

# *Caenorhabditis elegans* Eyes Absent Ortholog EYA-1 Is Required for Stress Resistance

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**Abstract**—Eyes absent (*Eya*) is a highly conserved transcription cofactor and protein phosphatase that regulates multiple developmental processes throughout the metazoans. It is a dual function protein, working as a transcription factor in the nucleus and as a tyrosine phosphatase in the cytoplasm. In this study, we isolated EYA-1 of *Caenorhabditis elegans*, the only homolog of Eyes absent, and set up an effective feeding-based RNAi (RNA interference) against the gene. We found that knockdown of *EYA-1* decreased heat and oxidative stress tolerance and accelerated the onset of paralysis mediated by Aβ1-42 proteotoxicity and polyQ. Under heat stress (35°C), *EYA-1* knockdown shortened the mean lifespan by 16.8%, which could be attributed to decrease in heat shock protein-16.2 (*hsp-16.2*) expression. Under oxidative stress, *EYA-1* knockdown could shorten the mean lifespan by 18.7%, which could be attributed to intracellular ROS accumulation and the decrease of superoxide dismutase-3 (*sod-3*) protein expression. Moreover, *EYA-1* knockdown animals also showed increased lipofuscin accumulation under oxidative stress. Further studies demonstrated that *EYA-1* knockdown could not inhibit *daf-16* nuclear accumulation in wild-type worms in response to stress. On the other hand, EYA-1 deficiency did not further reduce stress resistance of *daf-16* mutants, which are stress sensitive. Quantitative real-time PCR results also showed that the expression of two *daf-16* target genes, *hsp-12.3* and *sod-3*, was downregulated in EYA-1 RNAi-treated worms under stress. All this evidence indicates EYA-1 is required for stress resistance of worms, and it might act downstream of *daf-16* to regulate expression of stress resistance-associated genes.

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**Key words:** EYA-1, *Caenorhabditis elegans*, RNA interference, stress resistance

*Eyes absent* (*eya*) encodes a highly conserved transcriptional coactivator and protein phosphatase. It was first identified in *Drosophila* as a gene required for eye development [1-3], and its homologs are vital in the development of different organisms ranging from insects to humans [4, 5]. In higher animals, target genes of *eya* are implicated in the development of multiple organs, such as the eyes, muscles, ears, heart, lungs, endocrine glands, placodes, pharyngeal pouches, craniofacial skele-

ton, and parathyroid [6]. In *Drosophila*, specific and recessive *eya* mutations result in elimination of compound eyes in viable flies, whereas *eya* null mutations are lethal to embryos [1]. Mouse mutants for *eya* display abnormal apoptosis and reduced cell proliferation during the development of multiple tissues, such as kidney, muscle, and ear [7]. In humans, *eya* is also implicated in several diseases, such as the multi-organ developmental disorder branchio-oto-renal syndrome [8], congenital cataracts [9], and late-onset deafness [7], and it is overexpressed in numerous types of cancer [10, 11].

The genome of the nematode *Caenorhabditis elegans* contains a homolog of the “eye specification” gene, *EYA-1*. Furuya et al. [12] reported that loss of EYA-1 function by

**Abbreviations:** GFP, green fluorescent protein; HSP-16.2, heat-shock protein; NGM, nematode growth medium; RNAi, RNA interference; ROS, reactive oxygen species.

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RNA interference (RNAi) and deletion mutations resulted in early larval mortality with incomplete penetrance, which was associated with defects in the differentiation and morphogenesis of several tissues and organs. Hirose et al. [13] reported that the *C. elegans* Six-family homeodomain protein (CEH-34) and the eyes absent ortholog (EYA-1) promote programmed cell death of a specific pharyngeal neuron. In this study, we isolated EYA-1 of *C. elegans*, the only homolog of Eyes absent (Eya), and set up an effective feeding-based RNA interference against the gene. Importantly, we found that although knockdown of *EYA-1* in *C. elegans* could not change the lifespan of the worms under normal culture conditions, it could cause a significant shortening of the lifespan under stress condition. Therefore, we suggested that EYA-1 may be required for stress resistance in *C. elegans*. Our results also indicate that in *C. elegans*, EYA-1 is involved in *daf-16*-mediated stress resistance. Moreover, knockdown of *EYA-1* reduced stress resistance by downregulating the expression of stress-resistance-associated genes, such as *sod-3*, *hsp12.3*, and *hsp-16.2*. We believe that these findings will provide new insights into the role of EYA proteins.

## MATERIALS AND METHODS

**Strains of *C. elegans*.** All *C. elegans* strains were maintained at 20°C, except that of temperature-sensitive strain CL4176. The strains used were: N2 Bristol (wild type), CL2070 (*hsp16.2::gfp*), CF1553 *muIs84 [pAD76(sod-3::gfp)*], BA17, *fem-1(hc17)* (fertile at 20°C, infertile at 25°C), CL4176 (*dvIs27[pAF29(myo-3/Aβ 1-42/let UTR) pRF4(rol-6(su1006))]*), AM140 (*rmIs132[P(unc-54) Q35::YFP]*), TJ356 *zIs356 [daf-16::gfp + rol-6]* and CF1038 *daf-16(mu86)*.

**Molecular cloning of *EYA-1*.** We first considered full sequences of the *EYA-1* gene of *C. elegans* from www.wormbase.org and amplified the coding sequence with primers EYaf: 5'-AAAATGCTTCCAGATTCTGAGGGTCAAAA-3' and EYAr: 5'-ATTATCCGCCCAACAAAAGTTGTCCA-3'. PCR products of 1501-bp length were cloned into the vector pBluescript II KS. Sequencing analysis revealed that our sequences are identical to that in the database.

**Knockdown of *EYA-1*.** The full-length coding sequence of EYA-1 was cloned into the pPD129.36 vector. Plain p129.36 vector was used as the control throughout the study. *Escherichia coli* HT115 (DE3) were employed as hosts for the expression of double-stranded RNAs (dsRNAs), and 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce dsRNA expression. The efficiency of RNAi-mediated knockdown was confirmed by Western blot with anti-EYA-1 antibodies (prepared in Dr. Zhao's lab).

**Preparation of *C. elegans* protein extracts and Western blot analysis.** Worms in a mixed population were

collected and extracted by sonication in whole cell extraction buffer containing 25 mM 2-glycerol phosphate (pH 7.3), 5 mM EDTA, 5 mM EGTA, 0.1 M NaCl, 1% Triton X-100, 10 mM β-mercaptoethanol, and a cocktail of protease inhibitors (Roche Applied Science, Germany). Samples containing 20 μg of total protein were separated on 10% SDS gel and transferred to polyvinylidene difluoride (PVDF) membrane for Western blot analysis using the enhanced chemiluminescence method.

