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# The lifespan-promoting effect of acetic acid and Reishi polysaccharide

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ABSTRACT

Using *Caenorhabditis elegans* as a model organism, various natural substances and commercial health-food supplements were screened to evaluate their effects on longevity. Among the substances tested, acetic acid and Reishi polysaccharide fraction 3 (RF3) were shown to increase the expression of the lifespan and longevity-related transcription factor DAF-16 in *C. elegans*. We have shown that RF3 activates DAF-16 expression via TIR-1 receptor and MAPK pathway whereas acetic acid inhibits the trans-membrane receptor DAF-2 of the insulin/IGF-1 pathway to indirectly activate DAF-16 expression. In addition, a mixture of acetic acid and RF3 possesses a combined effect 30–40% greater than either substance used alone. A proteomic analysis of *C. elegans* using 2-DE and LC–MS/MS was then carried out, and 15 differentially expressed proteins involved in the lifespan-promoting activity were identified.



## 1. Introduction

Attempts to search for genes involved in aging and longevity have been made in model organisms of lower eukaryotic systems such as yeast, *Drosophila melanogaster* fruitflies and the nematode *Caenorhabditis elegans*.[1] Although aging is a fundamental process with great innate diversity, we now know that aging, like many other biological processes, is subject to regulation by pathways that have been conserved during evolution. Changing a single gene within these pathways can cause an experimental animal to age normally but more slowly than usual.[2] *C. elegans* has been commonly used as a model in recent years. It is a small worm, just 1 mm in length that lives in soil and has been used extensively for aging studies, mainly because of its short and consistent lifespan (average 14–20 days at 20 °C)[3] and better characterized aging-related pathways than other organisms. The lifespan-control mechanism of *C. elegans* is associated with the well-known conserved insulin/IGF-1 *daf-2* signaling pathway,[1,2] which includes the DAF-2 trans-membrane receptor, a series of intracellular kinases and the DAF-16 protein, which ultimately function to both positively and negatively regulate the aging process.[4–7] The DAF-16 protein, a homologue of human forkhead (FOXO) transcription factor involved in the regulation of various antioxidant enzymes such as superoxide dismutases (SOD), plays a major role in the lifespan regulation of *C. elegans*.[8,9] The insulin-like signaling cascade is an evolutionarily conserved pathway and is present in fruitflies and mice.[10–12] However, DAF-16 activity has also been shown to be modulated by the JNK signaling pathway, and linked to SIR-2.1 deacetylase, HSF-1, LIN-14, and SMK-1 in the nucleus.[13–17]

Our previous study[18] has shown that the polysaccharide fraction (RF3) isolated from the aqueous extract of Reishi mushroom (*Ganoderma lucidum*) possesses an immuno-modulating effect through interaction with the Toll-like receptor 4 (TLR4). When fed with RF3, tumor-implanted mice showed an increase in lifespan, presumably due to the activation of the host immune system.[19] Although it is still unclear whether there is any orthologue of TLR in *C. elegans*, the Toll-interleukin 1 receptor intracellular domain (TIR-1) has been shown to exist in this nematode and is associated with an antibacterial pathway.[20–22] Therefore, we speculated that RF3 might have a beneficial effect on the lifespan of *C. elegans*, which may in turn provide a molecular or genetic basis for the study of aging. We have also tested a series of commercial supplements, including some antioxidant vitamins, oriental herbal mushrooms, and vinegars (with acetic acid as its main component) as antioxidants and as immune-modulators.[23–28] Thus, using *C. elegans* as a model, we have developed a strategy to identify substances with longevity-promoting effects and to shed some insights into the underlying mechanism(s).

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## 2. Results

### 2.1. Lifespan analysis using wild-type *C. elegans*

To identify new substances with longevity-promoting properties, we used wild-type *C. elegans* (N2) as a live model organism. We measured its lifespan in the presence or absence of selected natural substances or commercial health-food products. These included antioxidant vitamins such as vitamins E and C, the vitamin B-complex group,[23–25] acetic acid, which is a major ingredient of vinegars,[26] some edible mushroom extracts such as RF3, the mycelium fractions of *Antrodia camphorata* (chang-chih, local herbal name) and *Hericium erinaceus* (lion's mane mushroom),[27,28] and others. When treated with some of the above-mentioned substances, the worm showed a significant extension of lifespan (Fig. 1A–C) as compared with the negative control group (no treatment). It was found that acetic acid possessed a prominent lifespan-extending effect. RF3 and the extracts of *A. camphorata* and *H. erinaceus* were also found to extend the lifespan of *C. elegans* by about 20–30%. However, the vitamin group showed a lower longevity effects than acetic acid or RF3 under similar experimental conditions.

### 2.2. Semi-quantitative RT-PCR and quantitative qRT-PCR analysis

To further investigate the mechanism of lifespan-extending effect, a series of experiments by means of RT-PCR (Table 1) were carried out to analyze the transcription profiles of some target genes in *C. elegans*, including *daf-2* and *daf-16*,[1–4] both involved in the pathways that affect lifespan, and the three genes *tir-1*, *rab-1* and *pmk-1* which are related to Toll-like receptors and the MAPK pathway of the immune system.[20–22] As shown in Figure 2A, all three mushroom extracts and acetic acid caused a significant increase in *daf-16* expression. Only one (*H. erinaceus*) of the three mushroom extracts[33–35] and acetic acid actually reduced *daf-2* expression (Fig. 2B). Neither RF3 nor the extract of *A. camphorata* significantly reduced *daf-2* expression, and yet both remarkably stimulated *tir-1* expression as opposed to the *H. erinaceus* extract (Fig. 2B). To understand whether the *tir-1* activation preceded the activation of MAPK pathway, we further analyzed in vivo *rab-1*, *pmk-1* and *clk-1* regarding their transcription levels after RF3 treatment. The result showed that RF3 stimulated the transcription of *tir-1* and *rab-1*/*pmk-1* of the MAPK pathway and exhibited no effect on the *clk-1* gene[36] expression (Fig. 2C), which had been shown to be involved in the control of mitochondrial energy metabolism. The time-course study revealed that the maximal induction of *tir-1* occurred on the first day after RF3 treatment; whereas the maximal induction level of *daf-16* was observed on day 2 (Fig. 2D). This is in accord with the well-known fact that activation of *daf*-16 expression occurs later inside the nucleus in the signaling pathway.

