The longevity effect of cranberry extract in *Caenorhabditis elegans* is modulated by *daf-16* and *osr-1*

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Abstract Nutraceuticals are known to have numerous health and disease preventing properties. Recent studies suggest that extracts containing cranberry may have anti-aging benefits. However, little is known about whether and how cranberry by itself promotes longevity and healthspan in any organism. Here we examined the effect of a cranberry only extract on lifespan and healthspan in *Caenorhabditis elegans*. Supplementation of the diet with cranberry extract (CBE) increased the lifespan in *C. elegans* in a concentration-dependent manner. Cranberry also increased tolerance of *C. elegans* to heat shock, but not to oxidative stress or ultraviolet irradiation. In addition,

we tested the effect of cranberry on brood size and motility and found that cranberry did not influence these behaviors. Our mechanistic studies indicated that lifespan extension induced by CBE requires the insulin/IGF signaling pathway and DAF-16. We also found that cranberry promotes longevity through osmotic stress resistant-1 (OSR-1) and one of its downstream effectors, UNC-43, but not through SEK-1, a component of the p38 MAP kinase pathway. However, SIR-2.1 and JNK signaling pathways are not required for cranberry to promote longevity. Our findings suggest that cranberry supplementation confers increased longevity and stress resistance in *C. elegans* through pathways modulated by *daf-16* and *osr-1*. This study reveals the anti-aging property of widely consumed cranberry and elucidates the underpinning mechanisms.

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Introduction

Delaying aging and the onset of age-related diseases is one of the major challenges in human societies worldwide. Dietary intervention is an effective non-genetic way to combat the effect of aging and to improve health. Botanicals, such as blueberry and cranberry, contain numerous biologically active phytochemicals that are thought to be potent in preventing various diseases and promoting health (Liu 2004). The North

American cranberry (*Vaccinium macrocarpon*) and its constituent products have been shown to provide numerous health benefits to humans, including anti-microbial, anti-virus, anti-mutagenesis, anti-angiogenesis, and antioxidation (Pappas and Schaich 2009). The anti-microbial activity of cranberry has been widely recognized due to its potency in the prevention of urinary tract infections (Walker et al. 1997) and probably stomach ulcers (Burger et al. 2002), as well as improvement of oral hygiene (Steinberg et al. 2004). The anti-microbial effect is likely attributable to the ability of cranberry to interfere with the adhesion of pathogens to the surfaces of host cells (Howell 2007; Sobota 1984). In addition, in vitro studies suggest that cranberry phenolics are capable of reducing the level of low-density lipoprotein and preventing platelet aggregation and thrombosis, which result in a reduction of blood pressure and/or inflammation (Bodet et al. 2006; Wilson et al. 1998). Cranberries have also been shown to inhibit the growth and proliferation of tumors in a broad spectrum fashion (Baur and Sinclair 2006; Liu 2004; Verma et al. 1988). The anti-cancer properties can be attributed to a variety of phytochemicals in cranberry, including proanthocyanidin oligomers, flavonols, anthocyanin glycosides, and triterpenoids. These phytochemicals can regulate cell apoptosis, DNA repair, and anti-inflammation (Bodet et al. 2006, 2007a, b; Neto 2007b; Neto et al. 2008).

A recent study showed that a mixture of cranberry and oregano extracts had a prolongevity effect in the Mexican fruit fly (Zou et al. 2010). However, it has not been determined whether the cranberry extract alone can promote longevity in any organism and the mechanisms underlying the anti-aging property of cranberry has not been defined. Here we have undertaken a study to investigate the prolongevity property of the cranberry extract (CBE) in *Caenorhabditis elegans*. *C. elegans* is a genetically tractable multicellular model organism that is widely used to investigate the mechanisms of aging and aging interventions (Guarente and Kenyon 2000; Kenyon 2010; Pietsch et al. 2011; Wilson et al. 2006; Zou et al. 2007). A number of genes and pathways have been identified to modulate lifespan. Many of these lifespan determinants are evolutionally conserved among diverse species. For instance, DAF-16/FOXO is a major longevity determinant and highly conserved in organisms ranging from worms, flies, rodents to humans to

regulate various biological processes, including cell proliferation, apoptosis, stress resistance and carbohydrate, protein, and lipid metabolism, as well as β -cell development and function (Ai et al. 2010; van der Horst and Burgering 2007). The insulin/IGF signaling (IIS) pathway modulates lifespan in species ranging from worms and flies to mammals (Ziv and Hu 2010). In *C. elegans*, insulin-like ligands bind to DAF-2, the counterpart of mammalian insulin/IGF-1 receptor, to initiate the signaling cascade. Subsequently, phosphorylation of AGE-1, PDK-1, and AKT-1/2 inactivates DAF-16/FOXOs by sequestering the protein in the cytosol (Cohen and Dillin 2008; Hertweck et al. 2004; Lee et al. 2001; Morris et al. 1996). Mutations in *daf-2*, *age-1* and *akt-1/2*, or increasing expression of *daf-16* can lead to reduction of the IIS and extension of lifespan in *C. elegans*. We report here that a cranberry extract can increase both lifespan and thermotolerance in *C. elegans*, a species rich in genetic resources. The lifespan extension induced by CBE is not associated with changes in the worms' resistance to oxidative stress, ultraviolet (UV) irradiation, osmotic stress, or physiological functions, such as brood size and motility. Our genetic epistasis analyses suggest that CBE promotes longevity by acting through the insulin signaling cascade and DAF-16, and requiring components of the osmotic stress response pathway, OSR-1 and UNC-43. Our findings reveal the prolongevity property of cranberry and the molecular mechanisms by which cranberry promotes longevity.