**Immunohistochemistry.** Worms were pelleted by brief centrifugation and then fixed overnight at 48°C with 4% paraformaldehyde, dehydrated in graded ethanol, embedded in paraffin, and sectioned at 3 μm thick. Sections were first heated with 0.01 M sodium citrate buffer (pH 6) in a water bath at 95°C for 15 min, and then sequentially reacted with primary and secondary antibodies (biotin-conjugated horse anti-mouse IgG; Vector Laboratories, USA).

**Lifespan assays and stress resistance.** Fertile adults were transferred to fresh nematode growth media (NGM) plates and allowed to lay eggs for 2 h for producing age-synchronized groups of worms. Age synchronized young larvae (L1) were cultured on NGM plates containing *E. coli* cells carrying the vector control or EYA-1 RNAi and maintained at 20 or 25°C. Once the worms reached adulthood, they were transferred daily for 6 days until egg laying ceased to avoid overlapping of generations. After these 6 days, the worms were transferred every second day. The worms were scored as dead if they did not respond to touch stimulus.

Heat-shock assays were performed at 30 and 35°C, using 4-day-old adults. Both control and RNAi-treated worms were cultured at 20°C for 4 days and then transferred to an incubator set to 30 or 35°C. The number of dead worms was counted every 2 h at 30°C, and every half an hour at 35°C.

High concentrations of juglone (5-hydroxy-1,4-naphthoquinone), which is a generator of reactive oxygen species (ROS), have a damaging effect on cells and organisms. Age synchronized young larvae (L1) were cultured on NGM plates containing *E. coli* cells carrying vector control or EYA-1 RNAi and maintained at 20°C for 4 days. Then both control and RNAi-treated worms were transferred into 96-well plates containing 200 μM of juglone in S-medium. The number of dead worms was counted every hour. For all lifespan assays, every experiment was repeated three times and conducted in double-blind manner.

**Fluorescence quantification and microscopy of transgenic *C. elegans* (CL2070, *hsp-16.2::gfp*).** The expression of *hsp-16.2::gfp* (green fluorescent protein) in CL2070 worms was investigated by fluorescence microscopy. Both control and RNAi-treated worms were treated at 35°C for 1 h and allowed to recover for 24 h. For quantification, the worms were anesthetized with sodium azide (10 mM)

on a 5% agarose pad on a glass slide, and the fluorescence was viewed under a microscope (Olympus, Japan) with excitation at 488 nm and emission at 500 to 530 nm. The fluorescence intensity was analyzed using the Image-Pro Plus software (Media Cybernetics). Each experiment was repeated twice, and 15 worms per group were used in each experiment. The data represent mean  $\pm$  standard deviation ( $n = 30$ ) of the two biological experiments.

**Measurement of intracellular reactive oxygen species (ROS) in *C. elegans*.** Intracellular ROS in *C. elegans* were measured with H2DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) as a molecular probe. For ROS detection under normal culture conditions, both control and RNAi-treated worms were incubated at 20°C for 4 days. On day 4, for the ROS test under oxidative stress, worms were treated with 200  $\mu$ M juglone for 2 h. At the end of the specified treatment time, the worms were collected into 100  $\mu$ l phosphate-buffered saline (PBS) with 1% Tween 20 in Eppendorf tubes. They were then pipetted into the wells of a Costar 96-well microtiter plate (black, clear, flat-bottom wells) containing H2DCF-DA (final concentration 200  $\mu$ M in PBS). Samples were read in a Thermo Labsystems Fluoroskan Ascent microplate reader at 37°C with excitation 485 nm and emission 530 nm.

**Lipofuscin accumulation and fluorescence quantification of transgenic *C. elegans* (CF1553, *sod-3::gfp*).** Age synchronized BA17 or CF1553 worms were cultured on NGM plates containing *E. coli* cells carrying vector control or EYA-1 RNAi and maintained at 20°C for 4 days, followed by 200  $\mu$ M juglone for 2 h. Then individuals were anesthetized with sodium azide (10 mM) on 5% agarose pad on glass slides and the fluorescence was viewed under a microscope (Olympus, Japan) with excitations at 488 nm and emissions at 500 to 530 nm. The fluorescence intensity was analyzed using Image-Pro Plus software. Each experiment was repeated twice and 15 worms per group were used in each experiment. Data represent mean  $\pm$  standard deviation ( $n = 30$ ) of two biological experiments.

**Worm paralysis assays.** For strain CL4176 (*dvIs27[pAF29(myo-3/A $\beta$  1-42/let UTR) + pRF4(rol-6(su1006))]*), worms were prepared by synchronous egg laying and induced to express A $\beta$  as third-stage larvae by upshift from 15 to 25°C on NGM plates containing *E. coli* cells carrying vector control or EYA-1 RNAi. All paralysis plots were done in triplicate with 100 worms per condition. For strain AM140 (*rmIs132[P(unc-54)Q35::YFP]*), age synchronized worms were cultured on NGM plates containing *E. coli* cells carrying vector control or EYA-1 RNAi at 20°C. Paralyzed worms were identified as those failing to make forward or backward movement in response to stimulation by tail-prodding.

**Quantitative real-time PCR.** Total RNA was extracted from adult worms with TRIzol reagent (Invitrogen, USA), and cDNA was produced by oligo(dT) priming. The reverse transcription PCR (RT-PCR) primers were

as follows: *act-1*, 5'-GTCATGGTCCGGTATGGGACA-3' and 5'-TTCGTAGATTGGGACGGTGT-3'; *hsp-12.3*, 5'-ACAACCTCTTGCCAAACGA-3' and 5'-CTGCCATCCAACCTTGCTC-3'; *sod-3*, 5'-CAAAAG-CATCATGCCACCTA-3' and 5'-CTCCCAAACGT-CAATTCCA-3'. We used *act-1* as an internal control for equal RNA loading. The mRNA expression was assessed by quantitative real-time PCR on a Bio-Rad IQ5 multi-color real-time PCR detection system using SYBR green as the detection method. The gene expression data were analyzed using the comparative  $2^{-\Delta\Delta Ct}$  method, with *act-1* mRNA as the control.