### 2.3. Gene-silencing by RNAi analysis

To examine if the increased expression of *tir-1* was the exclusive and direct cause of *daf-16* induction when stimulated by RF3

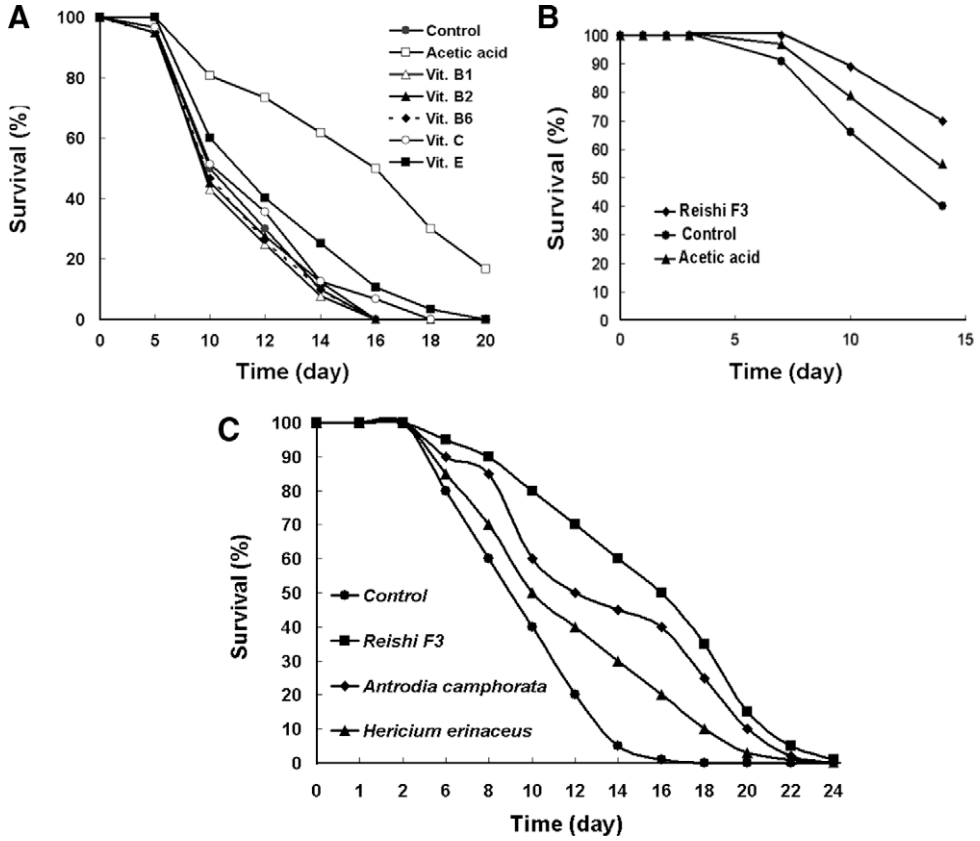



**Figure 1.** Effects of natural supplements and herbal mushrooms on the lifespan of *C. elegans*. The curves shown represent changes in percentage survival worms (at least 60 worms were initially transferred into each of three wells for triplicate assays) at different times (days) after treatments with each individual supplement. (A) Worms treated with water (●, control), acetic acid (□, 50 ppm), vitamin B1 (△, 50 ppm), vitamin B2 (▲, 50 ppm), vitamin B6 (♦, 50 ppm), vitamin C (○, 50 ppm), and vitamin E (■, 50 ppm). (B) Worms treated with water (●, control), acetic acid (▲, 50 ppm) and RF3 (♦, 100 ppm). (C) Worms treated with water (●, control), RF3 (■, 100 ppm) and crude extracts of *A. camphorata* (♦, 100 ppm) and *H. erinaceus* (▲, 100 ppm) herbal mushrooms. Lifespan assays were determined at 20 °C. Percentage survival was calculated from the number of living worms at indicated time periods (days) as compared with the original number of testing worms. *P* values were also calculated for each set of lifespan assay, which represented a direct comparison between the control group and each experimental treatment group during the same time period. These assays were carried out in triplicate and analyzed statistically with $^{*}P < 0.01$ for each set of experiments.

**Table 1**
Primers used in RT-PCR

| Primer | Sequence, starting from 5′ | Specificity[a] |
|---|---|---|
| CE-*daf16*-Forward | GAACTACCGTATACTCGC | DAF-16 transcription factor |
| CE-*daf16*-Reverse | CCGCCTGTCAACAGTCTC | DAF-16 transcription factor |
| CE-*daf2*-Forward | GGATTTGGAGATCGGTCTGG | DAF-2 receptor |
| CE-*daf2*-Reverse | TTTCGACACCTTGTTCCTGA | DAF-2 receptor |
| CE-*tir1*-Forward | GACTGACGTGATTGACCATA | Toll-interleukin 1 receptor intracellular domain |
| CE-*tir1*-Reverse | CCCTTGTGCATATTTATGAT | Toll-interleukin 1 receptor intracellular domain |
| CE-*clk1*-Forward | GAGTTGATCATGCTGGAGAG | Clock (biological timing) abnormality protein 1 |
| CE-*clk1*-Reverse | CTCCTCATCACGTAATCTTGT | Clock (biological timing) abnormality protein 1 |
| CE-*rab1*-Forward | TGAACCCTGAATACGACTAC | Rab family protein 1 |
| CE-*rab1*-Reverse | ATGCGTACCTATCGATTTCC | Rab family protein 1 |
| CE-*pmk1*-Forward | ATCATATACTTCATCCGACT | p38 MAP kinase 1 |
| CE-*pmk1*-Reverse | ATACACATCCTCGATATCAT | p38 MAP kinase 1 |
| CE-*act1*-Forward | GTAGACAATGGATCCGGA | Actin |
| CE-*act1*-Reverse | ACGATACCGTGCTCAATT | Actin |

[a] Gene specificity for the primer.

