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RME-1 is required for lifespan extension and increased resistance to stresses associated with decreased insulin/IGF-1-like signaling in *Caenorhabditis elegans*

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Abstract: The insulin/insulin-like growth factor (IGF)-1 signaling (IIS) pathway is a conserved lifespan-modulating genetic pathway. Many genes involved in lifespan extension associated with decreased signaling of the IIS pathway have been identified. In the present study, we found a novel gene required for the effect of the IIS pathway on the stress response and aging in *C. elegans*. Receptor mediated endocytosis (RME)-1 is expressed ubiquitously and known to be involved in cellular endocytic transport. Knockdown of *rme-1* abolished the lifespan-extending effect caused by decreased IIS. In addition, resistance to oxidative stress, heat shock and ultraviolet irradiation were significantly decreased when the expression of *rme-1* was blocked. The delayed age-related decline in motility observed in *age-1* mutants with defects in the IIS pathway was also modulated by RME-1. The expression of *sod-3*, which is positively correlated with the remaining lifespan of an individual, was decreased by *rme-1* knockdown. Our study demonstrates that RME-1 is required for the anti-aging effect associated with decreased IIS. We suggest that endocytic transport could be one underlying mechanisms for longevity via the IIS pathway.

Key words: *C. elegans*; insulin/IGF-1-like signaling pathway; lifespan; RME-1; stress response

INTRODUCTION

Aging in multicellular organisms can be defined as the universal, progressive and degenerative impairment of physiological functions. Molecular events observed in aging include the accumulation of oxidative damage on cellular macromolecules, such as DNA, proteins and lipids, dysfunction of mitochondria, and telomere shortening [1,2]. To explain the conserved underlying mechanisms of aging in various species, many theories of aging have been suggested, and include the free radical theory, the mitochondrial decline theory, the telomerase theory, etc. [1,3,4]. However, it is a common belief that there is no single theory of aging that can explain the entire aging process and each theory of aging explains specific phenomena observed in normal aging.

Studies on the aging process increasingly employ experimental molecular-genetic approaches to identify specific factors that may influence the rate of aging. The nematode *Caenorhabditis elegans* is one of the most widely used model organisms in aging studies due to its large brood size, relatively short lifespan and easy genetic manipulation. Genetic screening in *C. elegans* has found that reduced activity of the genes involved in the IIS pathway extends lifespan and increases resistance to environmental stressors. Mutation of *daf-2*, a transmembrane receptor for the IIS pathway, significantly extends both mean and maximum lifespan [5]. Other downstream signal transducers of the IIS pathway, *age-1*, *akt-1*/*akt-2* and *daf-16*, also modulate *C. elegans*' lifespan [6]. Interestingly, the longevity phenotype caused by a decrease in IIS is conserved in other experimental model organisms, including yeast, *Drosophila melanogaster* and mouse [7,8]. Recent studies have found many downstream targets of the insulin/IGF-1-like signaling involved in lifespan extension. Transcriptome profiling studies showed that the expression of genes involved in oxidative stress response, heat shock response and detoxification pathways are upregulated in long-lived *daf-2* mutants [8-10]. These findings suggest that the IIS

pathway controls resistance to environmental stressors and is one of underlying mechanisms of lifespan extension. The cellular pathways involved in the effect of the IIS pathway on aging are not yet understood fully.

Genetic screening for novel genes involved in endocytosis in *C. elegans* has identified several *rme* genes. RME-1 is a member of the Eps15-homology-domain protein family and is involved in the endocytic recycling compartment in delivering membrane proteins from recycling endosomes to the plasma membrane [11]. RME-1 binds and hydrolyzes ATP to form oligomers, which are necessary for endosome association [12]. RME-6 is required for the formation of chlathrin-coated pits and associated with transport from the plasma membrane to endosomes [13]. Mutations in *rme-6* lead to the accumulation of small endocytic vesicles below the plasma membrane [13]. RME-8 functions in receptor-mediated endocytosis in various cells and is required for development and viability in *C. elegans* [14]. In coelomocytes, the scavenger cells of *C. elegans*, RME-8 regulates the process of endocytosis before the lysosome [14]. RME-8 appears to modulate the initial gravity sensing and tropic growth response in *Arabidopsis* and is involved in the membrane trafficking of early endosomes, but not late endosomes, in humans [15,16].