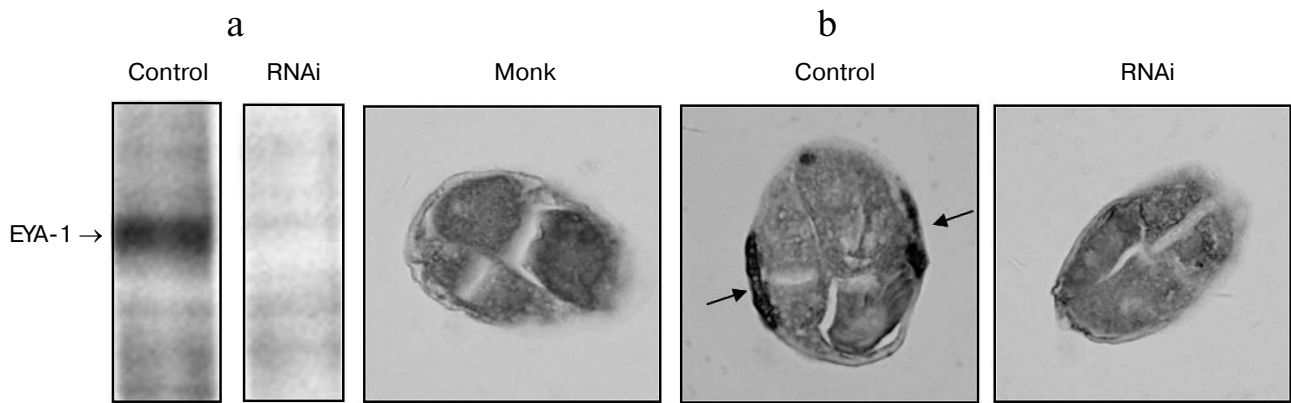
**Statistical analysis.** Statistical comparison between control and RNAi-treated worms was performed with unpaired *t*-test using the GraphPad Prism software (GraphPad Software, USA). Differences with  $p < 0.05$  were considered significant.

## RESULTS

In this study, the full-length coding sequence of EYA-1 was cloned into the pPD129.36 vector that contains the T7 promoter at both 5' and 3' ends to drive transcription. The wild-type N2 worms were fed with HT115 cells that contained the vector control or EYA-1 RNAi. EYA-1 protein expression was detected by Western blot and immunohistochemistry with anti-EYA-1 antibodies. As shown in Fig. 1a, treatment of worms with *E. coli* cells expressing dsRNA derived from a full-length EYA-1 cDNA resulted in almost total deletion of EYA-1. Moreover, the protein of EYA-1 was mainly expressed in body wall muscle cells in the control, but it decreased to near absence in RNAi-treated worms (Fig. 1b). These data indicated the high efficiency of the feeding-based RNAi in knocking down the gene expression of *EYA-1*.

Knockdown of *EYA-1* did not change the lifespan expectancy significantly at 20°C, but it shortened the lifespan of worms at 25, 30, and 35°C in a temperature-dependent manner compared with control (Fig. 2, b-d). Among the temperatures examined, knockdown of *EYA-1* caused the greatest effect on lifespan at 35°C, and it reduced adult mean lifespan to 16.8%. Therefore, we consider that EYA-1 of *C. elegans* may be required for thermotolerance.

*Hsp-16.2* can serve as a stress-sensitive reporter to predict longevity in *C. elegans* [14]. Higher levels of *hsp-16.2::gfp* predict longer mean remaining longevity of *C. elegans*. To study the effect of *EYA-1* knockdown on *hsp-16.2*, *C. elegans* strains CL2070 (*hsp-16.2::gfp*) were used. Compared with the control group, the RNAi-treated group showed lower *hsp-16.2::gfp* intensity in pictures taken with an Olympus fluorescence microscope (Fig. 2e). After quantification using Image-Pro Plus, the data showed that knockdown of *EYA-1* significantly downregulated *hsp-16.2::gfp* expression by 24.5% in CL2070 (Fig.



**Fig. 1.** RNAi-mediated knockdown resulted in almost total deletion of EYA-1. Normal N2 worms were fed with *E. coli* cells carrying the vector control, full-length *EYA-1*. a) Crude cell extracts containing equal amounts of total protein were subjected to Western blot analyses with anti-EYA-1 antibodies and then with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies. b) Paraffin-embedded worm sections were stained with anti-EYA-1 antibody and then with biotin-conjugated horse anti-mouse IgG. Arrows indicate that EYA-1 was expressed in the body wall muscle cells.

2f) ( $p < 0.001$  compared with the control). We consider that knockdown of *EYA-1* downregulates the expression of the *hsp-16.2::gfp* reporter gene in CL2070 under heat stress.