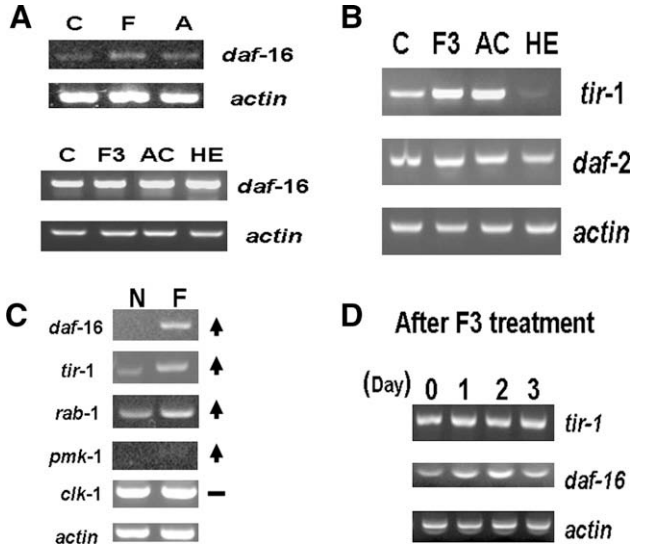



**Figure 2.** Semi-quantitative RT-PCR detection of transcripts for *daf-2*, *daf-16*, *tir-1*, *rab-1*, *pmk-1* and *clk-1* mRNA in *C. elegans* on day 2 after treatments. Specific primers for each gene were used in the reaction, and *act-1* was used as an internal control. Expression of the genes was normalized to this internal control in all RT-PCR data. (A) Expression of *daf-16* following different treatments. (Upper panel): C, No supplement (control); F, 100 ppm RF3; A, 50 ppm acetic acid, noting that upregulation of *daf-16* is more prominent for RF3 than acetic acid. (Bottom panel): C, No supplement (control); F3, 100 ppm RF3; AC, 100 ppm crude extracts of *A. camphorate* mycelia; HE, 100 ppm crude extracts of *H. erinaceus* mycelia. (B) Expression of *daf-2* and *tir-1* following treatments with F3, 100 ppm RF3; AC, 100 ppm crude extracts of *A. camphorate* mycelia and HE, 100 ppm crude extracts of *H. erinaceus* mycelia as compared with C, control without treatment; it is noted that acetic acid also showed downregulation of *daf-2* and *tir-1* similar to crude extract of HE mushroom (data not shown). (C) Expression of *daf-16*, *clk-1*, *tir-1* and *rab-1* and *pmk-1* (the last two are important genes involved in the MAPK pathway) after incubation of *C. elegans* with (F) or without (N) RF3 at a concentration of 100 ppm for 2 days. The arrows show the expression level of a specific mRNA is elevated after RF3 treatment, and the horizontal line indicates no change in expression. (D) Time-course analysis of transcription of *tir-1* and *daf-16* in response to RF3 stimulation. Notice that the maximal induction of *tir-1* occurred on the first day after RF3 treatment; whereas the maximal induction level of *daf-16* was observed on the day 2. All experiments were done in triplicate.

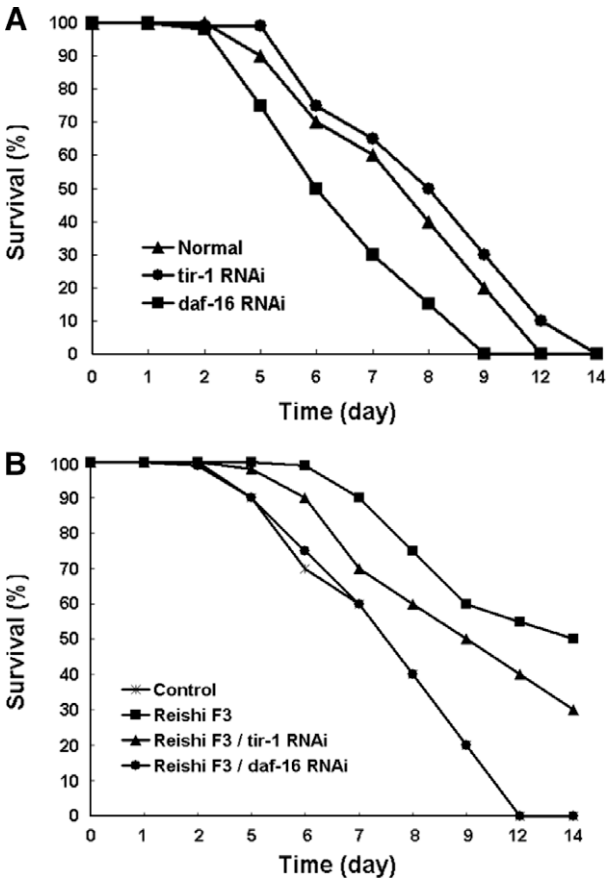



**Figure 3.** Effects of treatment with RNAi of *tir-1* and *daf-16* genes on lifespans of *C. elegans*. Lifespan analyses were performed using RNAi plates as described in Section 5. (A) Comparison of lifespan of normal worms (▲) and worms treated with RNAi of *tir-1* (●) and *daf-16* (■) genes. It is noted that *daf-16* RNAi shortened the lifespan of worms by ~25%, while *tir-1* RNAi extended that by ~18%. (B) Worms treated with RNAi of *tir-1* and *daf-16* genes in the presence of Reishi polysaccharide fraction 3 (RF3). Treatment of RNAi targeting *daf-16* completely prevented lifespan extension by RF3. Worms treated with both RF3 and *daf-16* RNAi (●) had lifespans similar to wild-type control group (*). On the other hand, worms treated with RF3 and *tir-1* RNAi (▲) exhibited a weaker lifespan extension effect than normal worms treated with RF3 (■), indicating that *tir-1* induction was unlikely as an exclusive route for *daf-16*-mediated lifespan extension of *C. elegans* by RF3 treatment. The worms had shorter lifespans than those RF3-treated worms without *tir-1* gene knock-out (■), but still much longer than the control group (>18%). *$P$ <0.01 for these studies.

polysaccharides, the effect of RF3 on the lifespan of *C. elegans* (Fig. 3) was analyzed by treatment with *tir-1* or *daf-16* RNAi bacteria.[37,38] The results showed that knock-down of DAF-16 expression could completely eliminate the lifespan-extending effect of RF3. Although the longevity-promoting effect of RF3 was reduced upon treatment with *tir-1* RNAi, the treated *C. elegans* still achieved a longer lifespan than untreated worms or those treated with RF3 and *daf-16* RNAi. This pointed to the fact that the receptors in *C. elegans* responding to RF3 were not limited to the receptors linked to TIR-1; there were probably other as-yet-unidentified receptors

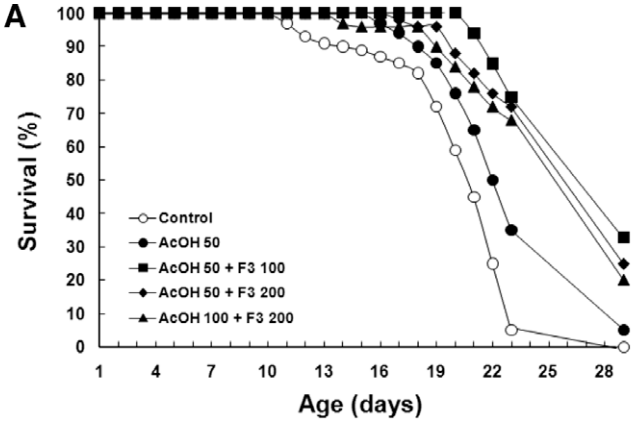
associated with the longevity effect of RF3 reported here.

