduced expression of *rpi* by RNAi in neuronal tissue and eyes also provided significant lifespan extension and better resistance to oxidative stress in *Drosophila*. The results demonstrated that the specific tissues like neuronal tissue and perhaps the photoreceptor neurons in the eyes may be the target sites of the beneficial effect of knockdown expression of *rpi*. Neurons have been proposed to be important for modulating longevity and stress resistance (Wolkow, 2002; Tatar *et al.*, 2003; Garelick & Kennedy, 2010). Overexpression of SOD in motorneurons increased resistance to oxidative stress and enhanced lifespan in *Drosophila melanogaster* (Parkes *et al.*, 1998). Activation of JNK signaling in neurons but not in muscle had better resistance to oxidative stress and extended lifespan (Wang *et al.*, 2003). Neuronal expression of *hsp26* and *hsp27* elevated lifespan and resistance to oxidative stress (Liao *et al.*, 2008). Mutations that affected the function

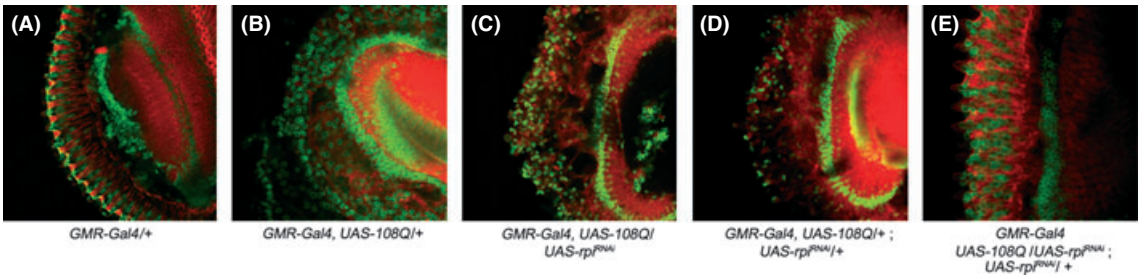

Fig. 6 The knockdown of *rpi* improves the internal structure of the fly eyes under polyglutamine toxicity. The structures of the 4-day-old fly eyes were assessed by the staining of phalloidin to indicate rhabdomeres (red) and anti-lamin to nuclear membrane (green). (A) The control fly with one copy of *GMR-Gal4* displays normal integrity of rhabdomere structure. (B) The expression of *UAS-108Q* under *GMR-Gal4* exhibits massive cell loss and collapsed structure with vacuoles. (C, D) The knockdown of *rpi* either on the second or on the third chromosome shows more rhabdomere staining, although the vacuoles still exist. (E) The knockdown of *rpi* with two copies of *UAS-rpi^{RNAi}* dramatically improves the structure of rhabdomere and decreases the size of vacuoles.