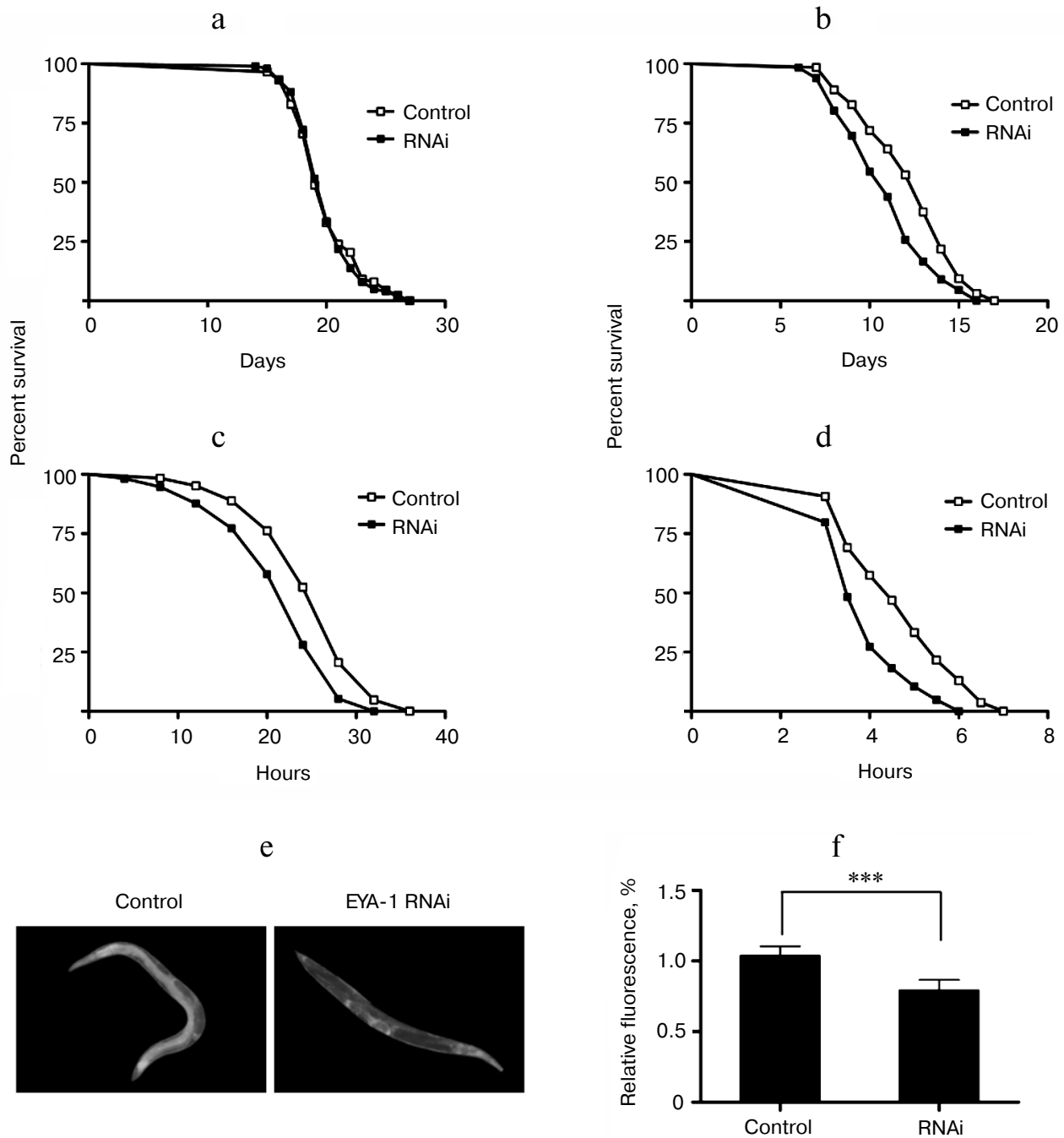
In *C. elegans* and other organisms, environmental conditions or genetic changes that result in increased lifespan are frequently associated with enhanced resistance to stress, including heat stress and oxidative stress [15]. To explore whether knockdown of *EYA-1* can affect *C. elegans* against vulnerability to oxidative stress, we performed oxidative stress resistance assays. We observed that knockdown of *EYA-1* caused a significant shortening of the lifespan in *C. elegans* under oxidative stress generated by juglone. The data showed that the mean survival rate was significantly shortened by 18.7% (Fig. 3a). Therefore, we consider that EYA-1 may be required for oxidative stress resistance in *C. elegans*. Furthermore, we determined intracellular ROS accumulation in 4-day-old adults, and an increase in ROS accumulation was found in RNAi-treated worms in comparison to control using a fluorescence well-plate reader to measure DCF fluorescence (Fig. 3b). According to the “free radical theory of aging” [15], ROS are a crucial factor for aging, and the intracellular amount of ROS can be correlated to stress. Thus, the increased amount of ROS in RNAi-treated worms may explain the decreased survival rate under oxidative stress conditions.

The mechanism by which EYA-1 affects oxidative stress was further studied using transgenic CF1553 worms expressing *sod-3::gfp*. Sod-3 is a typical scavenger enzyme in oxidative stress, and it catalyzes disproportionation of reactive superoxide anion. Fluorescence microscopy revealed a decreased expression of *sod-3* by *EYA-1* knockdown in CF1553. In subsequent studies, we quantified the *sod-3::gfp* expression using the Image-Pro Plus software. Knockdown of *EYA-1* significantly downregulated the

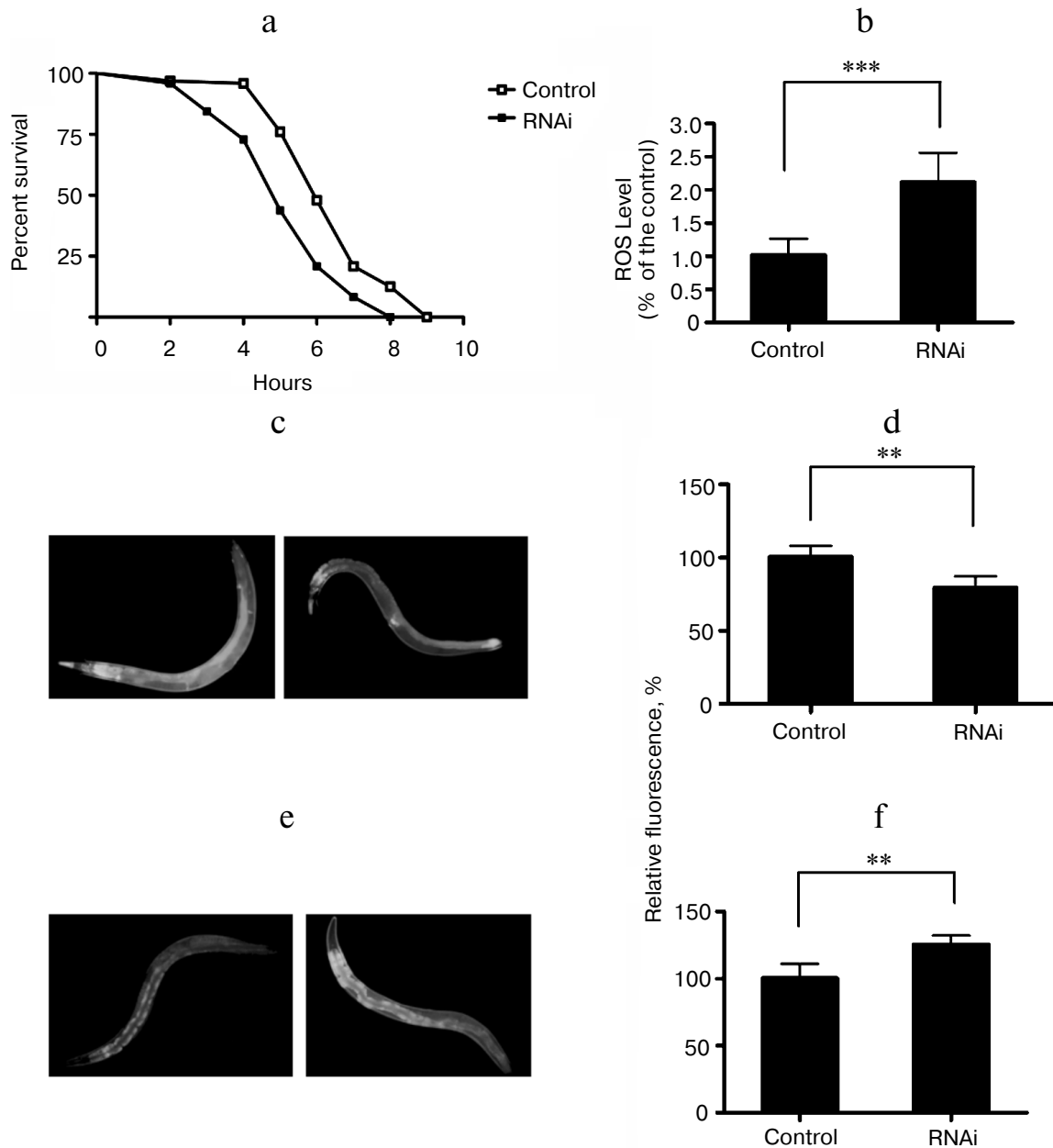
*sod-3::gfp* expression by 17.43% in CF1553 (Fig. 3d) ( $0.001 < p < 0.05$  compared with the control). We consider that knockdown of *EYA-1* downregulates the expression of *sod-3::gfp* in CF1553 under oxidative stress.