In this study, we examined the role of three *rme* genes, *rme-1*, *rme-6*, and *rme-8*, on the lifespan of *C. elegans*. We observed that RME-1 is specifically required for the lifespan extension associated with decreased IIS. In addition, the involvement of RME-1 in resistance to environmental stressors and age-related decline of motility was investigated. The results of this study will broaden our understanding of the cellular mechanisms involved in lifespan extension via the IIS pathway.

MATERIALS AND METHODS

Worm strains and culture

The N2 strain was used as the wild type in this study. Mutants *age-1* (*hx546*), *eat-2* (*ad465*), and *clk-1* (*e2519*) were purchased from the *C. elegans* Genetics Center (CGC, Minneapolis, St. Paul, MN, USA) and used for the longevity assay. The lifespan of the *age-1* mutant is extended due to a defect in the IIS pathway. The *eat-2* mutant is a genetic model of dietary restriction in *C. elegans*. The long-lived *clk-1* mutant shows a reduced mitochondrial electron transport chain reaction due to a defect in the biosynthesis of ubiquinone. The green fluorescent protein (GFP)-expressing strain CF1553 (muIs84 [P_{sod-3}::GFP, *rol-6*]) was also purchased from CGC. Worms were cultured at 20°C on nematode growth media (NGM) agar plates (1.7% agar, 2.5 mg/mL peptone, 25 mM NaCl, 50 mM KH_2PO_4 pH 6.0, 5 μg/mL cholesterol, 1 mM $CaCl_2$ and 1 mM $MgSO_4$) seeded with *Escherichia coli* OP50 as the food source. Age-synchronized worms were obtained by permitting five young adult (L4) worms to lay eggs on a fresh NGM plate at 20°C. After 4 h, all adult worms were removed from the plate and the eggs were maintained at 20°C for 3 days.

Gene knockdown by RNA interference (RNAi)

All clones used in RNAi experiments were contained in the Ahringer RNAi library and verified by sequencing [17]. An empty vector (EV) bacterial clone was used as a negative control for gene knockdown by RNAi. Worms were fed RNAi from the larval stages. Five L4/young adult worms cultured on NGM plates were transferred to a fresh NGM plate containing 100 μg/mL ampicillin, 12.5 μg/mL tetracycline, 0.4 mM isopropyl-β-D-thiogalactoside (IPTG, Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mg/mL 5-fluoro-2'-deoxyuridine (Sigma-Aldrich, St. Louis, MO, USA) and spotted with bacteria expressing each double-strand RNA. After laying eggs for 4 h, all five adult worms were removed. Sixty young adults were taken 3 days after hatching and transferred to a fresh plate with double-strand RNA expressing bacteria.

Longevity assay

Sixty age-synchronized worms were transferred to NGM plates containing 100 μg/mL ampicillin, 12.5 μg/mL tetracycline and 0.8 mM IPTG and seeded with the bacterial culture of a specific RNAi clone. 12.5 mg/L of 5-fluoro-2'-deoxyuridine were added to the NGM plates to inhibit internal hatching. Live worms were transferred to a fresh NGM plate every 2~3 days and counted every day until all the worms had died. Statistical analysis was performed using the log-rank test [18].

Resistance to oxidative stress

Sixty age-synchronized worms were transferred to a fresh NGM plate seeded with a bacterial culture of each RNAi clone and incubated for 24 h at 20°C. Then, all worms were transferred to NGM plates containing 20 mM paraquat (methyl viologen dichloride hydrate, Sigma-Aldrich, St. Louis, MO, USA) to induce oxidative stress *in vivo*. Thereafter, dead worms were counted three times per day until all the worms were dead. The log-rank test was used for statistical analysis [18].

Heat-shock stress resistance

Age-synchronized 3-day-old worms were picked from a NGM plate and transferred to a fresh NGM plate seeded with a bacterial culture of each RNAi clone. After 24 h of culture at 20°C for 24 h, all worms were moved to a 35°C incubator to induce heat-shock stress. After incubating at 35°C for 10 h, the worms were moved back to a 2°C incubator. Survival rates after 24 h of adaptation at 20°C were compared between EV and *rme-1* RNAi clones. The standard two-tailed Student's t-test was used for statistical analysis.