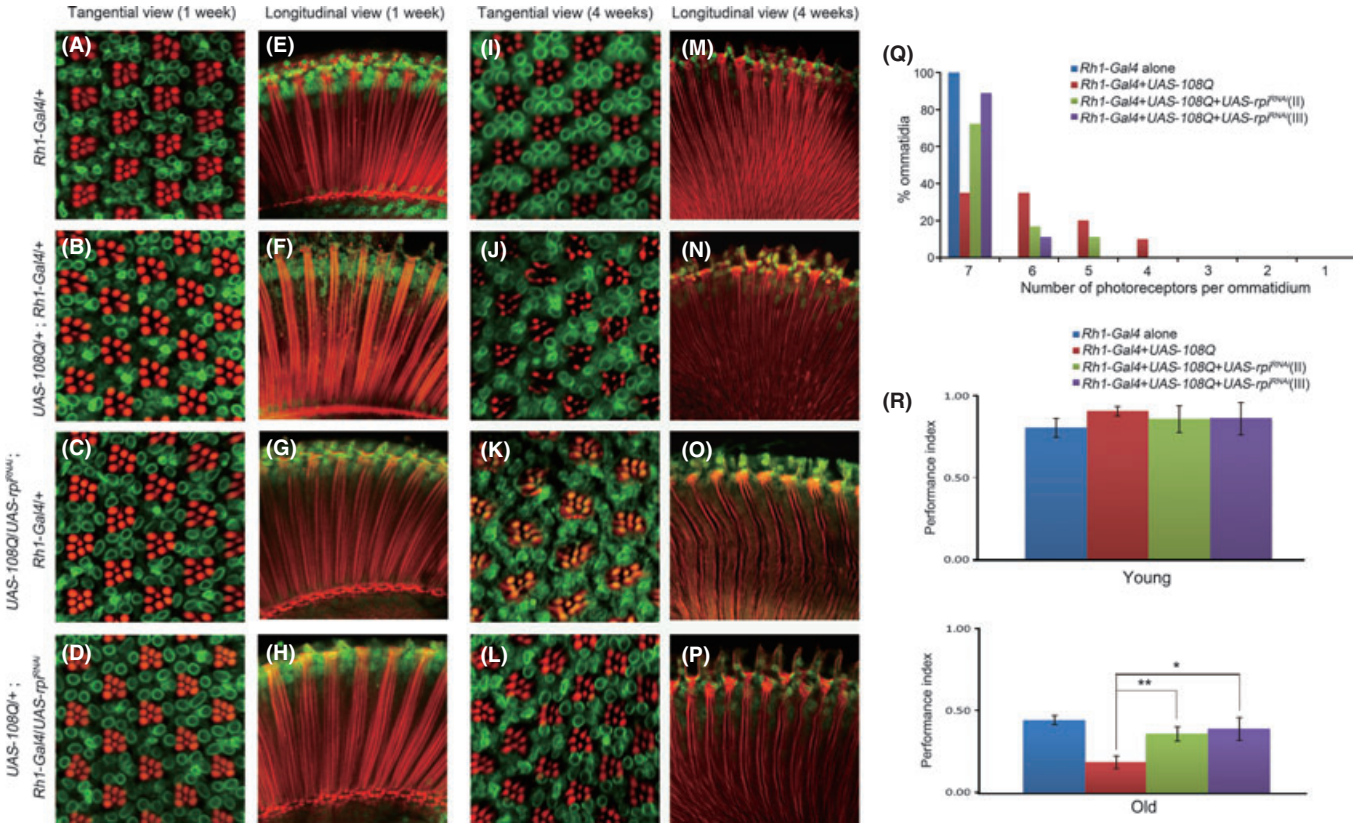

Fig. 7 The knockdown of *rpi* suppresses adult-onset polyglutamine toxicity in aged *Drosophila*. Phalloidin staining to rhabdomere (red) and anti-lamin staining to nuclear membrane (green) of the eyes of the 1-week young and 4-week-old flies with different transgenes were shown from A to P. The images from A to D (1-week) and I to L (4-week) are tangential view of the retina, and those from E to H (1-week) and M to P (4-week) are longitudinal view of the retina. (A, E, I, M) Both the 1- and 4-week control flies *Rh1-Gal4/+* show normal structures of ommatidia, which consist of an ordered array of R1-R6 photoreceptor neurons surrounding the central R7 cell, and a well lineup of rhabdomeres. (J, N) Severe degeneration of R1–R6 photoreceptor neurons and the disintegrated structures of rhabdomeres were observed only in the eyes of 4-week-old *Rh1-Gal4 > UAS-108Q*, (B, F) but not in those of the 1-week young ones. (C, D, G, H) The knockdown of *rpi* either on the second or on the third chromosome improved the structure of photoreceptor neurons and rhabdomeres under *Rh1-Gal4 > UAS108Q*. (Q) The knockdown of *rpi* mitigates the photoreceptor neurons loss caused by polyglutamine toxicity. The distribution of the number of photoreceptor neurons in ommatidia under the expression of different transgenes was shown. (R, the upper panel) The 1-week-old young flies show no differences in the phototaxis function among the fly lines under *Rh1-Gal4*. (R, the lower panel) The knockdown of *rpi* significantly rescues the impaired phototaxis function in the old flies under adult-onset polyglutamine toxicity.

of specific sensory neurons influenced longevity in *Caenorhabditis elegans* and *Drosophila* (Alcedo & Kenyon, 2004; Libert *et al.*, 2007).