Lipofuscin is the fine yellow-brown pigment in granules composed of lipid-containing residues of lysosomal digestion. It is a product of oxidative damage and autophagy that is found in liver, kidney, heart muscle, adrenals, neurons, and ganglion cells. In *C. elegans*, lipofuscin is detectable as autofluorescent granules in the intestine, and its accumulation is a well-established marker of the biological age of *C. elegans* [16]. To estimate the effect of RNAi treatment on lipofuscin levels, the *C. elegans* strain BA17 was used. In comparison to the control group, the RNAi-treated group showed a significantly increased accumulation of lipofuscin (Fig. 3f) ( $0.001 < p < 0.05$  compared with control).

Aggregation of misfolded proteins increases with aging and causes chronic proteotoxic stress, which is the main reason for a variety of age-related neurodegenerative diseases, such as Alzheimer’s disease and Huntington’s disease. As knockdown of *EYA-1* has a negative effect under conditions of heat and oxidative stress, we investigated if knockdown of *EYA-1* can also cause internal proteotoxic stress in *C. elegans*. Two *C. elegans* models of human proteotoxic diseases were exploited here: the strains CL4176 (*dvIs27 [pAF29(myo-3/Ab 1-42/let UTR) + pRF4(rol-6(su1006))]*) and AM140 (*rmIs132[P(unc-54) Q35::YFP]*). The strain CL4176 is engineered to provide temperature-inducible muscle expression of a human  $\beta$ -amyloid peptide ( $A\beta$ ) transgene, resulting in a readable paralysis phenotype of  $A\beta$  toxicity upon temperature upshift to 25°C. Synchronized eggs were cultured on NGM plates containing *E. coli* cells carrying vector control or EYA-1 RNAi at 15°C.  $A\beta$  induction was induced at L3 stage when the temperature was



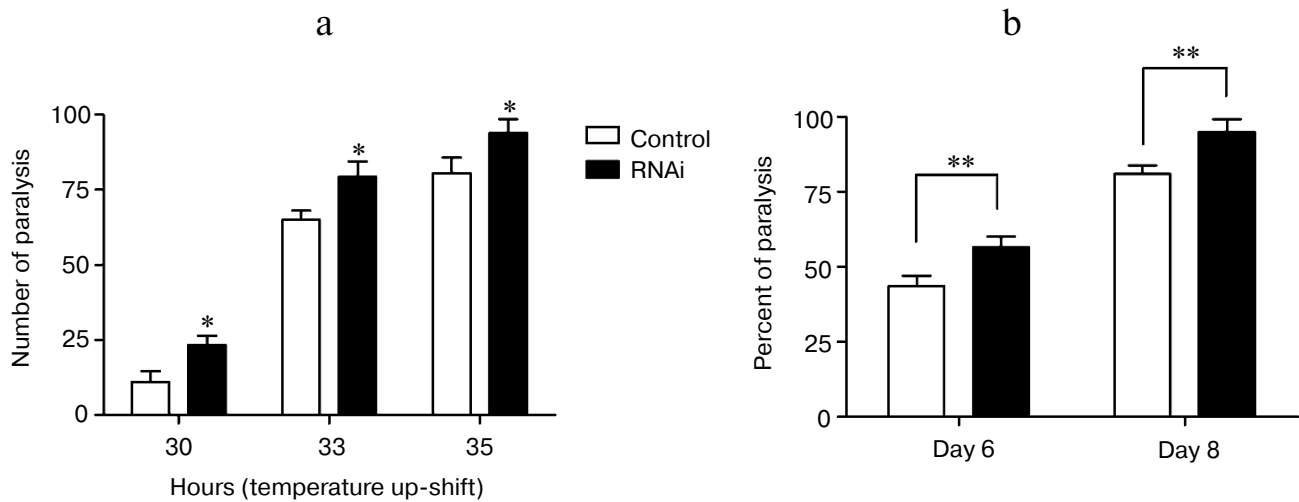
**Fig. 2.** Knockdown of *EYA-1* shortens the lifespan of worms under heat stress. a, b) Survival curves of normal N2 worms cultured on NGM plates containing *E. coli* cells that carry the vector control or *EYA-1* RNAi from day 0 to death at 20 (a) and 25°C (b). The number of dead worms was counted every day. c, d) Survival curves of normal N2 worms under heat stress. Worms were cultured on NGM plates containing *E. coli* cells that carry the vector control or *EYA-1* RNAi at 20°C for 4 days and then transferred to an incubator set to 30 (c) and 35°C (d). This time point was represented by 0. The number of dead worms was counted every 2 h at 30°C and every half an hour at 35°C. All survival curves are presented based on three individual experiments. The data were processed with the GraphPad Prism software ( $n = 250$ ), \*\*\*  $p < 0.001$ . e, f) Effect of *EYA-1* knockdown on the expression of *hsp-16.2::gfp* in *C. elegans*. Both control and RNAi-treated worms were cultured at 20°C for 4 days and then transferred to an incubator set to 35°C for 1 h and allowed to recover for 24 h. e) Image of *hsp-16.2::gfp* expression in control and RNAi-treated worms. f) Quantified *hsp-16.2::gfp* intensity (mean  $\pm$  standard deviation) in CL2070 was from three experiments in each group, with 20 worms in each experiment, as quantified by Image-Pro Plus; \*\*\* $p < 0.001$ .