Materials and methods

Strains and growth conditions

All strains were maintained at 20 °C on nematode growth medium (NGM) seeded with *Escherichia coli* OP50 feeding strain. One hundred microliters of OP50 was dropped on the center of the 60-mm NGM plates, which were allowed to dry overnight before the culture assays were carried out. Strains used in this study were: N2, Bristol (wild type), *daf-16* (*mgDf50*), *sir-2.1* (*ok434*), *osr-1* (*rm1*), *sek-1* (*ag1*), *unc-43* (*n1186*), *daf-2* (*e1370*), *jnk-1* (*gk7*), *age-1* (*hx546*), *daf-16* (*mgDf47*); *xrIs87*. All the strains were obtained from the *Caenorhabditis* Genetics Center, University of Minnesota, USA.

Cranberry extract preparation

The cranberry extract was kindly provided by R. Ghaedian (Decas Cranberry Products, Inc., Carver, MA) and was described in a previously published study (Zhu et al. 2011). To prepare cranberry supplemented food, the cranberry extract in powder was first dissolved in sterile distilled water. A 10 % fresh stock solution was prepared 1 day before the assay and then the appropriate dilutions were overlaid onto the NGM plates.

Lifespan assay

All lifespan assays were carried out at 25 °C. Synchronized populations were obtained by allowing 10–15 hermaphrodites lay eggs overnight, and the next day the parents were removed. The eggs were allowed to hatch and 30 L4/young adult worms per plate (NGM plate containing 50 µg/mL FUDR to prevent the growth of progeny) were used for each assay. All the assays were carried out in triplicates, and a minimum of two independent trials were performed for all conditions. The dead worms were counted starting the next day and exploding, protruding, bagging or contaminated worms were censored if applicable. We defined the day when we transferred the L4/young adult worms as day 0 of adult age. All statistical analyses were carried out using SPSS software (IBM SPSS Statistics). Kaplan–Meier lifespan analysis was carried out, and *p* values were calculated using the log-rank test. *P*<0.05 was accepted as statistically significant.

Stress assays

For stress assays, worms were pretreated with 2 mg/mL CBE for two generations at 25 °C on OP50 seeded NGM plates. Worms on regular NGM plates without CBE served as controls. Each assay was carried out in three independent trials, and the data were pooled and analyzed using Student's *t* test. *P*<0.05 was accepted as statistically significant.

For the oxidative stress assay, we first tested the survival of L4/young adult N2 worms at 25 °C on NGM plates containing serial dilutions of paraquat (0.1–10 mM) (Sigma-Aldrich, Corp. St. Louis, MO, USA). The concentration, 5 mM, that resulted in ~20 % of worm death on the second day (LD₂₀=

5 mM) was selected for subsequent cranberry protection assays. CBE pretreated L4/young adult worms and their controls were transferred onto NGM plates containing 5 mM paraquat, and the survival was counted daily until all worms died (i.e., the 10th day after paraquat exposure).

The UV-induced stress assay was carried out using a UV crosslinker (CL-1000 Ultraviolet Crosslinker UVP). We first determined the LD₂₀ by subjecting L4/young adult worms to UV irradiation in the range of 0.05 to 1.0 J/cm² for 20, 30, and 60 s, respectively. Their survival rates were monitored on the second day. We found that the LD₂₀ was at 0.05 J/cm² for 20 s, which was chosen for subsequent experiments. For the cranberry protection assay, CBE-pretreated L4/young adult worms and controls were transferred to CBE (2 mg/mL) plates and NGM plates, respectively, and were irradiated at 0.05 J/cm² for 20 s. The lifespan of the worms was followed at 25 °C.

The heat shock assay was performed as described previously (Pietsch et al. 2009; Strayer et al. 2003) with some modifications. CBE-pretreated L4/young adult stage worms and controls were transferred to CBE supplemented (2 mg/mL) plates and regular NGM plates, respectively. The plates were incubated at 37 °C for 3.5 h and then transferred back to 25 °C. The survival of the worms was monitored daily.

The osmotic stress assay was conducted as described previously (Solomon et al. 2004). For the acute osmotic stress assay, CBE-pretreated worms and their controls were exposed to high salt concentrations (300 and 500 mM NaCl) for 10 min and then transferred to regular unseeded NGM plates to monitor their motility behavior. For the chronic osmotic stress, CBE-treated and non-treated worms were subjected to a high salt concentration (500 mM) and then their mortality rate was monitored for the next 12 h.

Measurement of reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were measured using 2',7'-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Carlsbad, CA, USA) as described previously (Lee et al. 2009, 2010) with some modifications. CBE-pretreated and non-treated L4/young adult worms were exposed to 5 mM paraquat and collected at day 1 and day 4. Age-matched CBE-pretreated and non-treated adult worms without paraquat challenge were used as

controls. For each assay, approximately 1,000 worms were collected and washed three times with M9 buffer to remove bacteria. The worms were then washed with 100 μ L of PBS once and were immediately frozen by placing them in a -80°C freezer. The worm extracts were prepared by sonication and subsequent centrifugation at 13,000 rpm at 4°C for 30 min. The supernatant was transferred to a new tube and centrifuged again at 13,000 rpm at 4°C for 30 min. Ten microliters of the supernatant was used for protein quantification using the Bradford method. Supernatant containing 50 μ g of protein was pre-incubated with 250 μ M of DCF-DA in 100 μ L of PBS at 37°C for 1 h. Fluorescence intensity was measured with a fluorometer (molecular devices, SPECTRAmax Gemini XS, USA) at the excitation wavelength 485 nm and the emission wavelength 535 nm. The fluorescence was measured once every 30 min for 1.5 h. The time-dependent increase in the fluorescence was linear for this time frame. We therefore chose to use fluorescence intensity at 1 h for the quantification. The fluorescence intensity was normalized by subtracting the background fluorescence of 250 μ M DCF-DA solution in PBS at 1 h. This assay was carried out in three independent trials. The data were pooled and analyzed using Student's *t* test. $P < 0.05$ was accepted as statistically significant.