We observed that overexpression of *rpi* did not have any adverse effect on the lifespan and oxidative stress resistance. This may be due to the fact that the enzyme does not catalyze the rate-limiting step in the pathway it controls and that therefore effects are only observed when the enzyme levels are reduced to the point at which flux through the pathway is altered. Additional reports have shown that inhibition of *short neuropeptide F* (*sNPF*) expression in neurons by RNAi silencing extended lifespan but overexpression of *sNPF* did not shorten the lifespan in *Drosophila* (Lee *et al.*, 2008), suggesting that critical levels of gene expression are needed to promote longevity changes; however, that changes in only one direction may have effects on longevity.

Many progressive neurodegenerative diseases are caused by oxidative damage that leads to neuronal cell death. Polyglutamine toxicity has been shown to be involved in a number of progressive neurodegenerative disorders. It has been shown that polyQ can induce intracellular ROS and cause cell death (Wyttenbach *et al.*, 2002). Overexpression of Cu-Zn SOD significantly increased lifespan and oxidative stress resistance (Parkes *et al.*, 1998; Sun & Tower, 1999). Neuronal expression of *hsp27* not only increases tolerance to oxidative stress and lifespan, it also ameliorates Parkinsonism climbing disorder and mild polyglutamine toxicity (Liao *et al.*, 2008). Our finding that the reducing *rpi* expression in neurons leads to higher levels of NADPH and enhanced oxidative stress resistance and reduced sensitivity to polyglutamine toxicity suggests a new strategy for neuronal protection. RPI isomerizes ribulose-5-phosphate to ribose-5-phosphate in the nonoxidative phase of PPP that facilitates the synthesis of the nucleotides and nucleic acids. A recent report suggested that the lack of activity of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase isoform 3 (PFKFB3) in neurons causes glucose-6-phosphate (G6P) to be metabolized predominantly via PPP to generate NADPH against nitrosative stress (Herrero-Mendez *et al.*, 2009; Bolanos & Almeida, 2010). The knockdown of *rpi* may allow the excessive ribulose-5-phosphate to be metabolized to become fructose-5-phosphate (F6P) by nonoxidative phase of PPP (Wamelink *et al.*, 2008). The lack of PFKFB3 activity in neurons may redistribute the recycled F6P back to G6P for the re-entry of PPP and generate more NADPH in oxidative phase of PPP. Therefore, we hypothesize that lowered expression of *rpi* in neurons may allow the ribulose-5-phosphate via F6P to be recycled back into G6P and result in more NADPH to cope with oxidative stress. The oxidized glutathione (GSSG) needs a glutathione reductase, which

requires NADPH as a cofactor to direct the regeneration of reduced form of glutathione (GSH). The reduced GSH is the antioxidant used to protect cells from oxidative stress (Masella *et al.*, 2005). Exposure of cells to the antioxidant *N*-Acetyl-L-cysteine (NAC) or GSH protected against polyglutamine-induced cell death (Wyttenbach *et al.*, 2002).

In our studies, mRNA levels of *G6PD*, a rate-limiting enzyme for the generation of NADPH, was increased in both young and old flies with reduced *rpi*. In addition, both the G6PD activity and the reduced form of glutathione were increased by knockdown of *rpi* in *Drosophila* S2 cells. Likewise, overexpression of G6PD increases resistance to oxidative stress and enhances lifespan (Legan *et al.*, 2008). This may explain the mechanism through which lowered *rpi* expression in neurons enhances resistance to oxidative stress and suppression of polyglutamine toxicity. Interestingly, the rescue effect by the knockdown of *rpi* on 108Q toxicity was abrogated by the knockdown of *G6PD* (Fig. 5K). On the other hand, expression of two copies of UAS-*G6PD* can partially rescue 108Q toxicity (Fig. 5M). Together, it supports the notion that the effect by *rpi* knockdown depends on the expression of *G6PD*.

In summary, the knockdown of *rpi* in neurons provides enhanced resistance to oxidative stress, extends lifespan, and alleviates polyglutamine toxicity in *Drosophila* by at least one of the mechanisms to increase NADPH level through elevated G6PD activity. The development of new compounds that suppress ribose-5-phosphate isomerase enzyme activity may serve a new avenue for postponement of aging, a therapy for polyglutamine neurodegenerative diseases, and even on other age-related diseases like cancers.