Ultraviolet (UV) resistance

Sixty age-synchronized worms were prepared as previously mentioned and treated with each RNAi clone for 24 h. Then the worms were exposed to UV (20 J/cm^2/min) for 1 min in a 254 nm-UV crosslinker (BLX-254; Vilber Lourmat, France). Thereafter, dead worms were counted every day until all worms had died. The log-rank test was employed for statistical analysis [18].

Locomotion assay

The response to mechanical stimuli of each worm (n=100) was monitored and recorded at day 15, 20, and 25 after hatching. A worm that moved spontaneously without mechanical stimuli was grouped as "level 1". Worms that could move the whole body or only head part after being stimulated by a worm picker were recorded as "level 2" or "level 3", respectively.

Fertility assay

Five L4/young adult stage worms were transferred to a fresh NGM plate and permitted to lay eggs for 5 h. The eggs were maintained at 20°C for 2 days. A single worm was transferred to a fresh NGM plate containing 5 mM NAC every day until it laid no more eggs. Eggs spawned by a single worm were incubated at 20°C for 48 h, and the number of progeny produced was recorded on each day. The total numbers of progeny produced during the gravid period and fertilities on each day were compared between worms treated with EV or *rme-1* RNAi clones.

Expression of age-related reporters

Age-synchronized 3-day-old CF 1553 worms were fed with EV or *rme-1* RNAi clones for 7 days at 20°C. Then the worms were mounted on a glass slide coated with 2% agarose and anaesthetized with 1 M sodium azide. After covering the slide with a coverslip, expression of each reporter was observed using a confocal microscope (Olympus FV10i, Olympus, Tokyo, Japan). Total fluorescence intensity of a randomly selected single worm was quantified with a fluorescence multi-reader (Infinite F200, Tecan, Grödig, Austria).

RESULTS

RME-1 is specifically required for lifespan extension via the IIS pathway

To determine the role of *rme* genes in lifespan extension, we first examined the effect of *rme* knockdown on the lifespan of long-lived mutants. The expression of *rme-1*, *-6* and *-8* was inhibited using the double-strand RNA of each *rme* gene. We asked whether any of the *rme* genes were involved in known lifespan-extending mechanisms. In *age-1*, *rme-1* RNAi significantly decreased both mean and maximum lifespan among the three *rme* genes tested (Fig. 1a and Table 1). The mean lifespan of *age-1* decreased from 20.8 days to 13.1 days by *rme-1* knockdown ($p<0.001$). There was a 36.7% decrease in mean lifespan. The maximum lifespan was 33 and 25 days in the EV control and *rme-1* RNAi groups, respectively. However,

