ASM-3 Acid Sphingomyelinase Functions as a Positive Regulator of the DAF-2/AGE-1 Signaling Pathway and Serves as a Novel Anti-Aging Target

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Abstract

In C. elegans, the highly conserved DAF-2/insulin/insulin-like growth factor 1 receptor signaling (IIS) pathway regulates longevity, metabolism, reproduction and development. In mammals, acid sphingomyelinase (ASM) is an enzyme that hydrolyzes sphingomyelin to produce ceramide. ASM has been implicated in CD95 death receptor signaling under certain stress conditions. However, the involvement of ASM in growth factor receptor signaling under physiological conditions is not known. Here, we report that in vivo ASM functions as a positive regulator of the DAF-2/IIS pathway in C. elegans. We have shown that inactivation of asm-3 extends animal lifespan and promotes dauer arrest, an alternative developmental process. A significant cooperative effect on lifespan is observed between asm-3 deficiency and loss-of-function alleles of the age-1/PI 3-kinase, with the asm-3; age-1 double mutant animals having a mean lifespan 259% greater than that of the wild-type animals. The lifespan extension phenotypes caused by the loss of asm-3 are dependent on the functions of daf-16/FOXO and daf-18/PTEN. We have demonstrated that inactivation of asm-3 causes nuclear translocation of DAF-16::GFP protein, up-regulates endogenous DAF-16 protein levels and activates the downstream targeting genes of DAF-16. Together, our findings reveal a novel role of asm-3 in regulation of lifespan and diapause by modulating IIS pathway. Importantly, we have found that two drugs known to inhibit mammalian ASM activities, desipramine and clomipramine, markedly extend the lifespan of wild-type animals, in a manner similar to that achieved by genetic inactivation of the asm genes. Our studies illustrate a novel strategy of anti-aging by targeting ASM, which may potentially be extended to mammals.

Introduction

The nematode Caenorhabditis elegans serves as an excellent genetic model to study the mechanisms of cell signaling and organismal aging. In C. elegans, the DAF-2/IIS pathway plays a critical role in the regulation of animal lifespan, metabolism, reproduction and development [1–4]. In particular, reduced DAF-2/IIS signaling results in lifespan extension and stress resistance in adult animals, and dauer arrest in larvae [1–4]. The components of the IIS pathway are also highly conserved in various organisms including worms, flies and mammals [5]. In the IIS pathway, ligand engagement to the DAF-2 receptor tyrosine kinase, through activating the AGE-1/PI-3 kinase homolog, leads to the activation of PDK-1 and then AKT-1/2 serine/threonine kinases [3,4]. Activated AKT-1/2 phosphorylates the DAF-16/FOXO transcription factor, preventing the latter from entering into the nucleus [3,6–8]. Down-regulation of DAF-2 signaling leads to nuclear translocation and activation of DAF-16 [3,6,9]. Loss of daf-16 suppresses the phenotypes of lifespan extension and constitutive dauer arrest in the mutants defective in daf-2 signaling [1–3,10]. On the other hand, increased protein levels of DAF-16, either through transgene-mediated overexpression or through reduced protein degradation, extends animal lifespan [11–13]. Another negative regulator of the daf-2 pathway, daf-18, encoding the C. elegans homolog of human PTEN tumor suppressor, antagonizes daf-2 and age-1 signaling. Thus, inactivation of daf-18 suppresses lifespan extension and constitutive dauer arrest phenotypes of daf-2 or age-1 mutants [14,15]. However, additional regulators of the IIS pathway remain to be identified.