Experimental procedures

Fly strains and maintenance

All flies were raised on standard fly food at 25°C, 65% humidity, 12-hr light/dark cycle incubator. The fly lines used in the experiment *EP2456*, w^{1118}, *GMR-Gal4*, *Appl-Gal4*, *da-Gal4*, *GMR-Gal4/CyO;UAS-127Q* were originally derived from Dr. Seymour Benzer's lab (Caltech, USA). The RU486-induced *elav-GeneSwitch* (*elav-GS*) Gal4 line was provided by Dr. Pei-Yu Wang, where it was from Dr. Keshishian H. at Yale University. *EP2456* and all the Gal4 drivers (except *elav-GS* here) used in the lifespan assay were back-crossed with w^{1118} ten times and generated as the homozygous lines used in the experiment. The fly lines, *GMR-Gal4,UAS-108Q/Cyo* and *UAS-108Q;Rh1-Gal4* and *Rh1-Gal4* were reported previously (Chang *et al.*, 2011). Transgenic RNAi line *UAS-G6PD*^{RNAi} (#3337) targeting *glucose-6-phosphate dehydrogenase* (*G6PD, CG12529*) and *UAS-tal* ^{RNAi} (#106308) targeting *transaldolase* (*tal*, *CG2827*) were purchased from VDRC. The *G6PD* transgenic overexpression lines *UAS-G6PD*^{9G} and *UAS-G6PD*^{Fb} were kindly provided by Dr. William Orr (Legan *et al.*, 2008).

Oxidative stress and lifespan assays

Four-day-old flies were collected and sexed and kept on the standard fly food with 25 flies per vial overnight. For paraquat assay, the flies were fed with 10 mM paraquat in 5% sucrose solution and the number of deaths was counted 6 h until all the flies were dead. For hyperoxia assay, the flies were kept in a chamber with 95% oxygen adjusted by ProOx 110 (BioSpherix, Ltd., Lacona, NY, USA) and counted daily until all dead. Student's *t*-test was used to calculate *P* value to determine statistical significance. For lifespan assay, the flies were maintained in a 25°C/65% humidity-controlled incubator, transferred to new food every 3–4 days until all were dead. Log-rank test was used to calculate *P* value to determine statistical significance. At least three independent measurements were performed for each experiment.

Generation of the transgenic flies to express either the full-length coding sequence or the double-stranded RNA of *ribose-5-phosphate isomerase*

To generate *UAS-rpi* transgenic flies, the full-length coding sequence (CDS) of *rpi* (*CG30410*, which contains no intron) was amplified from the genomic DNA from Canton S by PCR using forward primer (5′-CAG**CTCGAG**TTAACCACATGTTATCTGGGTT-3′) and reverse primer (5′-GTC**ACTAGT**AACTCCGCCCA AGAACTACTTG-3′), and the purified CDS was subcloned into *Xho*I/*Spe*I sites of the transgenic vector *pINDY6* (Wang *et al.*, 2004). To generate *UAS-rpi*^{RNAi} transgenic flies, the 338-bp partial CDS fragment, which was analyzed by BLAST to avoid the off-target effects, was amplified by PCR with the primer set (5′-TCA**GAATTC**GGCGGTGGACCAGTGGGTGACTGA-3′ and 5′-TGA**GAATTC**AGTCGGCCACCACGATGAAGTGCTT-3′), and the purified partial CDS was subcloned into *EcoRI* site of *SympUAST* vector (Giordano *et al.*, 2002). The constructs were first verified by DNA sequencing to confirm that no point mutation derived from PCR amplification was introduced, and later used to generate the multiple independent transgenic lines. The ability of *UAS-rpi* to overexpress and *UAS-rpi*^{RNAi} to knockdown the expression of *rpi* upon Gal4 induction was verified by RT–PCR, and the folds of changes were listed in the Table S6 (Supporting information). All primer sequence is available upon request.