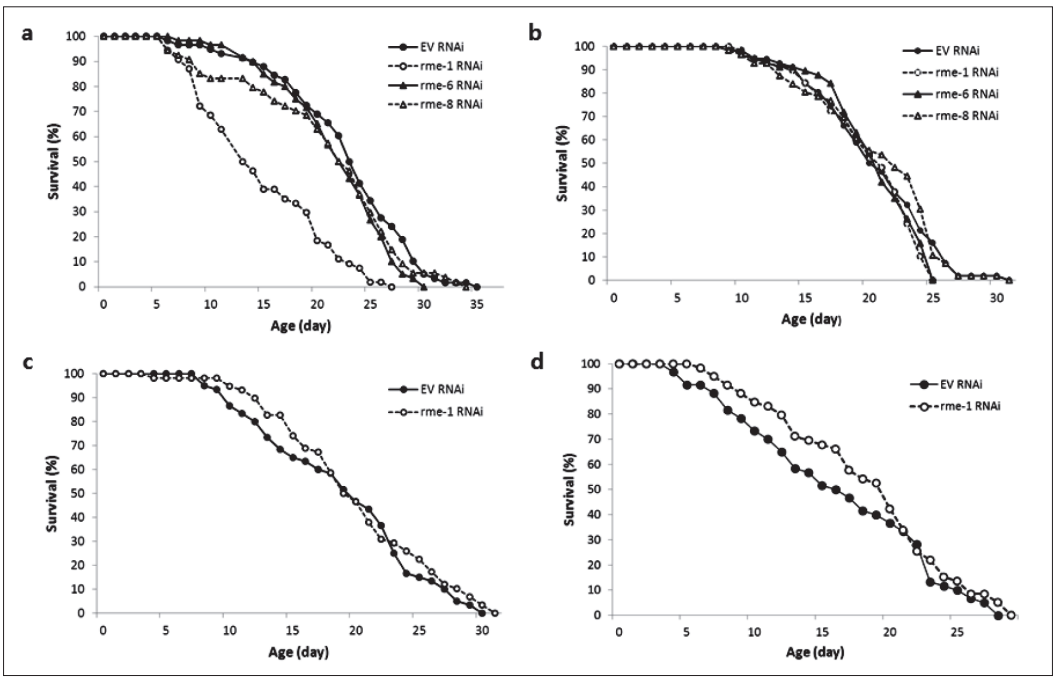

Fig. 1. Effect of *rme* gene knockdown on lifespan of *C. elegans*. The expression of *rme-1*, *-6*, and *-8* was inhibited using RNAi from larval stages and the lifespan of *age-1* (**a**), *eat-2* (**b**), *clk-1* (**c**), and wild-type N2 (**d**) was monitored. Knockdown of each *rme* gene tested showed no effect on lifespan in *eat-2*. However, RNAi of *rme-1* significantly decreased lifespan in *age-1* ($p<0.05$), while knockdown of *rme-6* and *-8* caused no change in lifespan. The lifespan of *clk-1* and N2 was not affected by knockdown of *rme-1* gene. EV – empty vector control.

Table 1. Effect of *rme* gene knockdown on lifespan of long-lived mutants and N2.

	RNAi	Mean lifespan (day)	Maximum lifespan (day)	p-value[a]	% effect[b]
age-1	EV	20.8	33		
	rme-1	13.1	25	<0.001	-36.7
	rme-6	19.8	28	0.113	-4.7
	rme-8	19.1	32	0.347	-8.2
	EV	19.6	30		
	rme-1	17.0	26	0.018	-13.0
eat-2	EV	18.2	30		
	rme-1	18.4	24	0.218	1.2
	rme-6	18.4	24	0.349	1.0
	rme-8	19.8	30	0.619	8.4
	EV	20.2	32		
	rme-1	18.0	29	0.040	-11.2
clk-1	EV	17.9	29		
	rme-1	18.3	30	0.354	1.9
N2	EV	16.3	27		
	rme-1	17.6	28	0.500	7.7

[a] p-value was calculated using the long-rank test by comparing the survival of EV with that of each *rme* gene in each strain. EV – empty vector control.

[b] % effects were calculated by {(C-A)/C} * 100, where A is the mean survival time of each *rme* gene and C is the mean survival time of EV in each strain.

the long lifespan of *eat-2* was not altered by the RNAi of *rme-1*, *-6*, or *-8* (Fig. 1b and Table 1). We monitored the role of *rme-1* in the long-lived *clk-1* mutant. The RNAi of *rme-1* had no effect on the lifespan of *clk-1* (Fig. 1c and Table 1). The lifespan of wild-type N2 was also not affected by *rme*-1 RNAi (Fig. 1d and Table 1). These findings suggest that among the three *rme* genes we examined, only *rme-1* is specifically required for the lifespan extension regulated by the IIS pathway.

Increased resistance to oxidative stress in *age-1* is abolished by *rme-1* knockdown

The free radical theory of aging suggests that the accumulation of free radicals, byproducts of cellular metabolism, causes oxidative damage to cellular macromolecules and the resulting damage is major causal factor of aging [3]. In *C. elegans*, the long-lived *age-1* mutant shows increased resistance to oxidative stress [19]. Dietary supplementation of antioxidants also promotes lifespan extension [20]. We investigated whether *rme-1* can modulate the increased resistance to oxidative stress observed in the long-lived *age-1* mu-

tant. The resistance to oxidative stress was significantly increased in *age-1* compared to the wild-type N2 (Fig. 2). Mean survival time was increased up to 2.8-fold; 49.8 h in N2 and 189.7 h in *age-1* ($p<0.001$). The increased survival under oxidative-stress conditions of *age-1* was significantly reduced by *rme-1* RNAi (Fig. 2). Mean survival time decreased from 189.7 h to 134.9 h ($p<0.001$, 28.9% decrease). As observed in the lifespan assay, the resistance to oxidative stress was not affected by *rme-1* RNAi in wild-type N2. An independent replicate experiment showed the same significant effect by *rme-1* RNAi in *age-1* (data not shown).