### 2.4. Study of mechanisms of lifespan extension by analysis of gene expression among different signaling pathways

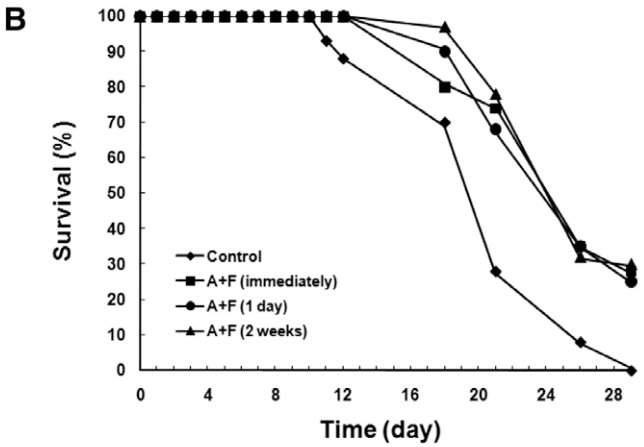
According to our previous reports,[18,19] RF3 was shown to modulate the immune system in animals through binding to the TLR2/TLR4 receptors, which initiate the pathway similar to that of lipopolysaccharide (LPS) endotoxins from bacteria.[33] Therefore, we also explored whether RF3 and LPS from *Escherichia coli* have the same effect on *tir-1* mediated MAPK-activation and *daf-16* expression in *C. elegans*. Feeding worms with RNAi bacteria followed by real-time RT-PCR analysis of mRNA (Fig. S1, Supplementary data) revealed that both RF3 and LPS could induce *tir-1* expression to a similar extent (slightly higher with LPS). On the other hand, while RF3 induced *daf-16* expression, the expression was strongly inhibited by LPS under similar conditions. Moreover, after knocking down *tir-1*, the inhibition of *daf-16* by LPS decreased. It is noted that no matter what treatment was applied, *rab-1* expression always increased when *tir-1* expression was inhibited. When using the crude extract from *A. camphorata* under identical conditions to those of RF3, the same results as with RF3 (data not shown) were obtained. Therefore, it is conceivable that the mechanisms by which RF3 and *A. camphorata* promote longevity might be similar (see Section 3 below).

### 2.5. Combined effect of RF3 and acetic acid on the lifespan of *C. elegans*

Since both acetic acid and RF3 promoted longevity in *C. elegans* through different mechanisms, we further investigated whether there was a greater effect when these two substances were combined. It is noteworthy that various mixtures with different proportions of acetic acid and RF3 all possess higher activities than either substance used alone (Fig. 4A). Among these, a solution of 50 ppm (w/w) acetic acid and 100 ppm RF3 displayed the highest activity, achieving a lifespan extension 1.4 times that of 50 ppm




| Treatment | Control | AcOH | F3 | AcOH 50 + F3 100 | AcOH 50 + F3 200 | AcOH 100 + F3 200 |
|---|---|---|---|---|---|---|
| Fold of lifespan | 1±0.02 | 1.23±0.02 | 1.35±0.01 | 1.74±0.03 | 1.57±0.02 | 1.39±0.04 |




| Treatment | Control | AcOH | F3 | AcOH 50 + F3 100 (immediately) | AcOH 50 + F3 100 (1 day) | AcOH 50 + F3 100 (2 weeks) |
|---|---|---|---|---|---|---|
| Fold of lifespan | 1±0.02 | 1.26±0.01 | 1.33±0.03 | 1.76±0.02 | 1.70±0.02 | 1.85±0.04 |

**Figure 4.** The cumulative effect of RF3 and acetic acid on lifespan enhancement of *C. elegans*. (A) Lifespan of *C. elegans* treated with mixtures of RF3 (F3) and acetic acid (AcOH) in different concentrations. The best result was achieved with a mixture containing 50 ppm AcOH and 100 ppm F3 (■) as compared with (1) control (○), (2) 50 ppm AcOH (●), (3) 50 ppm AcOH + 200 ppm F3 (♦), (4) 100 ppm AcOH + 200 ppm F3 (▲). (B) Effect of the mixture of RF3 and acetic acid on lifespan of *C. elegans* after storage for different periods. Control (♦), test after immediate mixing (■), test after mixing for 1 day (●), and test after mixing for 2 weeks (▲). The results show that long-term storage (up to two weeks) of the mixture of RF3 and acetic acid did not affect the efficacy on the lifespan extension of treated *C. elegans*. All tables under the survival curves refer to the lifespan extension induced by the mixture as compared with the control group. All the values shown are mean ± SD, calculated from triplicate experiments.

acetic acid and 1.3 times that of 100 ppm RF3 used alone, or about 1.8-fold lifespan extension when compared with that of untreated control. We also tested the long-term stability of RF3 in 5% acetic acid. Even after standing for two weeks, the mixture still maintained the same activity as that of the freshly-prepared RF3 solution (Fig. 4B).

### 2.6. Proteomic analysis of *C. elegans* under treatments of acetic acid and/or RF3

To investigate the changes in the proteome of *C. elegans* under the longevity-promoting treatment of acetic acid and RF3, we have analyzed the protein-expression profile of *C. elegans* under normal control and treated (100 ppm RF3, 50 ppm acetic acid or a mixture of both) conditions by using 2-DE coupled with mass spectrometric identification of the expressed proteins. The representative protein profiles from *C. elegans* treated with or without acetic acid and/or RF3 for three days (Fig. S2 of Supplementary data) revealed about 15 differentially expressed protein spots. The proteomic analysis showed a significant increase in the expression of seven proteins and decrease in the expression of eight proteins (Table 2). Their corresponding genes were similar to those identified by DNA microarray analysis reported previously,[5] which suggested that the insulin/IGF-1 signaling pathway upregulated a wide variety of genes, including stress–response, antimicrobial and metabolic genes and downregulated some specific life-shortening genes.

### 2.7. Green fluorescent protein (GFP)-expression assays

We have used transgenic *C. elegans* with the *gfp* gene conjugated to the *daf-16* gene to follow the change of green fluorescence as a function of survival time. The GFP fluorescence emitted from the transgenic worms is indicative of the expression of *daf-16*, which is diminished after silencing the *daf-16* gene by *daf-16* RNAi (Fig. 5A). Moreover, the green fluorescent intensity of transgenic worms carrying the *gfp* gene was found to increase with survival time from 6 to 10 days after feeding with acetic acid (50 ppm), Reishi F3 (RF3) (100 ppm) and LPS (10 ppm), respectively (Fig. 5B). We have also found that changes in the intensity of GFP fluorescence can be correlated with the lifespan of the treated worms, that is, normal and LPS-treated worms showed weaker fluorescence and a shorter life span (10–14 days). RF3- and acetic acid-treated worms showed stronger fluorescence with about 20–30% increase in lifespan (17–20 days), while the worms treated with a mixture of acetic acid and RF3 exhibited an increased effect on the GFP-fluorescence intensity with the longest survival time of up to 30 days. The qualitative analysis by GFP-expression assays is in general agreement with the results obtained from quantitative RT-PCR and lifespan analysis.