**Fig. 3.** Knockdown of *EYA-1* reduces resistance to oxidative stress in *C. elegans*. a) Survival curves of normal N2 worms cultured on NGM plates containing *E. coli* cells that carry the vector control or *EYA-1* RNAi at 20°C for 4 days, then both control and RNAi-treated worms were transferred into 96-well plate containing 200 μM of juglone in S-medium. This time point was taken as 0. The number of dead worms was counted every hour. The data were processed with the GraphPad Prism software ( $n = 250$ ),  $*** p < 0.001$ . b) Knockdown of *EYA-1* significantly increased the intracellular ROS level in *C. elegans* under oxidative stress. Both control and RNAi-treated worms were incubated at 20°C for 4 days, and after 2 h under oxidative stress induced by 200 μM juglone the RNAi-treated worms showed a significantly higher DCF (2,7-dichlorofluorescein) fluorescence in comparison to control ( $n = 120$ ),  $*** p < 0.001$ . All the data are presented based on three individual experiments. c, d) Effect of *EYA-1* knockdown on the expression of *sod-3::gfp* in *C. elegans*. Both control and RNAi-treated worms were cultured at 20°C for 4 days followed by 200 μM juglone for 2 h. c) Image of *sod-3::gfp* expression in control and RNAi-treated worms. d) Quantified *sod-3::gfp* intensity (mean ± standard deviation) in CF1553 was from three experiments in each group, with 20 worms in each experiment, as quantified by Image-Pro Plus;  $** 0.001 < p < 0.05$ . e) Intestinal autofluorescence images of lipofuscin in control and RNAi-treated worms. f) Quantified mean fluorescence intensity from intestinal lipofuscin (mean ± standard deviation) in BA17 was from three experiments in each group, with 20 worms in each experiment, as quantified by Image-Pro Plus;  $** 0.001 < p < 0.05$ .

shifted to 25°C. Paralysis at several time points was monitored. A moderate acceleration in paralysis was observed in RNAi-treated transgenic animals at both time durations (Fig. 4a), suggesting that knockdown of *EYA-1* can

exacerbate the Aβ toxicity. Worms expressing 35 glutamine residues conjugated to YFP in body wall muscle (*unc-54p::Q35::YFP*) were cultured on NGM plates containing *E. coli* cells carrying vector control or *EYA-1*



**Fig. 4.** Knockdown of *EYA-1* exacerbates proteotoxicity-mediated paralysis. a) The paralysis associated with muscle A $\beta$ 1-42 expression is exacerbated by *EYA-1* knockdown from hatching in the transgenic strain CL4176. Shown is the non-average paralysis percentages in three independent experiments with 100 animals/experiment at indicated time points after temperature upshift to 25°C; \*  $p < 0.05$ . b) Paralysis associated with Q35 expression is significantly deteriorated by *EYA-1* knockdown from hatching in the transgenic strain AM140. A representative assay of more than three independent experiments is shown. Error bars indicates SD among the paralysis percentages of three independent experiments; \*\*  $0.001 < p < 0.05$ .

RNAi at 20°C. As expected, knockdown of *EYA-1* accelerated the onset of *Q35::YFP*-induced paralysis (Fig. 4b). Thus, we suggest that EYA-1 is required for resistance to multiple stressors, including proteotoxic injury.

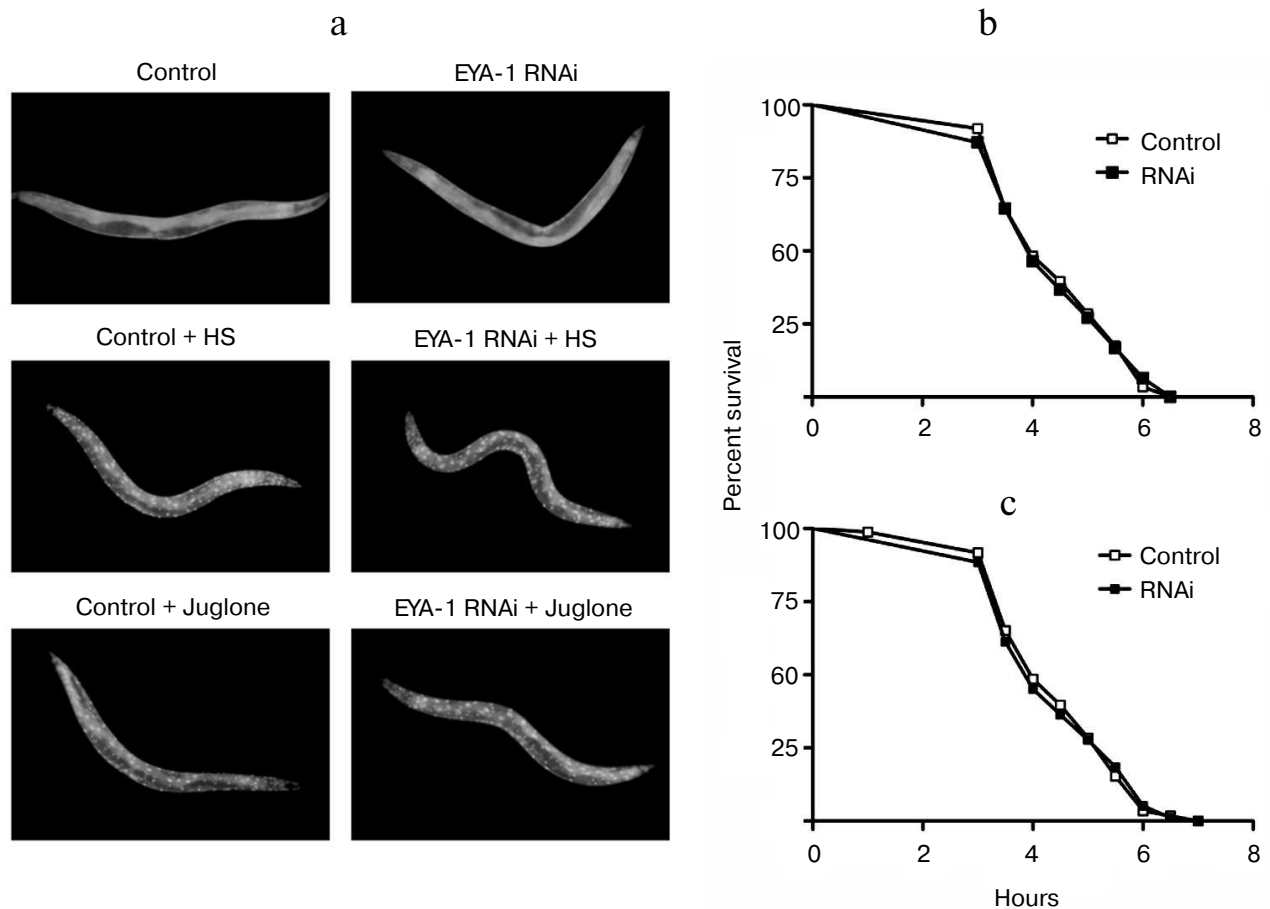
Daf-16/forkhead transcription factor, the downstream target of insulin-like signaling in *C. elegans*, is indispensable for lifespan regulation, stress tolerance, and resistance to proteotoxicity [17]. The daf-16 protein normally lies in the cytoplasm, and in response to stress daf-16 will translocate from the cytoplasm to the nucleus. In the nucleus daf-16 can activate transcription of a large number of genes including *hsp-16.2* and *sod-3*, thus increasing stress resistance and longevity [18]. To examine whether or not EYA-1 plays a role in directing daf-16 to the nucleus in response to stress, we used the TJ356 (*daf-16::gfp*) strain to assess the effect of *EYA-1* knockdown on daf-16 nuclear localization. As a result, both control and RNAi-treated group did not direct *daf-16::gfp* to the nucleus under normal culturing conditions, but both heat treatment at 35°C for 1 h and 200  $\mu$ M juglone for 1 h resulted in rapid nuclear localization of *daf-16::gfp* (Fig. 5a). Our results showed that knockdown of *EYA-1* could not inhibit daf-16 nuclear translocation in response to stress. Hence, EYA-1 is dispensable in directing daf-16 to the nucleus in response to stress. Consequently, we suggest that EYA-1 does not act upstream of the insulin-like signaling pathway to regulate stress resistance. Alternatively, it is also possible that EYA-1 does not act in other pathways, which can direct daf-16 to the nucleus in response to stress. On the other hand, we tested the lifespan in a *daf-16* null mutant strain, which is stress sensitive. As shown in Fig. 5 (b and