In mammals, acid sphingomyelinase (ASM) is a phosphodiesterase that hydrolyzes sphingomyelin to produce ceramide and phosphorylcholine [16]. When acting in lipid rafts, which are plasma membrane microdomains [17], ASM leads to the production of ceramide-enriched lipid rafts [16]. Because ceramides have a physical property of self-association, it is likely that ceramide-enriched lipid rafts can provide a unique local microenvironment for protein-protein interactions. Indeed, ASM-dependent and ceramide-enriched lipid rafts have been shown to facilitate the oligomerization and signaling of CD95 receptors, leading to apoptosis [18,19]. However, most recent studies have shown that CD95, previously known as death receptors, also possess pro-proliferation and pro-survival function in vivo under...
physiological conditions [20]. It is not known if ASM is involved in the pro-proliferation and pro-survival signaling of the CD95 receptors. In addition to ASM, neutral sphingomyelinase (NSM) can also hydrolyze sphingomyelin to produce ceramide, although the exact site of NSM action is not clear [21,22]. Ceramides can also be synthesized by a de novo biosynthetic pathway, which occurs in the endoplasmic reticulum and Golgi apparatus [21,22]. Ceramides produced through biosynthesis are also known to affect the cellular processes such as cell signaling, stress resistance, and apoptosis, although the detailed molecular mechanisms are not clear [21,22]. As ceramides produced by hydrolysis of sphingomyelin and through biosynthesis are localized in different cellular compartments, it is possible that they may have different cellular functions. In C. elegans, while the ceramides produced through de novo biosynthesis are known to be involved in the radiation-induced apoptosis in the germline [23], the roles of ceramides produced through hydrolysis of sphingomyelin are not clear, although C. elegans have been shown to contain ASM homologs but no NSM homologs [23,24].

In the current study, we used a genetic approach to study the role of asm in signal transduction in the C. elegans model system. Our results show that asm-3 is a critical and positive regulator of the DAF-2/IIS pathway and controls both animal lifespan and dauer formation. Using pharmacological agents, we further demonstrated that inhibition of CeASM, similar to genetic inactivation of the asm genes, leads to significant animal lifespan extension. Our studies illustrate a novel anti-aging strategy of targeting CeASMs to down-regulate the DAF-2/IIS pathway, which could potentially be extended to mammals.

Figure 1. Loss of asm gene activities extends animal lifespan in a daf-16 or daf-18 dependent manner. For RNAi experiments, the vector alone (L4440) was used as a control. (A) asm-3 RNAi extended animal lifespan with the mean lifespan 19% greater than that of the control in the rrf-3(pk1426) background (P<0.0001). (B) asm-3(ok1744) mutants had 14% longer lifespan than wild-type (N2) animals (P = 0.0141). (C) Knockdown of asm-1, asm-2 or asm-3 by RNAi each extended lifespan with the mean lifespan 12%, 10% or 19% greater than that of the vector control in the rrf-3(pk1426) background, respectively (P = 0.0068 for asm-1 RNAi, P = 0.0258 for asm-2 RNAi and P<0.0001 for asm-3 RNAi). (D) Experiments were carried out in the asm-3(ok1744);rrf-3(pk1426) background. asm-3(ok1744) mutation extended lifespan with the mean lifespan 15% greater than that of the control (P = 0.0018), and the lifespan of asm-3(ok1744) mutant was further enhanced by RNAi of asm-1 or asm-2 with the mean lifespan 30% or 28% greater than that of the control (P<0.0001 for asm-1 RNAi or asm-2 RNAi). Lifespan extension produced by asm-3 mutation was inhibited by daf-16 RNAi (P<0.0001). (E) daf-16(mgDf47) null mutation completely abolished lifespan extension phenotype of asm-3 mutants. (F) Lifespan extension phenotype by asm-1, asm-2 or asm-3 RNAi shown in (C) was completely abolished by daf-18(nr2037) null mutation in the rrf-3(pk1426);daf-18(nr2037) background. Mean lifespan, P values and other details for these experiments are listed in Table 1 and Table 2. doi:10.1371/journal.pone.0045890.g001
Results