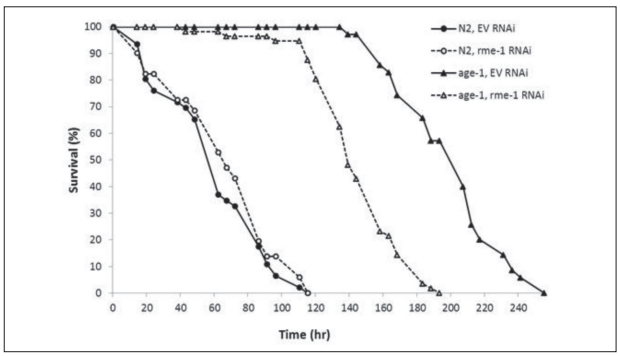

Fig. 2. RNAi of *rme-1* decreased survival under oxidative-stress conditions in *age-1*. RME-1 is required for increased resistance to oxidative stress in *age-1*. Oxidative stress was induced with 20 mM of paraquat in adult worms. Survival under oxidative-stress conditions is increased in *age-1* compared to wild-type N2. Increased resistance to oxidative stress by *age-1* mutation was significantly reduced by *rme-1* gene knockdown ($p<0.05$). RNAi of *rme-1* had no effect on resistance to oxidative stress in wild-type N2. EV – empty vector control.

RME-1 regulates the response to environmental stressors

Mutation of genes causing a decrease in IIS signaling lead to increased resistance to environmental stressors in addition to a longevity phenotype [21]. Having observed the requirement of RME-1 in increased lifespan and survival under oxidative-stress conditions, we investigated the role of RME-1 in response to other environmental stressors. We examined the effect of *rme-1* knockdown on response to heat shock and UV irradiation. The *age-1* mutant showed increased survival after 10 h of heat shock compared to wild-type N2. Only 2.8±2.04% (means±SEM) of N2 worms survived after heat shock, while 26.7±5.36% of worms were still alive after heat shock in *age-1* ($p=0.014$). However, the increased resistance to heat stress observed in *age-1* mutants was almost completely ablated when the expression of *rme-1* was blocked (Fig. 3a). The percentage of worms that survived reduced from 26.7±5.36 to 4.4±2.22% by *rme-1* knockdown in the *age-1* mutant. The % inhibition by *rme-1* RNAi was 93.2% in *age-1* ($p=0.019$).

Resistance to UV irradiation was also significantly enhanced in *age-1* mutants (Fig. 3b). Mean survival times after UV irradiation were 2.6 and 4.2 days in wild-type N2 and *age-1*, respectively. There was a 65% increase in survival after UV irradiation in *age-1* compared to the N2 strain ($p<0.001$). As observed in the response to heat stress, a significant reduction in resistance to UV irradiation was observed in *age-1* by the knockdown of *rme-1*. The mean survival time of *age-1* in which the expression of *rme-1* is prevented was 2.8 days. There was an 85.6% inhibition of sur-