Brood size and body bend assays

The brood size assay was carried out by transferring a single L4/young adult worm onto an NGM plate supplemented with or without 2 mg/mL CBE (triplicates). Each worm was allowed to lay eggs at 25°C for 24 h and was then transferred to a fresh plate until it ceased to lay eggs. The offspring were counted after they reached the L3 or L4 phase. This assay was carried out in three independent trials. The data were pooled and analyzed using Student's *t* test. $P < 0.05$ was accepted as statistically significant.

For the motility assay, worms were pretreated with or without 2 mg/mL CBE for two generations on NGM plates at 25°C . Ten CBE-pretreated or non-treated L4/young adult nematodes were placed onto individual NGM plates without OP50. The body bends per minute were counted. This assay was carried out in three independent trials. The data were pooled and analyzed using Student's *t* test. $P < 0.05$ was accepted as statistically significant.

DAF-16 translocation assay

The *daf-16(mgDf47); xrls87* worms carrying a DAF-16-GFP fusion construct (Lee et al. 2001) were treated with 2 mg/mL CBE for 1 h and were then observed under a fluorescence microscope (Nikon AZ100) to monitor the nuclear translocation of DAF-16-GFP.

Gene expression analysis by quantitative PCR

The nematodes were grown on NGM plates supplemented with or without 2 mg/mL CBE at 25°C until they reached the young adult stage. The worms were collected with M9 buffer. RNA was prepared using Absolutely RNA miniprep kit (Agilent technologies) and stored at -80°C . Complementary DNA was prepared by using Invitrogen Superscript first strand synthesis system for real-time polymerase chain reaction (RT-PCR) (Invitrogen). Quantitative PCR (qPCR) was performed using SsoFast EvaGreen Supermix and the iCycler iQ real-time PCR detection system according to the manufacturer's suggested protocol (Bio-Rad). The qPCR conditions were: 95°C for 3 min followed by 40 cycles of 10 s at 95°C and 30 s at 60°C . The *act-1* was used as an internal control to normalize the expression levels of target transcripts. Each qPCR experiment was repeated three times using independent RNA preparations. The data were pooled and analyzed using Student's *t* test, and $p < 0.05$ was accepted as statistically significant. The qPCR primers for *sod-3* are: 5'-CCAACCAGCGCTG-AAATTCATGG-3' (forward primer (F)) and 5'-GGAACCGAAGTCGCGCTTAATAGT-3' (reverse primer (R)). Primers for *daf-16* are 5'-CCAGACGGAAGGCTTAAACT-3' (F) and 5'-ATTGCGATGAAACGAGAATG-3' (R). Primers for *hsp-4* are 5'-TGACTCGTGCCAAGTTTGAG-3' (F) and 5'-GCTCCTTGCCGTTGAAGTAG-3' (R). Primers for *hsp-16.1* are 5'-GCAGAGGCTCTCCATCTGAA-3' (F), and 5'-GCTTGAAGTGCAGACATTG-3' (R). Primers for *hsp-16.49* are 5'-GCTCATGCTCCGTTCTCATATTCTGATTCAAATGC-3' (F) and 5'-GCAACAAAATTGATCGGAATAGAACGTGATGAG-3' (R). Primers for *hsp-70* are 5'-CGTTTCGAAGAACTGTGTGCTGATCTATTCCGG-3' (F), and 5'-TTAATCAACTTCCTCAACAGTAGGTCCTTGTGG-3' (R). Primers for *hsp-12.6* are 5'-ATGATGAGCGTTCCAGTGAT

GGCTGACG-3' (F), and 5'-TTAATGCATTTTCTTGCTTCAATGTGAAGAATTCC-3' (R). Primers for *act-1* are: 5'-CCAGGAATTGCTGATCGTATGCAGAA-3' (F), and 5'-TGGAGAGGGAAGCGAGGATAGA-3' (R).

Results

CBE supplementation extends the lifespan of *C. elegans* in a dose-dependent manner

To determine the longevity property of cranberry, we treated wild-type N2 worms with CBE at concentrations ranging from 0 to 10 mg/mL in the standard nematode growth medium (NGM) at 25 °C. CBE at 2 mg/mL increases mean lifespan from 12 to 15.9 days, while CBE at 10 mg/mL decreased the mean lifespan from 12 to 10.5 days (Fig. 1; Table 1). We found that CBE at ≥ 6 mg/mL reduced pH of the NGM ($\text{pH} \leq 6.0$) probably due to the acidogenicity of CBE. To test whether the acidogenicity of CBE caused the shortened lifespan, we neutralized CBE with a Tris base solution ($\text{pH} 9.0$) before supplementing it to worms. Controls were treated with the same amount of Tris buffer but at $\text{pH} 7.4$. We found that the neutralized CBE affected lifespan in a similar way as the original CBE (data not shown). Taken together, these findings

indicate that CBE supplementation can extend lifespan in *C. elegans*, and this lifespan extension is dose-dependent.

Considering that cranberry possesses antimicrobial properties, it is possible that CBE inhibits the growth of feeding *E. coli* OP50 under our assay conditions, thereby extends *C. elegans* lifespan via dietary restriction. To test this hypothesis, we cultured OP50 in LB medium with a series of concentrations of CBE (10, 5, 2, and 0.2 mg/mL). We found that CBE at these concentrations did not inhibit the growth of *E. coli* OP50 at any bacterial growth phases. CBE at 2 mg/mL (the optimal concentration for lifespan extension) even promoted the growth of OP50 as compared to the control medium (data not shown). Therefore, it is unlikely that CBE prolongs *C. elegans*' lifespan through its antimicrobial effect.