c), knockdown of *EYA-1* did not further reduce stress resistance of *daf-16* mutants, suggesting that *EYA-1* knockdown decreases stress resistance of the worms in a daf-16-dependent manner. In conclusion, we consider that EYA-1 may act downstream of daf-16 to carry out its functions in response to stress.

Daf-16 shows its functions by transcriptional regulation of a large number of target genes [18]. The role of EYA-1 in daf-16-dependent gene expression was tested in the context of two well-known daf-16 targets, *hsp-12.3* and *sod-3*. Hsp-12.3 is a small heat shock protein that is activated by daf-16 at the transcriptional level. Sod-3, one of the downstream effectors of daf-16, is also an important regulator of lifespan and stress resistance in *C. elegans*. Under heat stress conditions, EYA-1 RNAi led to decreased mRNA levels of two genes positively regulated by daf-16 (Fig. 6a). On the other hand, under oxidative stress conditions, EYA-1 RNAi led to decreased mRNA level of *sod-3* (Fig. 6a), which helped explain why knockdown of *EYA-1* caused a significant shortening of lifespan of *C. elegans* under stress conditions. Moreover, these results imply that EYA-1 is involved in daf-16-mediated stress resistance for carrying out its functions by transcriptional regulation of daf-16-dependent target genes.

## DISCUSSION

The Eyes absent (Eya) proteins, first identified in *Drosophila*, are now implicated in processes as disparate as organ development, innate immunity, DNA damage repair, photoperiodism, angiogenesis, and cancer metas-



**Fig. 5.** Knockdown of *EYA-1* cannot inhibit *daf-16* nuclear localization and shorten the lifespan of *daf-16* null mutants. a) *daf-16::gfp* distribution was observed in *daf-16::gfp* animals grown on NGM plates containing *E. coli* cells carrying vector control and *EYA-1* RNAi at 20°C (upper panel), or immediately after heat shock at 35°C for 1 h (HS) (middle panel), or 200  $\mu$ M juglone for 1 h (lower panel) ( $n = 20$ ). b, c) Survival curves of *daf-16* (*mu86*) mutants cultured on NGM plates containing *E. coli* cells that carry the vector control or *EYA-1* RNAi at 20°C for 4 days and then transferred to an incubator set to 35°C (b) or 96-well plate containing 200  $\mu$ M of juglone in S-medium (c). This time point was taken as 0. The number of dead worms was counted every half an hour ( $n = 250$ ). A representative assay of more than three independent experiments is shown.

tasis. It is expressed in the nuclei of a subset of anterior cells, which includes pharyngeal and body wall muscle cells, starting from the morphogenesis stage in embryogenesis [6]. In this study, we have cloned a *C. elegans* ortholog of Eya, *EYA-1*. We found that knockdown of *EYA-1* using feeding-based RNAi leads to a nearly total loss of *EYA-1* expression (Fig. 1a) but could not change the lifespan of *C. elegans* under normal culture condition (Fig. 2a). Interestingly, *EYA-1* knockdown caused a significant shortening of lifespan under heat stress and oxidative stress. Because *hsp-16.2* can serve as heat stress-sensitive reporter to predict longevity in *C. elegans* [14], the effects of *EYA-1* RNAi on the expression of *hsp-16.2* protein were investigated. RNAi-treated worms showed significantly lower expression of heat shock proteins as determined through analysis of *gfp* reporter expression, which might explain why knockdown of *EYA-1* decreased thermotolerance in the *C. elegans*. In *C. elegans* pretreat-

ed with juglone, a generator of ROS, a subsequently increased ROS accumulation was observed. Therefore, oxidative stress generated by juglone can result in a shortened lifespan in N2 worms. Does *EYA-1* knockdown reduce oxidative stress resistance of *C. elegans* to shorten its lifespan by accumulating ROS? We got a positive answer from this study. We found that *EYA-1* knockdown significantly upregulated ROS levels under oxidative stress (Fig. 3b). Combined, these results suggested that the sensitivity of RNAi-treated worms to oxidative stress is a specific sensitivity resulting from their decreased ability to detoxify ROS. In addition, *EYA-1* knockdown could downregulate the expression of *sod-3* protein in *C. elegans*, which might explain why *EYA-1* knockdown could significantly decrease the survival of *C. elegans* under oxidative stress. Another oxidative damage marker investigated in this study was lipofuscin, also called age pigment. It is a brown-yellowish autofluorescent material,