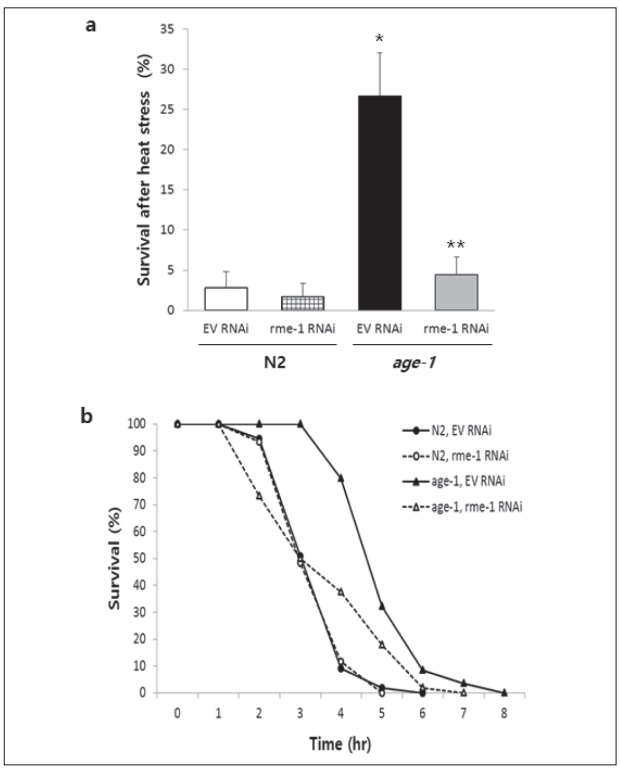

Fig. 3. RME-1 is required for increased resistance to environmental stressors of *age-1*. (**a**) Heat stress was induced by incubating adult worms at 35°C for 10 h. After heat shock, survival of worms was measured in each group. Data show the average of three independent experiments. (**b**) Time-course survival of worms after UV irradiation was monitored hourly. Increased resistances to both heat stress and UV irradiation were observed in *age-1* and abolished by *rme-1* knockdown. Error bars indicate SEM. * – significantly different from EV RNAi in N2 ($p<0.05$); ** – significantly different from EV RNAi in *age-1* ($p<0.05$).

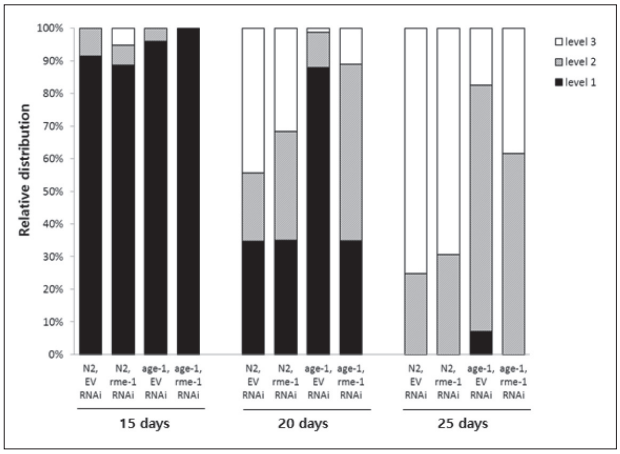

Fig. 4. Role of *rme-1* in age-related decline in motility. Time-course change of motility was determined in N2 and *age-1* at indicated days. Locomotion of each worm was classified into three different levels. ▪ level 1 = worms moved spontaneously without mechanical stimuli; ▪ level 2 = worms moved the whole body in response to mechanical stimuli; □ level 3 = worms moved only the head part in response to mechanical stimuli; EV – empty vector control.

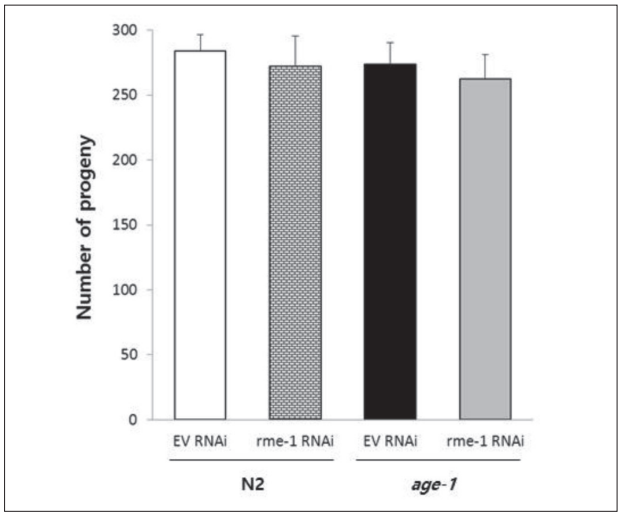

Fig. 5. Effects of *rme-1* knockdown on *C. elegans* fertility. Total number of progeny produced during gravid period was compared between EV and *rme-1* RNAi clones in N2 and *age-1* (n=10) groups. There was no significant difference in the total number of progeny produced among all groups tested. Error bars indicate SEM.

vival after UV irradiation by in *rme-1* RNAi samples (p=0.001). Repeated experiments showed the same significant reduction in resistance to UV irradiation by in *rme-1* RNAi in *age-1* samples (data not shown).