CBE supplementation alters worms' response to heat shock stress and osmotic stress but not to oxidative stress or UV irradiation

Lifespan extension is generally associated with elevated stress resistance (Broughton et al. 2005; Clancy et al. 2002; Larsen 1993; Lithgow et al. 1994). To test whether CBE enhances stress resistance, we exposed *C. elegans* to a series of environmental stressors. We first tested oxidative stress. Adult wild-type N2 worms were pretreated with and without 2 mg/mL CBE for two generations at 25 °C and then were transferred to NGM plates containing 5 mM of paraquat. Paraquat is a chemical commonly used to generate reactive oxygen species and induce oxidative stress in the cell (Fukushima et al. 1993; Yamada and Fukushima 1993). The survival was monitored over 10 days. We found that paraquat reduced the lifespan, but CBE supplementation did not increase worms' resistance to paraquat (Fig. 2a). Considering that paraquat is a very potent toxicant, paraquat treatment may not only induce ROS but also generate other toxic effects. It is possible that CBE supplementation is able to reduce paraquat-induced ROS, but fails to antagonize other toxic effects. To address this concern, we measured ROS levels in worms treated with or without CBE using the DCF-DA staining method. We found that CBE supplementation did not significantly reduce the ROS levels in 1-day and 4-day-old adult worms relative to age-matched non-supplemented control (Fig. 2b). Although paraquat treatment increased the

Fig. 1 CBE extends *C. elegans* lifespan in a dose-dependent manner. Wild-type N2 worms were treated with CBE at 0–10 mg/mL. Each lifespan experiment was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments are included in Table 1

Table 1 The lifespan of various worm strains at 25 °C

Strain	Mean $\pm$ SE	Median	No. of worms	<i>P</i> value
N2 ^a	11.83 $\pm$ 0.946	12.0	310	
N2+0.2 mg/mL CBE ^a	13.50 $\pm$ 0.95	13.0	325	0.187
N2+2 mg/mL CBE ^a	15.90 $\pm$ 1.20	16.0	298	0.001
N2+10 mg/mL CBE ^a	10.50 $\pm$ 0.76	10.0	303	0.190
N2 ^b	10.62 $\pm$ 0.96	11.0	267	
N2+2 mg/mL CBE ^b	16.73 $\pm$ 1.25	17.0	299	0.007
<i>daf-16(mgDf50)</i> ^b	8.66 $\pm$ 1.13	9.0	301	
<i>daf-16(mgDf50)</i> +2 mg/mL CBE ^b	9.12 $\pm$ 1.08	9.0	287	0.840
N2 ^b	11.88 $\pm$ 0.98	12.0	184	
N2+2 mg/mL CBE ^b	14.60 $\pm$ 1.29	15.0	191	0.047
<i>daf-2(e1370)</i> ^b	26.66 $\pm$ 1.72	27.0	167	
<i>daf-2(e1370)</i> +2 mg/mL CBE ^b	27.94 $\pm$ 1.73	28.0	183	0.978
N2 ^b	12.50 $\pm$ 0.77	12.0	193	
N2+2 mg/mL CBE ^b	15.00 $\pm$ 1.13	15.0	167	0.050
<i>age-1(hx546)</i> ^b	28.50 $\pm$ 1.37	28.0	189	
<i>age-1(hx546)</i> +2 mg/mL CBE ^b	27.70 $\pm$ 1.44	28.0	174	0.561
N2 ^c	12.50 $\pm$ 0.96	12.0	293	
N2+2 mg/mL CBE ^c	15.40 $\pm$ 1.11	15.0	271	0.040
<i>osr-1(rm1)</i> ^c	10.50 $\pm$ 0.87	10.0	300	
<i>osr-1(rm1)</i> +2 mg/mL CBE ^c	9.90 $\pm$ 1.11	10.0	282	0.890
<i>unc-43(n1186)</i> ^c	14.00 $\pm$ 1.00	14.0	307	
<i>unc-43(n1186)</i> +2 mg/mL CBE ^c	14.97 $\pm$ 1.12	15.0	282	0.336
<i>sek-1(ag1)</i> ^c	12.50 $\pm$ 1.04	12.5	199	
<i>sek-1(ag1)</i> +2 mg/mL CBE ^c	14.00 $\pm$ 1.15	14.0	198	0.197
N2 ^d	12.00 $\pm$ 0.82	12.0	263	
N2+2 mg/mL CBE ^d	16.60 $\pm$ 1.01	16.0	299	0.001
<i>jnk-1(gk7)</i> ^d	10.78 $\pm$ 0.05	11.0	275	
<i>jnk-1(gk7)</i> +2 mg/mL CBE ^d	14.50 $\pm$ 1.56	15.0	296	0.027
N2 ^d	12.57 $\pm$ 1.48	13.0	295	
N2+2 mg/mL CBE ^d	18.78 $\pm$ 1.02	19.0	284	0.006
<i>sir-2.1(ok434)</i> ^d	13.00 $\pm$ 1.91	13.0	293	
<i>sir-2.1(ok434)</i> +2 mg/mL CBE ^d	16.10 $\pm$ 1.18	16.0	297	0.025

Lifespan and standard error are shown in days. The lifespan experiments were repeated at least three times with similar results, and the data for representative experiments are shown. The lifespan data were analyzed using the log-rank test and *p* values for each individual experiment are shown

^aResults presented in Fig. 1

^bResults presented in Fig. 3

^cResults presented in Fig. 5

^dResults presented in Fig. 4

ROS level relative to the non-treated groups, CBE supplementation was not effective in reducing the ROS level in worms exposed to paraquat (Fig. 2b).