## 3. Discussion

Aging remains a fundamental and unsolved mystery in biology in spite of advances in the field using model organisms of lower eukaryotic systems such as yeasts, fruitflies and *C. elegans*.[1,2] Most of our knowledge about the regulation of aging comes from the study of aging-related genes and systematic RNAi screening.[37–39] A number of genes related to lifespan were first discovered in *C. elegans*. In particular, genetic disruption of the 'insulin-like signaling pathway' can extend longevity significantly in the nematode,[1,2] and to a lesser degree in other species, including fruitflies and mice. In another well-studied system of yeast,[40] the Sirtuin family of NAD(+)-dependent deacetylases, including the 'Silent Information Regulator 2.1 (SIR-2.1) in *C. elegans* and SIRT1 in human',[41] regulates lifespan and mediates calorie restriction. The sirtuin

**Table 2**
List of differentially expressed proteins in *C. elegans* treated with acetic acid and Reishi polysaccharide fraction RF3

| Spot | Protein name | Swiss-Prot entry | Score/match | Sequence coverage (%) | pI/mass (kDa) | Protein expression ratio (S/N)[a] | | | Regulation[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | AA | F3 | A+F | |
| Glycoproteins | | | | | | | | | |
| 1 | Vitellogenin-6 precursor (vit6) | P18948 | 346/11 | 6 | 6.85/193.197 | 0.01 | 0.03 | 0.10 | ↓ |
| 2 | Vitellogenin-6 precursor (vit6) | P18948 | 1232/28 | 16 | 6.85/193.197 | 0.50 | 1.52 | 0.53 | ↓ |
| Transport proteins | | | | | | | | | |
| 3 | Probable voltage-dependent anion-selective channel | Q21752 | 811/18 | 49 | 9.20/29.942 | 0.30 | 0.22 | 0.42 | ↓ |
| 4 | ATP synthase subunit alpha, mitochondrial precursor | Q9XXK1 | 900/19 | 25 | 8.98/57.725 | 0.52 | 0.20 | 0.55 | ↓ |
| Transferase | | | | | | | | | |
| 5 | Aspartate aminotransferase | Q17994 | 244/9 | 24 | 9.20/45.589 | 0.66 | 0.50 | 0.40 | ↓ |
| 6 | Nucleoside diphosphate kinase | Q93576 | 377/12 | 41 | 6.97/17.106 | 2.23 | 1.25 | 2.14 | ↑ |
| Motor proteins | | | | | | | | | |
| 7 | Myosin, essential light chain (mlc-3) | P53014 | 130/6 | 34 | 4.63/17.134 | 0.54 | 0.79 | 0.35 | ↓ |
| 8 | Myosin regulatory light chain 1 (mlc-1) | P19625 | 511/10 | 39 | 5.06/18.605 | 1.35 | 1.37 | 2.00 | ↑ |
| 9 | Myosin regulatory light chain 1 (mlc-1) | P19625 | 618/10 | 60 | 5.06/18.605 | 1.29 | 1.84 | 2.28 | ↑ |
| 10 | Tropomyosin isoforms a/b/d/f | Q22866 | 1746/34 | 76 | 4.66/32.984 | 0.93 | 0.64 | 0.40 | ↓ |
| Others | | | | | | | | | |
| 11 | Heat shock protein Hsp-12.2 | P34328 | 140/3 | 12 | 6.28/12.257 | 2.21 | 2.57 | 2.66 | ↑ |
| 12 | Guanine nucleotide-binding protein subunit beta-2-like 1 (rack-1) | Q21215 | 143/4 | 8 | 6.44/35.808 | 1.41 | 1.47 | 2.13 | ↑ |
| 13 | 60S ribosomal protein L5 (rpl-5) | P49405 | 142/4 | 11 | 9.76/33.366 | 1.82 | 0.89 | 1.99 | ↑ |
| 14 | Probable malate dehydrogenase, mitochondrial precursor (mdh-1) | O02640 | 771/21 | 41 | 9.39/35.098 | 0.30 | 0.32 | 0.62 | ↓ |
| 15 | Major sperm protein 3 | Q19832 | 196/5 | 33 | 7.77/14.228 | 1.58 | 1.68 | 2.14 | ↑ |

[a] S, stress condition (AA: 50 ppm acetic acid; F3: 100 ppm RF3; A+F: 50 ppm acetic acid and 100 ppm RF3); N, normal condition.
[b] Regulation trend of mixture (A+F).