Enhanced motility of *age-1* is dependent on RME-1

One of major tissues heavily damaged by age-related accumulation of free radicals is muscle tissue due to its high demand for mitochondrial energy. To examine the role of RME-1 in muscle aging, we monitored age-related changes in motility. Worms of the same age that could move freely without any mechanical stimuli (level 1), were more prevalent in *age-1* than the wild-type N2 strain (Fig. 4). We observed that 34.9% of animals could be classified as having "level 1" motility in the N2 strain and 88.0% of *age-1* mutants were scored in "level 1" at 20-days of age. On day 25, 7.2% of worms were still in "level 1" among *age-1* while no worms were in "level 1" in the N2 group. The percentage of worms that could move their whole body after mechanical stimuli (level 2) increased from 25.0% in N2 to 75.4% in *age-1* at 25-days of age. Interestingly, inhibition of *rme-1* expression abolished the enhanced motility observed in *age-1* mutants (Fig. 4). The percentage of worms classified as "level 1" in *age-1* was reduced to levels similar to those in the N2 group by knockdown of *rme-1*. On day 20, 35.0% of worms were in "level 1" and no worms were classified as "level 1" on day 25 when the expression of *rme-1* was prevented in *age-1*.

Total number of progeny is not affected by RME-1

Studies show that lifespan-modulating genetic or nutritional interventions may accompany changes in an organism's fertility. A gene expression profiling study demonstrated that genes involved in reproduction were downregulated under oxidative-stress conditions [22]. Therefore, we examined the role of RME-1 in *C. elegans*' reproduction. As shown in Fig. 5, the total number of progeny produced during the gravid period was not significantly affected by knockdown of *rme-1* in either the N2 strain or *age-1* mutants. The total number of progeny produced were 284.2±12.42 (means±SEM) in the N2 group with EV RNAi and 272.0±23.64 in the N2 group with *rme-1* RNAi. The *age-1* mutant produced 273.5±16.70 progeny with EV RNAi and 262.1±18.91 progeny with *rme-1* RNAi.

RME-1 modulates the expression of antioxidant gene

Superoxide dismutase (SOD) is an antioxidant enzyme that converts harmful superoxide into oxygen or hydrogen peroxide. The *C. elegans* genome has many

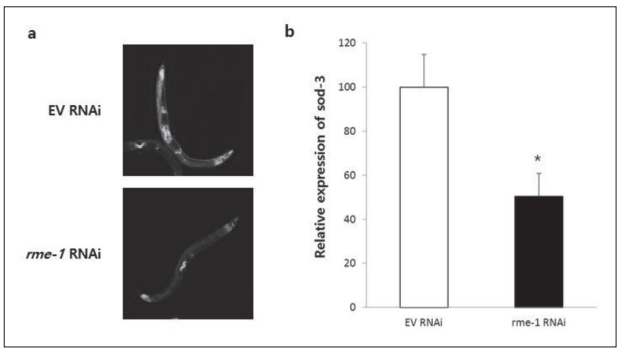

Fig. 6. Expression of *sod-3* was significantly reduced by *rme-1* knockdown in *age-1*. Age-synchronized 3-day-old young adult CF1533 worms containing transgenic GFP under *sod-3* promoter were cultured on NGM plates seeded with bacteria expressing double-strand RNA for 7 days. (**a**) Total GFP fluorescence of each whole worm was compared between EV and *rme-1* RNAi clones. (**b**) Quantification of GFP fluorescence intensity was performed using a fluorescence multi-reader. Data are mean fluorescence intensity per worm. Error bars indicate SEM. * – significantly different compared to EV RNAi ($p<0.05$); EV – empty vector control.

genes encoding SOD isoforms. *sod-1* and *sod-2* encode cytosolic Cu/Zn SOD and mitochondrial Mn SOD, respectively [23]. The expression of *sod-3*, an isoform of mitochondrial Mn SOD, is induced in long-lived *daf-2* mutants [24]. The expression of *sod-3* declines with normal aging and is positively correlated with an individual's remaining lifespan [25]. In this study, we asked whether the expression of *sod-3* is regulated by RME-1 using GFP fused to the promoter of *sod-3*. The expression of GFP significantly decreased in response to knockdown of *rme-1* (Fig. 6a). The fluorescence provided by to P_{sod-3}::GFP fusion protein was markedly reduced by RNAi of *rme-1*. The fluorescent intensity of worms in which the expression of *rme-1* was inhibited was 50.5±10.56% of that of the EV control ($p=0.013$) (Fig. 6b).