Next, we tested the worms' resistance to UV irradiation. Wild-type young adult worms were exposed under 0.05 J/cm² UV irradiation for 20 s. The survival was monitored at 25 °C over the next 10 days. CBE supplementation had no or little effect on the survival of worms after UV irradiation (Fig. 2c). Our findings indicate that CBE supplementation does not improve resistance to acute oxidative stress or UV irradiation in *C. elegans*.

To test the worms' resistance to heat shock, young adult N2 worms pretreated with or without CBE for two generations at 25 °C were shifted to 37 °C for 3.5 h and then back to 25 °C. Wild-type *C. elegans* pretreated with CBE showed statistically significant higher resistance to heat shock compared to CBE untreated controls (Fig. 2d), indicating that CBE supplementation can elevate resistance to heat shock in *C. elegans*. Increasing the expression levels of heat shock proteins (HSPs) is known to have a protective effect and enhance stress resistance in *C. elegans* (Rea et al. 2005; Walker and Lithgow 2003; Yokoyama et al.

2002). We next measured changes in the mRNA levels of several representative HSPs, including *hsp-4*, *hsp-12.6*, *hsp-16.1*, *hsp-16.49*, and *hsp-70*. The mRNA level of *hsp-12.6* was upregulated with CBE treatment relative to the non-supplemented controls (Fig. 2e). However, *hsp-4*, *hsp-16.1*, and *hsp-16.49* were down-regulated by CBE treatment, while *hsp-70* did not show a significant change (Fig. 2e). These findings suggest that other mechanisms in addition to the heat shock response underlie the CBE-induced thermotolerance.

A common approach to assess the health status in *C. elegans* is to measure its locomotion behavior, including body bending (de Bono and Maricq 2005). Old worms generally develop motor deficits and are less active than young ones (Dillin et al. 2002; Glenn et al. 2004; Hosono et al. 1980; Huang et al. 2004; Johnson et al. 1988). Here, we use body bending frequency to examine *C. elegans*' acute and chronic response to osmotic stress. To this end, young adult N2 worms were pretreated with or without 2 mg/mL CBE for two generations, and were then transferred to hyperosmolarity plates containing high concentrations of NaCl. For the acute response assay, we exposed worms to either 300 or 500 mM of NaCl for 10 min and then transferred the worms back to regular NGM plates to measure the frequency of body bending as a parameter to reflect the motility of the nematode. The motility of CBE-treated worms was dramatically reduced compared to non-treated controls (Fig. 2f). For the chronic response assay, worms were kept on the NGM plates containing 500 mM of NaCl, and dead worms were counted until all worms died. Surprisingly, the mortality rate of CBE-treated worms was higher relative to non-treated controls (Fig. 2g). Taken together, we conclude that CBE treatment increases worms' susceptibility to osmotic stress.

Influence of CBE treatment on reproductive capacity and motility

Considering that CBE promotes lifespan and thermotolerance in *C. elegans*, we investigated whether CBE alters the worms' other general physiological indexes. In this regard, we tested brood size as a reproductive parameter and motility of worms to reflect their general fitness after CBE treatment. CBE treatment did not affect total offspring quantity compared to non-supplemented controls (Table 2). For the motility assay, young adult worms pretreated with or without

CBE for two generations were individually transferred to fresh NGM plates, and the body bends per minute were counted (Hart 2006). CBE did not affect the worms' body bend frequency, namely motility, compared to non-supplemented controls (Table 2). These findings suggest that CBE-induced lifespan extension is not associated with any significant change in reproductive capacity and motility.

CBE supplementation modulates lifespan through the IIS pathway and DAF-16

To understand the molecular mechanisms by which CBE promotes lifespan, we investigated which longevity determinants CBE genetically interacts with. We first conducted a genetic epistasis assay to test the relationship between CBE and the IIS pathway/DAF-16 (Henderson and Johnson 2001; Kenyon et al. 1993; Lee et al. 2001; Lin et al. 2001). We supplied null *daf-16(mgDf50)* mutant worms with 2 mg/mL CBE and found that CBE did not increase the lifespan of the *daf-16(mgDf50)* mutant (Fig. 3a; Table 1). This finding suggests that the longevity effect of CBE requires the activity of DAF-16. Next, we tested DAF-2 and AGE-1, two major components of the IIS pathway. Mutant worms, *daf-2(e1370)* and *age-1(hx546)*, were treated with 2 mg/mL CBE, respectively. CBE at 2 mg/mL did not extend the lifespan of either *daf-2(e1370)* (Fig. 3b; Table 1) or *age-1(hx546)* (Fig. 3c; Table 1), when compared to the genotype matched non-supplemented controls. These findings suggest that CBE acts at least partly through the IIS pathway and requires DAF-16 to increase lifespan in *C. elegans*.