asm Genes Regulate Animal Lifespan as Novel Longevity Genes

The C. elegans genome encodes three ASM homologs, asm-1, asm-2, and asm-3. Among them, ASM-3 is most closely related to human ASM, and the two proteins share 42% identity in the predicted C-terminal catalytic domain (Figure S1). The asm-3 gene was initially discovered in our recent genome-wide RNAi screen for new genes regulating aging [55]. In the current study, we found that RNAi-mediated inactivation of asm-3 produces a lifespan extension phenotype (Figure 1A). The mean lifespan of animals treated with asm-3 RNAi was 19% longer than that of the vector control (Table 1, Set #1). To verify lifespan extension phenotype in asm-3 mutant, we also investigated a chromosomal mutation of asm-3, asm-3(ok1744), a putative null allele with a 1558 bp deletion and a 7 bp insertion in the predicted catalytic domain (Figure S2). Consistent with RNAi experiments, the asm-3(ok1744) mutants also extended animal lifespan by 14% as compared to the wild-type (Figure 1B; Table 2, Set #1). In C. elegans, two other paralogs, asm-1 and asm-2, encode polypeptides highly homologous to ASM-3 (Figure S3). We thus examined their roles in lifespan regulation. When asm-1 or asm-2 was inactivated by RNAi knockdown, a modest lifespan extension phenotype was observed (Figure 1C), with the mean lifespan 12% or 10% greater than that of the control, respectively (Table 1, Set #2). We speculated that there may be functional redundancy between asm-3, asm-1 and asm-2. Indeed, double inactivation of asm-3 and asm-1 or of asm-3 and asm-2 further extended lifespan, with the mean lifespan 30% or 28% greater than that of the control, respectively (Figure 1D; Table 1, Set #3). These results suggest that asm-3, asm-1 and asm-2 each contributes to the regulation of animal lifespan, and inactivating two asm genes produces an additive effect on lifespan extension. Together, our results demonstrate the importance of the asm gene family in the regulation of lifespan and highlight asm-3 as the most prominent member in this process.

Genetic Interactions of asm-3 with Negative Regulators of the daf-2/IIS Pathway

To investigate whether asm-3 functions in the daf-2/IIS pathway, we examined genetic interactions between asm-3 and major components of the daf-2/IIS pathway. While partial loss-of-function daf-2(e1370) mutants had dramatically extended lifespan at 20°C [1], we found that the lifespan of daf-2 mutants was not further extended by the loss of asm-3 (Figure 2A; Table 2, Set #3). In the asm-3(ok1744);daf-2(e1370) double mutant, it is possible that daf-2 signaling was reduced to below threshold levels by the daf-2(e1370) mutation, and thus the asm-3(ok1744) mutation could not further dampen the daf-2 signaling outputs. Thus, this result suggests that asm-3 and daf-2 function in the same pathway. We also examined genetic interaction between asm-3 and age-1. The age-1 gene encodes a homolog of mammalian PI-3 kinase catalytic subunit [26]. Partial loss-of-function age-1(mg305) mutants have a dramatically extended lifespan phenotype [27]. Remarkably, our study showed that asm-3(ok1744);age-1(mg305) double mutants had mean lifespan 67% greater than that of age-1(mg305) single mutants, or 259% greater than that of wild-type animals (Figure 2B; Table 2, Set #4). Consistently, we also observed a cooperative effect on animal lifespan extension when asm-3 was inactivated in another partial loss-of-function age-1 mutant, age-1(hs546) [26] (data not shown). Subsequently, we analyzed the genetic interaction between asm-3 and akt-1. It has been previously reported that akt-1, encoding a C. elegans homolog of mammalian p53 regulatory subunit of PI-3 kinase, functions in the same pathway as age-1 [28]. While partial loss-of-function akt-1(n809) mutants exhibited longer lifespan phenotype than wild-type animals, silencing of asm-3 in the akt-1(n809) mutants further extended the mean lifespan by 21% (Figure 2C; Table 1, Set #5). These genetic results indicate that loss of asm-3 cooperates with an age-1 or akt-1 loss-of-function mutation to extend animal lifespan, suggesting that asm-3 potentiates age-1/akt-1 signaling. We also tested asm-3 interaction with pdk-1, which encodes a kinase that acts downstream of AGE-1 but upstream AKT-1/2 in the DAF-2/IIS pathway [29]. Loss of asm-3 did not further extend the lifespan in the partial loss-of-function pdk-1(sa709) [29] mutant background (Figure 2D; Table 2, Set #5). This result suggests that asm-3 and pdk-1 likely function in the same pathway. In addition, we tested the genetic interaction of asm-3(ok1744) with a null mutation of akt-1, akt-1(mg306) [30]. We observed that the asm-3(ok1744) mutation shortened the lifespan of the longer lived akt-1(mg306) mutant animals, but the akt-1(mg306) mutation did not seem to affect the lifespan extension phenotype of the asm-3(ok1744) mutant (Figure 2E; Table 2, Set #6). The fact that the asm-3(ok1744) mutation can suppress the lifespan extension phenotype of the akt-1(mg306) mutant suggests that there may be additional genetic interactions between the gene families of akt and asm. Taken together, these results show that asm-3 plays an important role in the daf-2/IIS pathway to regulate animal lifespan.