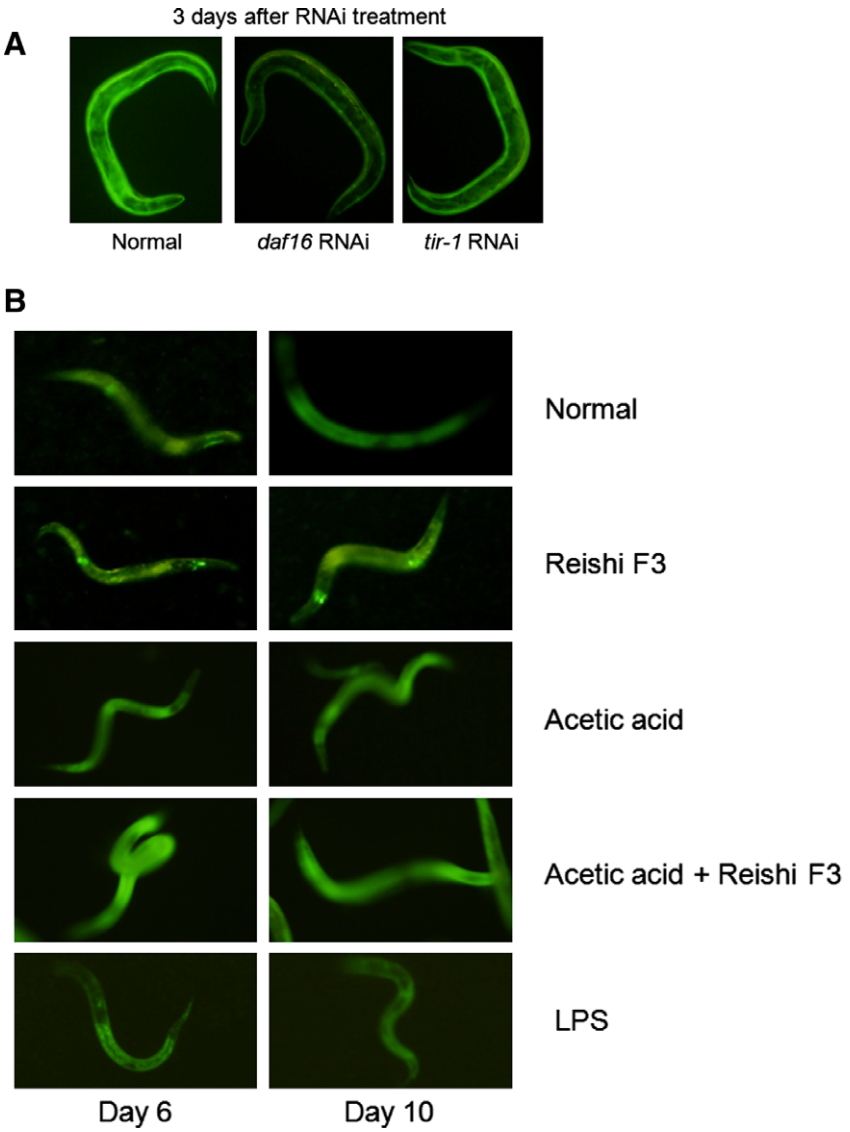



**Figure 5.** Expression of GFP as a biomarker for DAF-16 of *C. elegans* by transgenic worms carrying *daf-16::gfp* conjugated genes. (A) Comparison of GFP green fluorescent images of normal, *daf-16* RNAi and *tir-1* RNAi worms. It is noted that GFP fluorescence emitted from the transgenic worms is specific as an indicator for DAF-16 expression, which was diminished after silencing *daf-16* gene by *daf-16* RNAi. The *tir-1*-silenced worms still maintained partially the GFP green fluorescence. (B) Comparison of GFP green fluorescent images of transgenic worms expressing GFP at day 6 (left) and day 10 (right) after feeding with acetic acid (50 ppm), Reishi F3 (RF3) (100 ppm) and LPS (10 ppm), respectively. The green fluorescent intensities of transgenic worms carrying *gfp* gene was found to increase with survival time from 6 to 10 days. The green fluorescent intensities of C. elegans treated with acetic acid and RF3 are apparently stronger than control and LPS-treated samples. It is noteworthy that worms treated with a mixture of acetic acid and RF3 exhibiting a conspicuously greater effect on the emitted intensity of green fluorescence also possess the longest survival time of up to 30 days.

family can enhance pancreatic insulin secretion in mice following a glucose challenge, suggesting again the potential to regulate mammalian lifespan through the insulin signaling pathway.[42] To date, these two signaling systems coupled with dietary restriction appear to account for the lifespan in most experimental organisms.[43] To ask whether the aging and longevity pathways described above can be used to evaluate the anti-aging activity of herbal mushrooms and vinegars, we have carried out a series of investigations and discovered that the lifespan-promoting effect of RF3 and acetic acid is based on their action on the signaling pathways of longevity.

### 3.1. Mechanisms underlying the lifespan extension effects of Reishi RF3 and acetic acid

Regarding the mechanism(s) of RF3 and acetic acid, we have constructed various primers (Table 1) corresponding to the genes of signaling pathways such as *tir-1*, *rab-1*, *pmk-1*, *clk-1*, *daf-2* and *daf-16* (Fig. 2A–D) and their RNAi (Fig. 3A and B) in order to validate that the signal transduction pathways of these genes and their corresponding proteins affect aging process. As shown in Figure 2A, our treatment with acetic acid, RF3 and the water-soluble extracts of two other fungal mushrooms caused a significant increase in *daf-16* transcription and lifespan extension (Fig. 1A–C). These results are in accord with previous studies[29,30] that increasing lifespan is associated with stimulation of the expression of DAF-16 and antioxidant enzyme SOD in *C. elegans*. Although previous studies have attributed *daf-16* induction to the decrease or loss of *daf-2* function,[31,32] we found that only acetic acid and one (*H. erinaceus* species) of the three extracts actually caused a significant reduction in *daf-2* expression (Fig. 2B).[33–35] Neither RF3 nor *A. camphorata* exhibited a significant decrease in *daf-2* expression, and yet they were shown to possess impressive lifespan-promoting effect when compared with that of acetic acid and the extract of

*H. erinaceus* mushroom. Our previous studies[33,44,45] have shown that RF3 can stimulate spleen cell proliferation and cytokine expression via the Toll-like receptors TLR4/TLR2-mediated protein kinase (PKs) signaling pathways. The results obtained from RF3 and *A. camphorata* certainly distinguish the lifespan-extending mechanism of RF3 from that of the well-established insulin/IGF-1 signaling pathway via DAF-2 trans-membrane receptor. In addition, we showed previously that RF3 can exert immuno-modulating activities by stimulating the expression of inflammatory cytokines from spleen cells via PTK-mediated phosphorylation, followed by induction of PKs and activation of MAPKs such as ERK, JNK, and p38.[33] It has also been verified that interaction of RF3 with TLR4/TLR2 followed by signaling through p38 MAPK is involved in the induction of the transcription factor Blimp-1, a master regulator capable of triggering the changes of a cascade of gene expression during plasmacytic differentiation, whereas signaling through ERK, p38 MAPK, JNK, and IKK complex is involved in RF3-mediated immunoglobulin secretion. These results establish the signaling and molecular mechanisms of RF3 and provide a molecular and cellular basis for the study of immune-modulation. It is also conceivable that the lifespan-promoting or longevity effect of RF3 may be related to its activities to promote the expansion of hematopoietic stem cells.[18,45] Although it is not clear whether there is any orthologue of Toll-like receptors in *C. elegans*, a Toll-interleukin 1 receptor intracellular domain (TIR-1) has been demonstrated to exist in this primitive worm. TIR-1 has also been shown to be associated with aging and innate immunity in *C. elegans*.[20–22] In this report, we showed that *tir-1* activation indeed preceded the activation of MAPK. The result apparently showed that RF3 stimulated the transcription of *tir-1* and *rab-1*/*pmk-1* of the MAPK pathway and exhibited no effect on *clk-1* gene expression (Fig. 2C), which was shown to be involved in the control of mitochondrial energy metabolism and ATP biosynthesis.[36]

Feeding worms with RNAi bacteria followed by real-time RT-PCR detection of mRNA (Fig. S1, Supplementary data) revealed that both RF3 and LPS could induce *tir-1* expression to a similar extent. However, while RF3 induced *daf-16* expression, the expression was strongly inhibited by LPS under similar conditions. Meanwhile, after knocking down *tir-1*, the repression of *daf-16* by LPS was weakened, indicating the antagonistic pharmacological actions between RF3 and LPS. When using the crude extract from *A. camphorata* under identical conditions to those of RF3, we obtained the same results as with RF3 (data not shown). Therefore, the mechanisms by which RF3 and *A. camphorata* promote longevity might be similar and distinguishable from that of acetic acid. On the other hand, *rab-1* expression always increased when *tir-1* expression was inhibited as revealed by RNAi and RT-PCR. It is therefore suggested that RF3 and the extract of *A. camphorate* probably interact with at least two different types of receptors on the cell surface. One of the receptors increased *tir-1* expression, followed by downstream signal transduction to reduce *rab-1* expression. However at the same time, they may also bind to another unknown receptor, which could induce *rab-1* expression followed by activation of the MAPK pathway, resulting in an increase of *daf-16* activity in the nucleus and extension of the lifespan of *C. elegans*. In contrast, for acetic acid and the extract of *H. erinaceus*, the MAPK pathway appeared to be not involved. Their longevity-promoting effect is probably mediated by a pathway related to *daf-2* expression through several unidentified signaling steps and increased expression of *daf-16* as proposed in Figure 6. It is worth noting that acetic acid, in contrast to benzoic acid and citric acid concurrently tested on *C. elegans* (data not shown), uniquely possessed a prominent lifespan-extending effect comparable to RF3. Acetate is known to be involved in different aspects of carbohydrate and lipid metabolisms via the important and universal metabolic intermediate acetyl-CoA. The biosynthetic pathway has been reported in some microbes, invertebrates and rats,[46,47] which involve acetate thiokinase (acetyl-CoA synthetase; EC 6.2.1.1) to convert acetate + ATP + CoA→acetyl-CoA + AMP + PPi. Therefore the lifespan-promoting effect of acetic acid and various vinegars may be through acetyl-CoA and the related metabolic and hormonal regulation pathways of fatty acid shown to be essential for lifespan