Considering that the IIS pathway regulates lifespan in *C. elegans* by limiting DAF-16 nuclear localization (Henderson and Johnson 2001; Lee et al. 2001; Lin et al. 2001), we postulated that CBE treatment may affect DAF-16 nuclear localization and transcriptional activities. To this end, we examined subcellular localization of DAF-16 using transgenic worms with a DAF-16-GFP fusion transgene (Honda and Honda 1999; Lin et al. 2001; Murphy et al. 2003). Consistent with our prediction, we observed a significant increase of DAF-16-GFP in the nucleus after a 1-h treatment of 2 mg/mL of CBE (Fig. 3d). We then measured changes of the mRNA levels of *daf-16* and *sod-3*. *Sod-3* is one of the DAF-16 direct target genes and encodes a Fe/Mn superoxide dismutase (Honda and Honda 1999; Murphy et al. 2003). The mRNA level of

Fig. 2 CBE treatment results in the alterations of stress response to specific environmental stimuli. **a** The CBE-treated N2 worms showed similar survival in response to 5 mM paraquat as compared to non-treated worms. **b** The CBE-treated N2 worms showed similar intracellular ROS level in response to 5 mM paraquat as compared to non-treated worms. **c** The CBE-treated N2 worms showed similar survival after UV irradiation as compared to non-treated worms. **d** The CBE-treated N2 worms exhibited increased survival after 3.5 h at 37 °C when compared to non-treated worms. **e** The transcript levels of *hsp-4*, *hsp-12.6*, *hsp-16.1*, *hsp-16.49*, and *hsp-70* in N2 worms treated with and without 2 mg/mL CBE were quantified using qRT-PCR. Controls are set to 1. **f** The CBE-treated N2 worms showed reduced motility after 10 min exposure in 300 mM and 500 mM NaCl relative to non-treated worms. **g** The CBE-treated N2 worms showed an elevated mortality rate after 12 h exposure in 500 mM NaCl compared to non-treated worms. At least triplicate samples were examined for each stress assay. “Fraction alive” indicates the average survival among the multi-replicates and *error bars* represent the standard deviation. *P* value was calculated using Student’s *t* test. ***p*<0.05 when compared to corresponding control. Each of the stress assays was repeated at least three independent times with similar results

daf-16 was increased by three~four fold, and the expression of *sod-3* was robustly elevated by eight~ten-fold in CBE-treated worms compared to non-supplemented controls (Fig. 3e and f). These findings suggest that CBE treatment promotes longevity partly through increasing DAF-16 nuclear localization and transcriptional activities.

CBE-mediated lifespan extension does not require SIR-2.1 and JNK signaling

SIR-2.1, functioning as a protein deacetylase, has been implicated in lifespan modulation (Berdichevsky et al. 2006; Burnett et al. 2011; Rogina and Helfand 2004; Tissenbaum and Guarente 2001; Viswanathan et al. 2005). We examined whether SIR-2.1 is involved in lifespan extension induced by CBE. To this end, we

measured the lifespan of *sir-2.1(ok434)* null mutant worms treated with 2 mg/mL CBE. CBE treatment significantly extended the lifespan of *sir-2.1(ok434)* worms (Fig. 4a; Table 1), suggesting that SIR-2.1 is dispensable for CBE to extend lifespan.

The JNK signaling has been shown to modulate lifespan, and phosphorylation of JNK-1 is required for JNK signaling to extend lifespan in *C. elegans* (Oh et al. 2005). We investigated whether lifespan extension by CBE treatment depends on JNK signaling. To test this hypothesis, we supplied a loss-of-function mutant *jnk-1(gk7)* with 2 mg/mL CBE. Consistent with previous work (Oh et al. 2005), *jnk-1(gk7)* worms showed a decreased lifespan compared to control N2 worms (Fig. 4b; Table 1). However, CBE treatment was still able to increase the lifespan of *jnk-1(gk7)* mutant worms, suggesting that CBE promotes longevity independent of JNK signaling.

CBE requires OSR-1 to promote longevity in an UNC-43 dependent manner

It has been reported that blueberry extracts promote longevity through the OSR-1/UNC-43/SEK-1 osmotic stress response pathway in *C. elegans* (Wilson et al. 2006). We tested whether CBE treatment may target the same pathway. To test this possibility, we supplied *osr-1(rm1)* mutant worms with 2 mg/mL CBE and found that CBE treatment was not capable of increasing the lifespan of *osr-1(rm1)* mutant worms (Fig. 5a; Table 1). Since *osr-1(rm1)* worms are short-lived (Pietsch et al. 2009; Saul et al. 2010), it is possible that *osr-1(rm1)* mutant worms are sick in general, which may mask any apparent longevity benefit of CBE supplementation. To address this issue, we assessed several general health parameters in *osr-1(rm1)* worms, including body size, motility, and touch

Table 2 CBE supplementation does not influence the motility and reproductive capacity of *C. elegans*

	Temperature (°C)	Control ± SE	CBE treated ± SE	<i>P</i> value	Total number of worms tested
Brood size	16	213±14.5	208±15.0	0.64	9 ^a , 9 ^b
	20	339±15.5	394±17.5	0.35	9 ^a , 9 ^b
	25	296±15.0	254±15.0	0.73	9 ^a , 9 ^b
Motility	25	8.16±2.56	6.46±2.70	0.45	30 ^a , 30 ^b

These data represent the average of three independent trials for each test

^a Control worms

^b Assay worms

Fig. 3 CBE modulates the lifespan of *C. elegans* through the IIS pathway and DAF-16. **a** *Daf-16(mgDf50)* mutant worms were treated with 2 mg/mL CBE. **b** *Daf-2(e1370)* mutant worms were treated with 2 mg/mL CBE. **c** *Age-1(hx546)* mutant worms were treated with 2 mg/mL CBE. Each lifespan experiment was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments are included in Table 1. **d** Transgenic worms over-expressing DAF-16::GFP (*daf-16(mgDf47);xrls87*) were treated with 2 mg/mL CBE at 25 °C for 1 h. DAF-16::GFP exhibited nuclear accumulation in treated worms. Photos show the DAF-16::GFP expression pattern and DIC images of young adults. **e** The transcript levels of *daf-16* in N2 worms treated with and

without 2 mg/mL CBE were quantified using qRT-PCR. The data from three independent experiments were pooled to calculate the mean RNA level normalized to the internal control *act-1*. The standard errors of the mean (SEM) are shown. The normalized mean RNA level of *daf-16* in non-treated N2 worms was set as 1. ** $p < 0.05$ when compared to non-treated control. **f** The transcript levels of *sod-3* in N2 worms treated with and without 2 mg/mL CBE were quantified using qRT-PCR. The data from three independent experiments were pooled to calculate the mean RNA level normalized to the internal control *act-1*. The standard errors of the mean (SEM) are shown. The normalized mean RNA level of *sod-3* in non-treated N2 worms was set as 1. ** $p < 0.05$ when compared to non-treated control