Loss of asm-3 Results in an Increased Resistance to Environmental Stress

Many long-lived mutants of the daf-2/IIS pathway have strong resistance toward environmental stress such as oxidative...
transduction pathway [3,10]. Dauer, a hibernation-like state, is similar to the reports of enhanced resistance observed in the Pathway 2 (e1370) plays an important role in regulation of stress response, and the stress sensitive to environmental stress responses, and the stress wild-type animals (Figure S4B). From those two stress response more resistant against heat stress of 35 °C when compared to S4A). In addition, the asm-3(ok1744) stress response, as reported previously [31–35]. We found that oxidative stress test and high temperature (35 °C) for the heat oxidative stress or heat stress condition. We used paraquat, a chemical to produce reactive oxygen species towards paraquat as compared to the wild-type animals (Figure S4A). In addition, the asm-3(ok1744) mutant adult animals were more resistant against heat stress of 35°C when compared to wild-type animals (Figure S4B). From those two stress response assays, we also found that daf-16(mgDf47) mutants were very sensitive to environmental stress responses, and the stress resistance phenotypes of the asm-3(ok1744) mutant was daf-16-dependent (Figure S4A, S4B). Thus, these data suggest that asm-3 plays an important role in regulation of stress response, similar to the reports of enhanced resistance observed in the daf-2(e1370), age-1(hx546) or age-1(mg305) mutants [31–34,36,37].

asm-3 Regulates Dauer Arrest by Modulating the daf-2/IIS Pathway

In C. elegans, dauer arrest is controlled by a variety of signal transduction pathway [3,10]. Dauer, a hibernation-like state, is normally induced by starvation, high population density or high temperature [3,10]. Mutants with reduced signaling in the daf-2/IIS pathway form dauer even under favorable growth conditions [3,10]. We investigated whether asm-3 participates in the daf-2/IIS pathway to regulate dauer formation. As previously reported [28,38,39], temperature-sensitive daf-2(e1370) mutants are prone to induction of dauer arrest at 25°C and less so at 22.5°C. Consistent with previous reports, our results showed that the daf-2(e1370) mutants formed dauer constitutively at non-permissive temperature 25°C, but formed dauer less efficiently at semi-permissive 22.5°C (Figure 3A; Table S1). However, the dauer arrest at 22.5°C in daf-2(e1370) mutants was markedly enhanced from 46% to 86% by the presence of the asm-3(ok1744) allele (Figure 3A; Table S1). In addition, age-1(mg305) mutants possessed a constitutive dauer phenotype at 25°C [27] but rarely displayed dauer arrest phenotype at 22.5°C (Figure 3B; Table S1). On the other hand, loss of asm-3 by itself did not induce dauer arrest at 25°C (Figure 3A, 3B; Table S1). Therefore, these data indicate that the asm-3 gene activity potentiates daf-2 and age-1 signaling to regulate dauer arrest. In comparison, because dauer formation phenotype of the pdk-1(sa709) mutant was so penetrant at 27°C [29] (Figure 3C; Table S1), we could not detect any effects of asm-3(ok1744) mutation on pdk-1(sa709) at this temperature (Figure 3C;

Table 1. Summary of adult lifespan assays after RNAi-mediated gene inactivation.