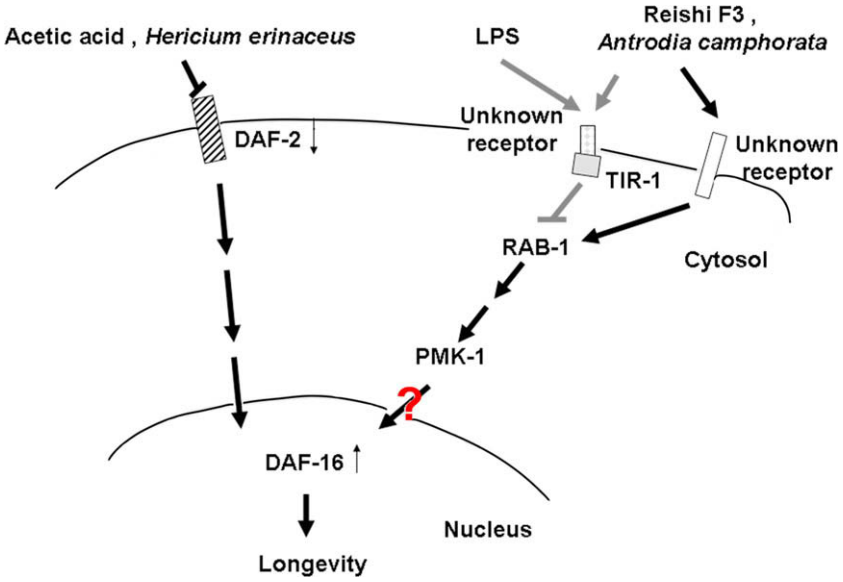



**Figure 6.** The proposed scheme illustrates the putative signaling pathways for *daf-16*-mediated longevity in *C. elegans* after stimulation by acetic acid, RF3, and extracts of *A. camphorata* or *H. erinaceus*, respectively. Reishi polysaccharide fraction RF3 probably attaches to at least two different types of receptors on the cell surface. One of the receptors increased *tir-1* expression leading to downstream signal transduction to reduce *rab-1* expression. However at the same time, they may also bind to another unknown receptor, which could induce *rab-1* expression followed by activation of the MAPK pathway, resulting in an increase of transcription factor *daf-16* activity to extend the lifespan of *C. elegans*. For acetic acid and extract of *H. erinaceus*, the MAPK pathway appeared to be not involved. Their longevity-promoting effect was probably mediated by a pathway which reduced the trans-membrane receptor *daf-2* expression, and thereby indirectly increased the downstream expression of *daf-16*. The upward and downward arrows in thin line show the pathways leading to elevated or reduced expression levels of some key factors like DAF-2 and DAF-16, and the question mark (**?**) indicates some unidentified factors or routes between PMK-1 and DAF-16.

extension.[48] In this regard, the longevity-promoting effect of acetic acid may be analogous to that of calorie restriction in *Saccharomyces cerevisiae* via the elevation of acetyl-CoA and the concomitant increase in the activity of mitochondrial respiration in Krebs cycle.[49] It could also be involved in the regulation of NAD(+)-dependent deacetylase activity. Detailed characterization of the signaling pathway concerning the longevity-promoting effect induced by acetic acid is worthy of further investigation.

### 3.2. Proteomic analysis of *C. elegans* under treatment of acetic acid and/or RF3

The proteomic analysis showed a significant increase in the expression of seven proteins and a decrease in the expression of eight proteins (Table 2). However, the DAF-16 transcription factor and DAF-2 trans-membrane receptor were not detected by the current proteomic methodology probably due to their scarce and transient expression levels. Finally, among the downregulated proteins, we have found a noticeable decrease in one glycoprotein called vitellogenin-6 precursor, confirming the role of vitellogenin involved in the lifespan extension of *C. elegans*. Previous reports also indicated that vitellogenin genes *vit-2* and *vit-5* were downregulated in *daf-2* (−) animals and upregulated in *daf-16* (−) animals.[5] Other proteins, which include transport proteins, transferases and some motor-related proteins, were also found to decrease after treatment with acetic acid and/or RF3. In addition to downregulated proteins, the stress-related heat shock protein 12.2 of the Hsp20/α-crystallin family, was found to be upregulated under these treatments. It is possible that these proteins may participate cumulatively in extending the lifespan of *C. elegans* after treatments.

## 4. Conclusion

The nematode *C. elegans* has proven to be a very useful experimental organism for the study of longevity.[50] More than 70 genes have been found to influence the lifespan in this organism,[5] and of these the insulin/IGF-1 signaling pathway is the best studied. The recent advances in aging studies suggest that the DAF-2/insulin/IGF-I-like receptor and the DAF-16/FOXO transcription factor control the ability of the organism to deal with oxidative stress, immune-modulation and energy metabolism associated with aging and lifespan. By observing the lifecycle of this nematode worm in the presence of some natural substances or synthetic compounds, it is feasible to achieve the goal of extending lifespan by selecting the right combination of active principles like acetic acid in vinegar and the polysaccharide fraction RF3 from Reishi mushroom. The study also addresses an urgent need to develop an efficient high throughput system such as GFP-expression assays (Fig. 5A and B) for identification of new substances which possess longevity effects on model organisms and possibly on humans.

## 5. Experimental section

### 5.1. Strains, bacteria, natural products and chemicals

We used the wild-type N2 strain of *C. elegans* in all experiments. The worms were maintained and cultured on nematode growth medium (NGM) agar plates or in liquid medium with *E. coli* OP50 as a food source. Worms were harvested and washed with M9 buffer (22 mM $KH_2PO_4$, 42 mM $Na_2HPO_4$, 86 mM NaCl and 1 mM $MgSO_4$). Contaminating food and worm debris were removed by sucrose floatation. In addition to OP50, we used two *E. coli* strains for RNAi (III-1N20 for *tir-1* RNAi and I-5M24 for *daf-16* RNAi). RF3 and its composition, crude mycelium-powders of *A. camphorate* and *H. erinaceus* were prepared as described before.[33–35] Each solution was diluted with deionized water when performing assays to make test solutions of desired concentrations.