NADPH measurement

Ten flies of each specific strain were frozen at –80°C in a freezer. The frozen flies were washed with cold phosphate-buffered saline (PBS) and extracted with cold NADP/NADPH extraction buffer. All the sample preparation was always kept on ice and according to the protocol in NADP/NADPH quantitation kit (Cat.# K347-100; BioVision, Mountain View, CA, USA). The values of the samples at O.D. 450 nm were measured and used to calculate NADPH concentration of each sample according the standard curve. The NADPH value of each sample was normalized by its own protein concentration. At least three independent measurements were carried out. Student's *t*-test was used to calculate *P* value to determine statistical significance.

Whole-mount retina immunostaining

The procedure was adapted from the method by Sang & Ready, (2002). The fly eyes of each sample at specific time points were fixed in 4% paraformaldehyde in PBS for 1 h and washed three times in PBST (PBS plus 0.3% Triton X-100) with 10 min each time. Then the eyes were incubated with TRITC-phalloidin (Sigma, St. Louis, MO, USA) to stain F-actin and incubated with 1:20 diluted anti-lamin monoclonal antibodies (ADL67.10, Developmental Studies Hybridoma Bank) in PBST plus 5% goat serum at 4°C overnight. After the eyes were washed three times in PBST, they were incubated with 1:100 diluted FITC-conjugate anti-mouse IgG (Jackson ImmunoResearch) for 4 h, washed three times in PBST, mounted with the mounting medium (VECTASHIELD), and examined by the confocal microscopy with Zeiss 510 (Thornwood, NY, USA).

Phototaxis assay

The 4-day-old male flies of each strain were collected at a density of 30 flies per vial, maintained on the regular fly food, and transferred to new

fly food every 3–4 days to age the flies to the specific time for the whole-mount retina staining and the phototaxis assay (Benzer, 1967). In a dark-room, a 10-watt fluorescent lamp was used as a light source. Approximately 100 flies of the same strain were pooled into a test tube, placed in the countercurrent apparatus, and left to relax for 2 min, then banged down in the apparatus and the empty tube side was placed toward to the light source and given 10 s for flies to react, and slide the tubes to collect the flies walking toward to the light. Performance index (PI) was calculated by the number of the flies walked to the light side divided by the total number of flies. Three independent measurements were performed. Student's *t*-test was used to calculate *P* value to determine statistical significance.

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Author Contributions

C-T Wang, Y-C Chen, Y-Y Wang, M-H Huang, T-L Yen, H Li, C-J Liang, T-K Sang, and S-C Ciou carried out the experiments and analyzed the data; H-D Wang and C-H Yuh designed the experiments, analyzed the data, and together with C-Y Wang and T. J. Brummel discussed the data and wrote the manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1 Knockdown or overexpression of *rpi* by *elav-GS* Gal4 does not alter lifespan in *Drosophila*.

Fig. S2 Increase mRNA level of *glucose-6-phosphate dehydrogenase* (*G6PD*) was detected in longevity mutant both young and old *EP2456*.

Fig. S3 G6PD activity and reduced glutathione (GSH) level were increased in the rpi knockdown *Drosophila* S2 cells.

Fig. S4 The alleviation of polyglutamine toxicity by *rpi* knockdown depends on the expression of *transaldolase*.

Table S1 A summary of lifespan and oxidative stress response in w^{1118} and *EP2456.*

Table S2 A summary of the response to 10 mM paraquat oxidative stress by transgenic flies carrying *UAS-rpi*^{RNAi} with different Gal4 drivers.

Table S3 The summary of the lifespan of the transgenic flies carrying *UAS-rpi*^{RNAi} with *Appl-Gal4*, *GMR-Gal4*, *da-Gal4* drivers.

Table S4 A summary of lifespan among the five independent *rpi* RNAi lines (*l-rpi*) under *Appl-Gal4* and *GMR-Gal4* drivers.

Table S5 A summary of the lifespan of transgenic flies carrying *UAS-rpi* with *Appl-Gal4*, *GMR-Gal4*, *da-Gal4* drivers.

Table S6 A summary of the fold change of *rpi* expression level in the transgenic flies carrying *UAS-rpi*^{RNAi} or *UAS-rpi* with *Appl-Gal4*, *GMR-Gal4*, *da-Gal4* drivers.

Table S7 A summary of the lifespan of the transgenic flies carrying *UAS-rpi*^{RNAi} or *UAS-rpi* with RU486 inducible *elav-GS* Gal4 driver.

Data S1 Experimental procedures.

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