| Genotype                  | RNAi          | Mean Lifespan (Days) ± SEM | Relative Mean Lifespan (%) | *Relative Mean Lifespan (%) | P value | * P value |
|---------------------------|---------------|----------------------------|---------------------------|---------------------------|---------|----------|
| Set #1                    |               |                            |                           |                          |         |          |
| rrf-3(pk1426)             | L4440         | 16.1±0.47                  | 100                       | –                         | –       | –        |
| asm-3                     |               | 19.1±0.59                  | 119                       | –                         | <0.0001 | –        |
| Set #2                    |               |                            |                           |                          |         |          |
| rrf-3(pk1426)             | L4440         | 19.6±0.54                  | 100                       | –                         | –       | –        |
| asm-1                     |               | 22.0±0.52                  | 112                       | –                         | 0.0068  | –        |
| asm-2                     |               | 21.6±0.57                  | 110                       | –                         | 0.0258  | –        |
| asm-3                     |               | 23.4±0.66                  | 119                       | –                         | <0.0001 | –        |
| Set #3                    |               |                            |                           |                          |         |          |
| rrf-3(pk1426)             | L4440         | 17.4±0.57                  | 100                       | –                         | –       | –        |
| asm-3(ok1744);rrf-3(pk1426) | *L4440         | 20.1±0.61                  | 115                       | 100                       | 0.0018  | –        |
| asm-1                     |               | 22.7±0.64                  | 130                       | 113                       | <0.0001 | 0.0019  |
| asm-2                     |               | 22.3±0.65                  | 128                       | 111                       | <0.0001 | 0.0072  |
| daf-16                    |               | 12.8±0.20                  | –                         | 64                        | –       | <0.0001 |
| Set #4                    |               |                            |                           |                          |         |          |
| rrf-3(pk1426);daf-18(nr2037) | L4440         | 9.9±0.60                   | 100                       | –                         | –       | –        |
| asm-1                     |               | 10.0±0.55                  | 103                       | –                         | 0.7936  | –        |
| asm-2                     |               | 10.4±0.46                  | 106                       | –                         | 0.609   | –        |
| asm-3                     |               | 10.1±0.49                  | 102                       | –                         | 0.9618  | –        |
| Set #5                    |               |                            |                           |                          |         |          |
| aap-1(mg889)              | L4440         | 42.41±1.79                 | 100                       | –                         | –       | –        |
| asm-3                     |               | 51.33±2.23                 | 121                       | –                         | <0.0001 | –        |

RNAi treatments, initiated at L1 stage, were continued throughout the assay time. Lifespan assays were performed at 20°C. Each experiment was individually grouped and statistical analyses of the data sets were carried out using the vector (L4440) control as the reference in each group (P value). In Set #3, the second set of statistical analyses was carried out for all the survivor data derived from the asm-3(ok1744);rrf-3(pk1426) strain, using the corresponding vector (L4440) control in this strain marked as ‘*’ as the reference for this subgroup (*P values). Each set of the lifespan experiments was repeated at least two independent times and similar results were obtained. Data from representative sets of experiments are shown. Greater than 50 worms were counted for each RNAI-inducing condition in each experiment.

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stress [4,31–35] and heat stress stimuli [33,34,36,37] and this response is regulated by DAF-16/FOXO [31–35]. Thus, we tested whether asm-3 could regulate stress response under the oxidative stress or heat stress condition. We used paraquat, a chemical to produce reactive oxygen species in vivo, for the oxidative stress test and high temperature (35°C) for the heat stress response, as reported previously [31–37]. We found that asm-3(ok1744) mutant adult worms had increased resistance towards paraquat as compared to the wild-type animals (Figure S4A). In addition, the asm-3(ok1744) mutant adult animals were more resistant against heat stress of 35°C when compared to wild-type animals (Figure S4B). From those two stress response assays, we also found that daf-16(mgDf47) mutants were very sensitive to environmental stress responses, and the stress resistance phenotypes of the asm-3(ok1744) mutant was daf-16-dependent (Figure S4A, S4B). Thus, these data suggest that asm-3 plays an important role in regulation of stress response, similar to the reports of enhanced resistance observed in the daf-2(e1370), age-1(hx546) or age-1(mg305) mutants [31–34,36,37].