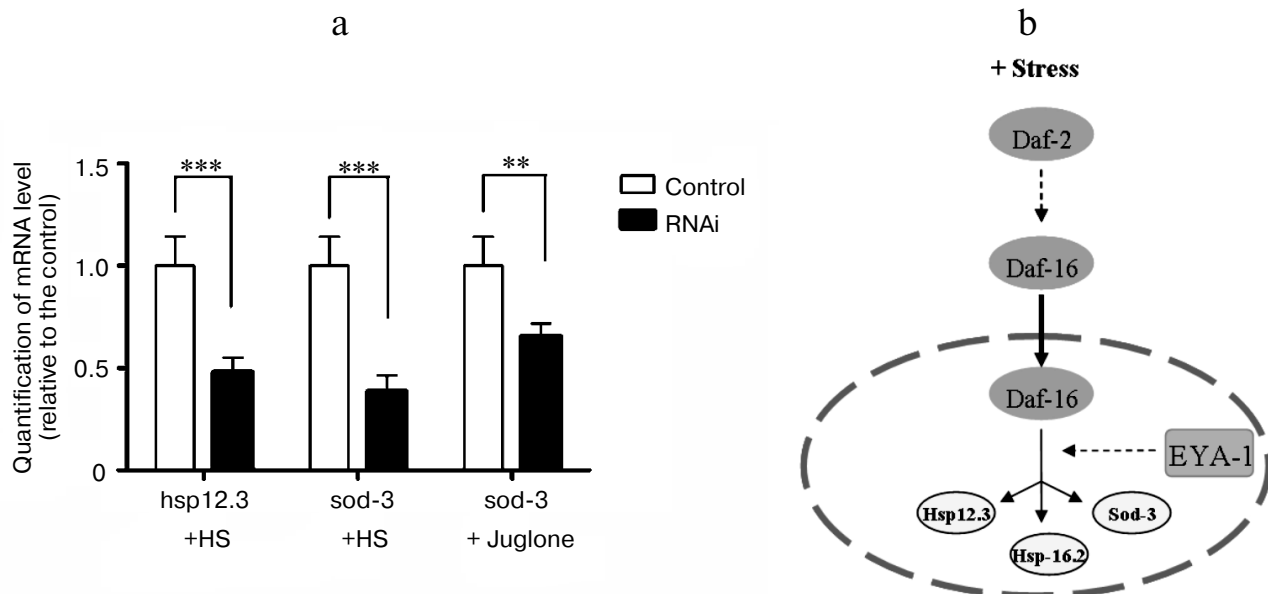


which accumulates in postmitotic cells [19]. The current study showed that knockdown of *EYA-1* could increase the amount of lipofuscin in the worms' intestines (Fig. 3e). Previously, oxidative stress, a condition describing the production of oxygen radicals beyond a threshold for proper neutralization by antioxidant, has been implicated as a pathologic condition in several neurodegenerative disorders, such as Alzheimer's disease and Huntington's disease [20]. The present study demonstrated that *EYA-1* RNAi accelerated the onset of paralysis mediated by A $\beta$ 1-42 proteotoxicity and polyQ in CL4176 and AM140, respectively. Oxidative stress is involved in Alzheimer' and Huntington' pathogenesis; moreover, accumulation of ROS along with the aggregation of toxic amyloid species and Q35-YFP (Q35) is proposed to exacerbate the condition in Alzheimer and Huntington patients [21-23]. Previous study has shown that *EYA-1* knockdown reduced oxidative stress resistance in *C. elegans*, which might be one of the contributing factors for the increased onset of paralysis in the worms.

In *C. elegans*, *daf-16* regulates a wide variety of genes involved in longevity, stress tolerance, resistance to proteotoxicity, metabolism, and development [16-18]. In response to stress, *daf-16* translocates from the cytoplasm to the nucleus. Once *daf-16* enters the nucleus, it enhances the expression of numerous target genes to prevent damage from harmful stresses, which would then confer increased stress resistance and help to maintain normal life in *C. elegans*. Approaching the question on the mode of action of

*EYA-1*, we used transgenic worms TJ356 (*daf-16::gfp*) in which a reporter protein (green fluorescent protein, *gfp*) is fused to the predicted last amino acid of the *daf-16* polypeptide chain [24]. Under normal conditions *daf-16::gfp* remains in the cytoplasm, but under thermal stress (35°C, 1 h), or oxidative stress (200  $\mu$ M juglone, 1 h), a strong nuclear localization of *daf-16::gfp* was observed in control and RNAi-treated worms (Fig. 5a). These results suggest that *EYA-1* knockdown could not impair stress-induced *daf-16* nuclear localization. However, the lifespan of *daf-16* null mutants showed that knockdown of *EYA-1* decreased stress resistance to heat shock and oxidative stress in a *daf-16*-dependent manner (Fig. 5b). Therefore, we suggest that *EYA-1* may act downstream of *daf-16* to carry out its functions in response to stress. The *daf-16* transcription factor is known to upregulate a large number of genes that increase stress resistance and extend the lifespan of worms. Knockdown of *EYA-1* has been shown to reduce the stress resistance of *C. elegans* in a *daf-16*-dependent manner, and these effects may be due to inhibition of *daf-16*-dependent gene expression that reduces stress resistance. As expected, quantitative real-time PCR results suggested that knockdown of *EYA-1* significantly downregulated the expression of the stress resistance-associated genes *hsp-12.3* and *sod-3* (Fig. 6a), which in turn explained why knockdown of *EYA-1* significantly shortened the lifespan of the worms under stress.

In conclusion, *EYA-1* is involved in *daf-16*-mediated stress resistance, and it may act downstream of *daf-16*



**Fig. 6.** *EYA-1* is required for optimal *daf-16*-dependent gene expression. a) Normal N2 worms were cultured on NGM plates containing *E. coli* cells that carry the vector control or *EYA-1* RNAi at 20°C for 4 days and then transferred to 35°C for 2 h or 200  $\mu$ M juglone for 2 h. Relative mRNA levels of *hsp-12.3* and *sod-3* were measured by quantitative RT-PCR. The representative of three independent experiments with similar results is shown. Error bars indicate standard deviation among three replicates in RT-PCR; *t*-test, \*\* 0.001 < *p* < 0.05, \*\*\* *p* < 0.001. b) Sketch: *EYA-1* presumably acts downstream of *daf-16* and function during stress. See text for details.

through regulating stress-resistance-associated genes such as *hsp-12.3* and *sod-3* to carry out its functions (Fig. 6b). Our findings reveal a novel role of the EYA-1 protein in stress resistance.

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