DISCUSSION

In the present study, we identified a novel gene, *rme-1*, which specifically mediates anti-stress response and lifespan-extension caused by decreased IIS in *C. elegans*. Knockdown of *rme-1* significantly reduced the lifespan of *age-1*, but had no effect that of *eat-2* and *clk-1*. Previous studies revealed that RME-1 regulates the intracellular transport of membrane proteins, including GLUT4, the transferrin receptor, and the major histocompatibility complex class I [26,27]. These findings suggest that the transport of a key protein modulating lifespan extension to the plasma membrane may be mediated by RME-1 under reduced IIS. Lifespan extension regulated by the IIS pathway requires the transcription factor DAF-16 [10]. Nuclear localization of DAF-16 caused by decreased IIS signaling regulates the transcription of various antioxidant genes, including SOD [10]. We observed a partial inhibition of increased resistance to oxidative stress in *age-1* by knockdown of *rme-1* and a significant downregulation of SOD-3 by *rme-1* knockdown. These findings suggest that RME-1 may work upstream of antioxidant genes induced by DAF-16. Transcriptome profiling of downstream targets of DAF-16 identifies several interesting genes involved in cellular stress-responses, antimicrobial responses and metabolism. Further studies of the relationship between the targets of DAF-16 and RME-1 will broaden our understanding of the role of RME-1 in lifespan extension. RME-1 is also required for the increased resistance to heat stress and UV irradiation observed in the long-lived *age-1* mutants. These results support the hypothesis that there is a positive correlation between stress responses and longevity in *C. elegans*.

RME-1 is necessary for the increased locomotion observed in long-lived *age-1* mutants. Supplementation of silymarin, a natural flavanone derivative, extends lifespan and reduces susceptibility to stress in *C. elegans* [28]. In addition, silymarin increases locomotion rate and response to stimuli [28]. Genetic intervention modulating lifespan and response to stress also regulates age-related changes in muscle function. Mutations in *sesn-1*, a worm ortholog of ROS-scavenging Sestrin, lead to a shortened lifespan and reduced resistance to various stressors, including hydrogen peroxide, heat and heavy metals [29]. Locomotion was significantly decreased in *sesn-1* mutants. Body bending was also lower in *sesn-1* mutants and recovered by overexpression of *sesn-1* [29]. These studies suggest that increased resistance to environmental stressors may be correlated with retardation of the age-related decline in movement and muscle function in *C. elegans*. Our data suggest that RME-1 is necessary for a delayed decline in motility in *age-1* mutants, possibly through an increased resistance to environmental stressors.

The disposable soma theory of aging suggests that the allocation of limited cellular resources between cellular maintenance and reproduction modulates organism aging and lifespan [30]. Ablation of germline cells significantly extends lifespan in *C. elegans* [31]. Dietary supplementation of resveratrol exhibits a longevity phenotype and reduced reproduction [32]. However, extracts from *Acanthopanax sessiliflorus* confers increased resistance to environmental stressors and lifespan without the accompanying reduced fertility [33]. A recent study showed that N-acetyl-L-cysteine increases both lifespan and total number of progeny produced in *C. elegans* [34]. In this study, we observed that there was no significant difference in reproduction between wild-type N2 and long-lived *age-1*. Further, knockdown of *rme-1* does not affect the fertility of individual worms. These findings indicate that RME-1 modulates lifespan extension without the accompanying alteration in fertility.

Recent studies showed that increased autophagy is required for the lifespan extension controlled by the IIS pathway in *C. elegans*. Genes involved in autophagy, such as *bec-1*, *agt-7* and *atg-12*, are essential for the dauer and longevity phenotypes conferred by decreased IIS [35,36]. The autophagy-related gene also regulates resistance to oxidative stress and aging in *Drosophila melanogaster* [37,38]. Since RME-1 is involved in endocytic recycling, we can hypothesize that RME-1 may contribute to the lifespan extension associated with decreased IIS through an increase in autophagy.

The present study shows that *rme-1* is required for the longevity phenotype modulated by the insulin/IGF-1-like signaling pathway. RME-1 is also necessary for increased resistance to environmental stressors. RME-1 is required for endocytic recycling transport. Therefore, our study suggests endocytic transport might be one of the underlying mechanisms for longevity via decreased activity of the IIS pathway. Further studies are required to identify direct target proteins regulated by RME-1 and to prove the role of endocytosis in the longevity phenotype regulated by the IIS pathway.

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Authors' contribution: Chul-Kyu Kim performed all experiments in this study and wrote the manuscript. Sang-Kyu Park designed the experiments, and reviewed and edited the final version of the manuscript.

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