asm-3 Regulates Dauer Arrest by Modulating the daf-2/IIS Pathway

In C. elegans, dauer arrest is controlled by a variety of signal transduction pathway [3,10]. Dauer, a hibernation-like state, is normally induced by starvation, high population density or high temperature [3,10]. Mutants with reduced signaling in the daf-2/IIS pathway form dauer even under favorable growth conditions [3,10]. We investigated whether asm-3 participates in the daf-2/IIS pathway to regulate dauer formation. As previously reported [28,38,39], temperature-sensitive daf-2(e1370) mutants are prone to induction of dauer arrest at 25°C and less so at 22.5°C. Consistent with previous reports, our results showed that the daf-2(e1370) mutants formed dauer constitutively at non-permissive temperature 25°C, but formed dauer less efficiently at semi-permissive 22.5°C (Figure 3A; Table S1). However, the dauer arrest at 22.5°C in daf-2(e1370) mutants was markedly enhanced from 46% to 86% by the presence of the asm-3(ok1744) allele (Figure 3A; Table S1). In addition, age-1(mg305) mutants possessed a constitutive dauer phenotype at 25°C [27] but rarely displayed dauer arrest phenotype at 22.5°C (Figure 3B; Table S1). However, loss of asm-3 dramatically enhanced dauer arrest of age-1(mg305) mutants at 22.5°C from 3% to 99% (Figure 3B; Table S1). On the other hand, loss ofasm-3 by itself did not induce dauer arrest at 25°C (Figure 3A, 3B; Table S1). Therefore, these data indicate that the asm-3 gene activity potentiates daf-2 and age-1 signaling to regulate dauer arrest. In comparison, because dauer formation phenotype of the pdk-1(sa709) mutant was so penetrant at 27°C [29] (Figure 3C; Table S1), we could not detect any effects of asm-3(ok1744) mutation on pdk-1(sa709) at this temperature (Figure 3C;
mutants are known to constitutively form dauer at 25°C (Figure 3E; Table S1). These results thus indicate that dauer formation of the constitutive dauer arrest phenotype of \( \text{asm-3} \) (Figure 3C, 2D; Figure 3D, 2E). Taken together, our genetic analyses suggest that \( \text{asm-3} \) regulates dauer formation. The genetic interactions of \( \text{asm-3} \) with the presence of the \( \text{akt-1} \) (mg306) null mutants, which were shown to form dauer efficiently at 27°C, loss of \( \text{asm-3} \) partially suppressed dauer formation of \( \text{akt-1} \) (mg306) mutants at 27°C (Figure 3D; Table S1). The genetic interactions of \( \text{asm-3} \) with \( \text{pdh-1} \) or \( \text{akt-1} \) in dauer formation appear to be consistent with those in lifespan regulation (Figure 3C, 2D; Figure 3D, 2E). Taken together, our genetic analyses suggest that \( \text{asm-3} \) acts in the daf-2/IIS pathway to regulate dauer formation.

In addition to the daf-2/IIS pathway, the daf-7/TGF-\( \beta \)-like signaling pathway is also involved in the regulation of dauer formation. The daf-7 gene encodes a ligand related to mammalian transforming growth factor beta (TGF-\( \beta \)) [41]. The daf-7 (e1372) mutants are known to constitutively form dauer at 25°C [41]. We found that, at either 25°C or 22.5°C, loss of \( \text{asm-3} \) did not affect the constitutive dauer arrest phenotype of daf-7 (e1372) mutants (Figure 3E; Table S1). These results thus indicate that dauer regulation via the daf-7/TGF-\( \beta \) signaling pathway does not require \( \text{asm-3} \) gene activity. These results suggest that the involvement of \( \text{asm-3} \) in the daf-2/IIS pathway is specific. Loss of \( \text{asm-3} \) Genes Results in Nuclear Localization of DAF-16

In live animals, the downstream signaling output of the DAF-2/IIS pathway can be assayed by the intracellular localization of DAF-16/FOXO. DAF-16 is normally sequestered in the cytoplasm after phosphorylation by AKT-1/AKT-2, while mutations causing reduced signaling in the daf-2/IIS pathway lead to translocation of DAF-16 to the nucleus [3,9]. To further investigate whether \( \text{asm-3} \) directly regulates the molecular signaling cascade of DAF-2 to DAF-16, we used a strain carrying a daf-16::gfp transgene to monitor the intracellular distribution of DAF-16::GFP fusion protein [9]. We constructed mutant strains of \( \text{raf-3(pk1426);daf-16::gfp} \) and \( \text{asm-3(ok1744);raf-3(pk1426);daf-16::gfp} \) with the presence of the \( \text{raf-3(pk1426)} \) allele to enhance the RNAi efficiency. We found that inactivation of individual \( \text{asm} \) genes each induced nuclear localization of DAF-16.