### 5.2. Lifespan analysis after treatments

Lifespan assays were performed in 12-well tissue culture plates. Each well contained 1–2 mL of NGM medium, supplemented with 1 mg/mL erythromycin to prevent bacterial growth, and 100 μL solution containing test substances at the desired concentrations. The supplement treatments were performed either at L4 or adult-only stage. Before treatments, 100 μM of 5-fluoro-2′-deoxyuridine (FUDR, Sigma, St. Louis, MO, USA) was added to the culture medium to prevent any progeny of test worms from developing. Briefly, worms were grown to the L4/young adult stage on NGM plates seeded with OP50 bacteria, then at least 60 worms were transferred into each of three wells for each assay to be carried out in triplicate. All the plates with gentle gyratory shaking were maintained at 20 °C, as described previously.[5,36] The lifespans of worms under different treatments were estimated by scoring live and dead worms at different time intervals until reaching the level of total death, that is, zero% survival. A worm was considered dead when it failed to respond to plate tapping or a gentle touch with a platinum wire as monitored under microscope (Olympus 1x71, NY, USA). The ANOVA program was used for statistical analysis to determine means and percentiles. In all assays, *P* values were calculated using the log-rank (Mantel-Cox) method.

### 5.3. RNA preparation and reverse transcriptase-PCR (RT-PCR)

Total RNAs from batches of *C. elegans* were isolated from tested samples in the NGM medium at appropriate time intervals using an RNeasy mini kit (Qiagen). Subsequently, RNA (500 ng) from each sample was reverse-transcribed and PCR was performed using a Superscript One-step RT-PCR kit (Invitrogen). One PCR cycle consisted of the following: 94 °C for 15 s, 50–56 °C for 30 s and 72 °C for 45 s, repeating the heating process for 40 cycles and a final extension for 7 min at 72 °C. Primer sequences were designed and listed in Table 1. PCR products were run on 1.8% agarose gels and stained with 0.4 μg/mL ethidium bromide. Stained bands were visualized under UV light and photographed with a camera (Nikon E4500).

### 5.4. Quantitative RT-PCR (qRT-PCR) analysis

For each experiment, approximately 100 L4 larvae or young adult worms were assayed. Total RNAs were isolated from animals incubated at 20 °C for the purpose of measuring *tir-1* and *rab-1* mRNA levels on day 1 after treatment. However, if the total RNA was used in tests for *daf-16* mRNA levels, the animals were harvested on day 2. Isolation, purification, and reverse transcription of *C. elegans* RNA were carried out according to published protocols.[37] Quantitative reverse transcriptase-polymerase chain reactions (qRT-PCR) were performed in an RG-3000 real-time PCR System (Corbett Research, Australia) and analyzed using Rotor-Gene Real-time analysis software 6.1 (Corbett Research); mRNA levels of *act-1* were used for normalization of experimental data. Primer sequences are available upon request.

### 5.5. RNA interference (RNAi) analysis

In the whole-organism RNAi analysis, eggs were added to agar plates seeded with the gene-specific RNAi bacteria (*E. coli* strain HT115) essentially according to the previous report.[38] In experiments before treatments, eggs were added to plates seeded with OP50 for them to grow to L4 worms, and then transferred to

freshly-prepared liquid NGM medium which contained gene-specific RNAi bacteria. 100 μM of FUDR was also added to the medium to prevent any progeny of test subjects from developing. After two days, animals were fed with substances to be tested. All the lifespan measurements of animals with RNAi were carried out as described above. In all experiments, the pre-fertile period of adulthood was used as $t = 0$ for lifespan analysis. ANOVA was used for statistical analysis and to determine means and percentiles. In all assays, *P* values were calculated using the log-rank (Mantel-Cox) method.

### 5.6. 2-DE and image analysis

L4 larvae or young adult worms were transferred to freshly-prepared liquid NGM medium and cultured at 20 °C for four days. 100 μM FUDR, 50 ppm acetic acid, and 100 ppm RF3 were then added to the medium. After three-day treatment, the worms were solubilized in lysis buffer containing 8 M urea, 0.5% Triton X-100 and protease inhibitor cocktail, frozen in liquid nitrogen, and then pulverized into fine powders with a mortar. The homogenates were sonicated and the supernatants after centrifugation were collected and used as protein-loading samples. 150 μg total protein as estimated by protein-content determination using 2-D Quant Kit (Amersham Biosciences), was loaded onto immobilized pH gradient (IPG) gel strips (pH 3–10, 13 cm, Amersham Biosciences). The IPG strips were rehydrated overnight. For the first-dimensional separation, IEF was carried out using Ettan IPGphor II (Amersham Biosciences) at 300–8000 V for 16 h. After IEF, the IPG strips were equilibrated for 10 min each in two equilibration solutions (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol containing 100 mg dithiothreitol (DTT) or 250 mg iodoacetic acid, respectively), and the second-dimensional electrophoresis was conducted at 130–250 V for 5–6 h. The gels were stained by Sypro-Ruby overnight. The 2-DE gel images were scanned using a fluorescence image scanner Typhoon 9400 (Amersham Biosciences) and analyzed by using PDQuest software (Bio-Rad). Intensity levels were normalized between gels as a proportion of the total protein intensity detected for the entire gel.

### 5.7. In-gel digestion and LC–MS/MS

Based on the 2-DE analysis of samples under different treatments, we selected 15 differentially expressed proteins (based on at least twofold protein-expression change between control and treated samples) for further identification by LC–MS/MS (nano ESI-Q/TOF) at the proteomic core facility of the Institute of Biological Chemistry, Academia Sinica. The protein spots were cut from 2-D gels, and then destained three times with 25 mM ammonium bicarbonate buffer (pH 8.0) in 50% acetonitrile (ACN) for 1 h. The gel pieces were dehydrated in 100% ACN for 5 min and then dried for 30 min in a vacuum centrifuge. Enzyme digestion was performed by adding 0.5 μg trypsin in 25 mM ammonium bicarbonate per sample at 37 °C for 16 h. The peptide fragments were extracted twice with 50 μL 50% ACN/0.1% TFA. After removal of ACN and TFA by centrifugation in a vacuum centrifuge, samples were dissolved in 0.1% formic acid/50% ACN and analyzed by LC–nanoESI-MS/MS. Proteins were identified in NCBI databases based on MS/MS ion search with the MASCOT program.

### 5.8. Green fluorescent protein (GFP)-expression assays

The transgenic worms carrying *daf-16::gfp* transcriptional reporter construct were used for GFP-expression assays. To test whether the expression of GFP exemplified specifically the expression of DAF-16, we used *daf-16* and *tir-1* RNAi to determine its specificity. RNAi assays were as described in the above section. Fluorescent images of the worms were taken at appropriate time intervals depending upon the lifespan analysis after treatments with RF3 and/or acetic acid under a fluorescent microscope (Olympus 1 × 71, NY, USA) and the affiliated digital camera (Olympus DP controller). All the experiments were repeated in duplicate with consistent and similar image results.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.09.002.

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