Fig. 4 CBE-mediated lifespan extension does not require SIR-2.1 and JNK signaling. **a** The *sir-2.1(ok434)* mutant worms were treated with 2 mg/mL CBE. **b** The *jnk-1(gk7)* mutant worms were treated with 2 mg/mL CBE. Each lifespan experiment shown here was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments are included in Table 1

response. *osr-1(rm1)* worms did not significantly alter any of these general health parameters when compared

Fig. 5 CBE requires OSR-1 to extend lifespan and is partially dependent on CaMKII and p38 MAP kinase pathway. **a** The *osr-1(rm1)* mutant worms were treated with 2 mg/mL CBE. **b** The *unc-43(n1186)* mutant worms were treated with 2 mg/mL CBE. **c** The *sek-1(ag1)* mutant worms were treated with 2 mg/mL CBE. Each lifespan experiment represented here was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments are included in Table 1

to the wild-type N2 worms (data not shown). These findings suggest that CBE requires OSR-1 to extend the lifespan in *C. elegans*.

We further tested whether CBE modulates lifespan in *C. elegans* through the CaMKII/p38 MAPK pathway (Solomon et al. 2004). OSR-1 negatively regulates the activity of CaMKII/p38 MAPK pathway. Elevated OSR-1 decreases the activities of UNC-43 and SEK-1, two major components of the CaMKII/p38 MAPK pathway. We measured the lifespan of *unc-43(n1186)* or *sek-1(ag1)* deletion mutant worms treated with CBE. Only *unc-43(n1186)* showed an extension in mean lifespan from 12.5 to 14 days when compared to N2 worms, while the lifespan of *sek-1(ag1)* mutants is similar to the N2 worms (Fig. 5b and c; Table 1). We next performed a genetic epistasis assay to further determine the role of the CaMKII/p38 MAPK pathway in CBE-induced lifespan extension. *unc-43(n1186)* and *sek-1(ag1)* mutant worms were fed 2 mg/mL CBE. CBE treatment increased the mean lifespan from 12.5 to 14 days in *sek-1(ag1)* worms but did not increase the mean lifespan (14 vs. 14.97 days) in *unc-43(n1186)* worms (Fig. 5c and b; Table 1). These findings suggest that CBE modulates lifespan at least partly through OSR-1 and one of its downstream effectors, UNC-43, but not the p38 MAPK signaling.

Discussion

Numerous studies in humans and model organisms have shown that nutraceuticals made from various botanicals can provide a variety of health benefits, including prevention of cancer, reduction of inflammation, and delay of age-related functional decline (Lin et al. 2003; Liu 2004; Shukitt-Hale et al. 2007; Zhu et al. 2011). Our study suggests that the North American cranberry (*V. macrocarpon*) is a potentially effective nutraceutical for promoting healthy aging in *C. elegans*. CBE consumption substantially extends lifespan and elevates resistance to heat shock stress in wild-type worms. CBE supplementation requires the activity of DAF-16 through the IIS pathway to modulate lifespan and stress response. Noticeably, supplementation of CBE increases DAF-16 nuclear localization and its transcriptional activities. In addition, CBE consumption also requires OSR-1 through UNC43, but not SEK-1, to modulate lifespan extension and stress response. In contrast, SIR-2.1 and JNK

signaling are not required for CBE-induced lifespan extension. Based on these findings, we propose that CBE supplementation modulates DAF-16 and OSR-1 activities to promote longevity and increase stress response in *C. elegans* through the IIS pathway and UNC-43, respectively (Fig. 6).

Our experiments demonstrate that CBE supplementation increases *C. elegans* lifespan in a dose-dependent manner, and 10 mg/mL CBE shortened *C. elegans* lifespan regardless of the acidic pH. It is known that cranberry contains abundant quercetin and many other unique phytochemicals (Pappas and Schaich 2009). Previous reports had shown that phytochemicals, such as quercetin, tannic acid, and rosmarinic acid, can promote longevity at relative low concentrations, but are detrimental at high concentrations in *C. elegans* (Pietsch et al. 2011; Saul et al. 2010). Thus, it is very likely that some phytochemicals in CBE are beneficial only at lower concentrations.

Cranberry and its constituent products have been widely consumed by humans due to their numerous health benefits (Howell 2007; Neto 2007a). Our findings ascribe the benefit of cranberry consumption to prolongevity function and show the correlation between

Fig. 6 A putative model for cranberry's mode of action in *C. elegans*. Cranberry modulates multiple longevity-related pathways to promote healthy aging and stress resistance. On the one hand, cranberry activates OSR-1 and consequently suppresses UNC-43 to promote longevity. On the other hand, cranberry suppresses DAF-2 and AGE-1 and activates DAF-16 to promote longevity