Table 2. Summary of adult lifespan assays in various mutant backgrounds.

| Genotype | Mean Lifespan ± SEM (Days) | Relative Mean Lifespan (%) | P value | *P value |
|----------|-----------------------------|-----------------------------|---------|---------|
| Set #1   |                             |                             |         |         |
| N2 (wild-type) | 15.3±0.58                   | 100                         | –       | –       |
| \( \text{asm-3(ok1744)} \) | 17.4±0.69                   | 114                         | 0.0141  | –       |
| Set #2   |                             |                             |         |         |
| N2 (wild-type) | 14.8±0.57                   | 100                         | –       | –       |
| \( \text{asm-3(ok1744)} \) | 17.1±0.80                   | 116                         | 0.0077  | –       |
| \( \text{daf-16(mgDf47)} \) | 13.6±0.47                   | 92                          | 0.0303  | –       |
| \( \text{asm-3(ok1744);daf-16(mgDf47)} \) | 13.7±0.4                   | 93                          | 0.0165  | 0.8974  |
| Set #3   |                             |                             |         |         |
| N2 (wild-type) | 16.2±0.56                   | 100                         | –       | –       |
| \( \text{asm-3(ok1744)} \) | 18.7±0.80                   | 116                         | 0.005   | –       |
| \( \text{forge}(e1370) \) | 39.4±1.34                   | 244                         | <0.0001 | –       |
| \( \text{asm-3(ok1744);forge}(e1370) \) | 40.1±1.51                   | 248                         | <0.0001 | 0.463   |
| Set #4 (FuDR) |                             |                             |         |         |
| N2 (wild-type) | 19.4±0.22                   | 100                         | –       | –       |
| \( \text{asm-3(ok1744)} \) | 21.2±0.49                   | 109                         | 0.005   | –       |
| \( \text{age-1(mg305)} \) | 41.7±0.98                   | 215                         | <0.0001 | –       |
| \( \text{asm-3(ok1744);age-1(mg305)} \) | 69.6±1.52                   | 359                         | <0.0001 | <0.0001 |
| Set #5   |                             |                             |         |         |
| N2 (wild-type) | 14.4±0.46                   | 100                         | –       | –       |
| \( \text{asm-3(ok1744)} \) | 16.7±0.64                   | 116                         | 0.0056  | –       |
| \( \text{pdh-1(sa709)} \) | 21.1±1.25                   | 147                         | <0.0001 | –       |
| \( \text{asm-3(ok1744);pdh-1(sa709)} \) | 21.7±1.07                   | 151                         | <0.0001 | 0.8404  |
| Set #6   |                             |                             |         |         |
| N2 (wild-type) | 15.7±0.56                   | 100                         | –       | –       |
| \( \text{asm-3(ok1744)} \) | 18.1±0.87                   | 115                         | 0.0039  | –       |
| \( \text{akt-1(mg306)} \) | 21.8±0.83                   | 139                         | <0.0001 | –       |
| \( \text{asm-3(ok1744);akt-1(mg306)} \) | 14.8±0.81                   | 95                          | 0.8422  | <0.0001 |

All the assays were carried out at 20°C and on regular NGM plates. For Set #4, assays were carried out on plates containing FuDR (50 μg/ml) to avoid the internal hatching of the \( \text{asm-3(ok1744);age-1(mg305)} \) mutant animals. Each experiment was individually grouped and statistical analyses were carried out for the experiment data set and the wild-type control data set in each group (P values). In addition, statistical analyses were carried out for the survival data of the assayed single mutant (marked as *P values). In Set #6, P value between \( \text{asm-3(ok1744)} \) and \( \text{asm-3(ok1744);akt-1(mg306)} \) is 0.064. Each set of the lifespan experiments was repeated at least two independent times and similar results were obtained. Data from representative sets of experiments are shown. Greater than 50 worms were counted for each strain in each experiment.