CBE consumption and activation of DAF-16/FOXO. A critical regulation of DAF-16/FOXO activity is its phosphorylation and nuclear exclusion by the IIS pathway. Mammals have several FOXOs, the counterpart of DAF-16, which are key regulators of diabetes and obesity due to their ability to regulate energy metabolism (Michael et al. 2000; Samuel et al. 2006). In insulin-responsive tissue, FOXO1 regulates the expression of genes involved in gluconeogenesis, energy metabolism, and oxidative stress, thereby affecting the whole body energy homeostasis. When nutrient and insulin levels are low, FOXO1 promotes expression of gluconeogenic enzymes to maintain glucose level. Conversely, in the fed state, pancreatic β cells secrete insulin to inactivate FOXO1 and partially suppress gluconeogenic enzyme expression in the liver (Puigserver et al. 2003). In addition, FOXO1 also plays a vital role in the formation of skeletal muscle and adipose tissues, two major organs for the maintenance of energy homeostasis (Ahima and Flier 2000; de Lange et al. 2007). Moreover, negative signaling to FOXO1 under particular pathophysiologic conditions, such as metabolic dysfunction and insulin resistance, leads to deleterious effects, including hyperglycemia and glucose intolerance. Our study shows that DAF-16 is required for CBE-induced lifespan extension. Therefore, it is likely that CBE modulates metabolic homeostasis to promote longevity and stress resistance. This raises an intriguing possibility that CBE supplementation can be implemented as a prevention method to fight against metabolic dysfunction diseases, such as diabetes and obesity.

OSR-1 is a master regulator to prolong *C. elegans* survival in hyperosmotic environments (Solomon et al. 2004). OSR-1 couples with SEK-1/MAPKK through UNC-43/CAMKII to promote resistance to chronic osmotic stress. Wilson et al. found that blueberry polyphenols induced longevity requires the OSR-1/UNC-43/SEK-1 pathway in *C. elegans* (Wilson et al. 2006). Our genetic study indicates that OSR-1 is essential for CBE-induced lifespan extension in *C. elegans*, but only UNC-43, not SEK-1, is involved in this prolongevity process. This is not surprising considering that individual components in the OSR-1/UNC-43/SEK-1 pathway have been reported to differentially mediate the prolongevity effects of different phytochemicals. For instance, SEK-1, but neither OSR-1 nor UNC-43, is required for tannic acid-induced longevity (Saul et al. 2010), while SEK-1 and UNC-43, but not OSR-1, are

required for quercetin-induced longevity in *C. elegans* (Pietsch et al. 2009). In addition, SEK-1 can regulate pathogen resistance independent of UNC-43 and OSR-1 (Kim et al. 2002). Future experiments are needed to investigate how phytochemicals in CBE differentially regulate downstream effectors of OSR-1 to modulate lifespan and stress resistance.

Our findings indicate that CBE supplementation increases *C. elegans* resistance to heat shock. However, CBE treatment does not improve worms' resistance to oxidative stress or UV irradiation and even renders worms more sensitive to osmotic stress when compared to the non-supplemented controls. These results suggest that CBE treatment selectively enhances worms' resistance to environmental stresses. A number of botanical extracts containing high levels of polyphenols have been shown to differentially promote health aging and stress resistance in diverse species. For instance, supplementation with blueberry polyphenols increases thermotolerance in *C. elegans* (Wilson et al. 2006). Blueberry extract promotes longevity in *C. elegans* and *Drosophila* (Peng et al. 2012; Wilson et al. 2006). Cocoa contains abundant flavonoids and promotes longevity and resistance to oxidative stress in worms (Martorell et al. 2011). Nectarine and açai extracts promote the survival in *Drosophila* under oxidative stress and fed high-fat diets (Boyd et al. 2011; Sun et al. 2010). Ginkgo biloba extract is widely used to improve health of the elderly due to its capacity to promote resistance to oxidative stress with its considerable volume of flavonoids (Abdel-Wahab and Abd El-Aziz 2012; DeFeudis and Drieu 2000; Wu et al. 2011). Similarly, CBE contains a high number of polyphenols, such as the proanthocyanidins, the flavonols, and the anthocyanins (Pappas and Schaich 2009). Thus, the ability of CBE to increase resistance to heat stress could be due to the similar polyphenols existing in blueberry or unique phytochemicals in cranberry. In addition, a number of experiments have shown that different phytochemicals may interact and synergize to exert their biological functions (Apostolidis et al. 2006; Liu 2004; Seeram et al. 2004). Considering that different botanical extracts have unique phytochemical profiles, CBE-induced thermotolerance may also be due to the synergistic effects generated by the interaction of unique cranberry phytochemicals. Moreover, lifespan extension induced by CBE requires DAF-16/FOXO, which is known to regulate different target genes in response to

different stress stimuli (Honda and Honda 1999; Kenyon 2005; van der Horst and Burgering 2007). Therefore, it is possible that CBE fine tunes the regulation of a subset of DAF-16 target genes, and therefore improves the survival under restrictive temperature.

We have found that CBE treatment induces susceptibility to chronic osmotic stress in *C. elegans*. This susceptible phenomenon may be due to the depression of CaMII/p38 MAPK signaling by CBE, based on our observation that *osr-1* is upregulated by CBE treatment (data not shown). This is consistent with recent studies indicating that flavonoids elevate *osr-1* expression level in *C. elegans* (Xue et al. 2011). Further systematic studies are warranted to determine the mechanisms underlying differential effects of CBE supplementation on stress resistance in worms.

Our study highlights the prolongevity and stress resistance properties of CBE and reveals the molecular mechanisms by which CBE modulates lifespan and stress response in *C. elegans*. Although health benefits of CBE have been studied for many years, our study is the first to thoroughly investigate the properties of CBE on longevity and stress response, and the first to systematically analyze the genetic requirements for CBE-mediated effects. Since both IIS/DAF-16 and OSR-1 are highly conserved from *C. elegans* to mammals, our findings have important implications in utilizing CBE to promote healthy aging and combat age-related diseases in humans.

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