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GFP in the rrf-3(pk1426);daf-16::gfp background (Figure 4A; Figure S5). As expected, age-1, daf-2, or akt-1 RNAi, which were used as positive controls, all resulted in the nuclear localization of DAF-16::GFP (Figure 4A; Figure S5). In contrast, control (L4440) RNAi and daf-18 RNAi led to a diffused distribution of DAF-16::GFP in cytoplasm, while daf-16 RNAi greatly decreased fluorescence intensity of DAF-16::GFP (Figure 4A; Figure S5). We also tested the effects of multiple knockdowns of asm genes. We found that combined inactivation of 2 or 3 asm genes caused a further increase in the nuclear localization of DAF-16::GFP in the asm-3(ok1744);rrf-3(pk1426);daf-16::gfp mutant background (Figure 4B; Figure S6). In addition, the nuclear translocation of DAF-16::GFP in the asm-3(ok1744) mutant was abolished by daf-16 RNAi or daf-18 RNAi (Figure S6). These results are consistent with the observations that daf-16 and daf-18 gene activities are required for the asm-mediated lifespan regulation (Figure 1D-1F). Taken together, these results indicate that asm-3 regulates DAF-16 through controlling its intracellular localization.

Loss of asm-3 Increases Endogenous DAF-16 Protein Expression

The activity and protein levels of the DAF-16/FOXO transcription factor are essential for the lifespan regulation in the daf-2/Insulin/IGF-1 signaling pathway [11–13]. Increased protein levels of endogenous DAF-16 have been reported for the longer-lived sir-1 mutants [12] or eak-7 mutant [42], and overexpression of the DAF-16 homolog in Drosophila leads to extension of lifespan (Figure 2). We therefore examined the DAF-16 protein levels in animals where the asm-3 gene was inactivated. We observed that endogenous DAF-16 protein levels were elevated by the asm-3(ok1744) mutation or by asm-3 RNAi (Figure 4C, 4D; an increase of 35% or 100%, respectively). Interestingly, we also observed that either daf-2(e1370) mutation or daf-2 RNAi led to a significant increase in DAF-16 protein levels (Figure 4C, 4D; an increase of 120% or 180%, respectively). As controls, wild-type animals or animals treated with vector (L4440) RNAi were used, respectively. Taken together, our results indicate that ASM-3 and DAF-2 each negatively regulates the endogenous DAF-16 protein expression

Figure 2. Effects of asm-3 on lifespan regulation in various mutants defective in the daf-2 signaling. (A) Loss of asm-3 did not further increase the lifespan of daf-2(e1370) mutants (P = 0.463). (B) asm-3(ok1744) mutation enhanced the mean lifespan of the longer-lived age-1(mg305) mutants by 67% (P<0.0001). (C) Silencing of asm-3 in the aap-1(m889) mutant background further extended the mean lifespan by 21% compared to control (L4440) RNAi (P<0.0001). (D) asm-3 mutation did not affect lifespan of pdk-1(sa709) mutant animals (P = 0.8404). (E) Effects of asm-3(ok1744), akt-1(mg306), and asm-3(ok1744);akt-1(mg306) mutations on lifespan regulation. asm-3 mutation inhibited the lifespan extension of akt-1(mg306) mutant (P<0.0001), but akt-1 mutation did not seem to affect the lifespan extension of asm-3(ok1744) mutant (P = 0.064). Mean lifespan, P values and other details for these experiments are listed in Table 1 and Table 2.
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ASM-3: An Essential Component of the DAF-16/FOXO Signaling Pathway

Previous studies have shown that DAF-16/FOXO transcription factor acts as a master regulator of aging in C. elegans [11–13]. However, the mechanisms by which DAF-16/FOXO transcription factor regulates aging are not fully understood. Here, we report that the acid sphingomyelinase (ASM) gene, asm-3, encodes a novel anti-aging target in C. elegans. We observed that loss of asm-3 results in increased lifespan, which is consistent with the previous studies [14–16]. In addition, we found that combined inactivation of 2 or 3 asm genes caused a further increase in the nuclear localization of DAF-16::GFP in the asm-3(ok1744);rrf-3(pk1426);daf-16::gfp mutant background. These results suggest that ASM-3 negatively regulates DAF-16 through controlling its intracellular localization.
levels. The increased DAF-16 protein levels, in addition to the nuclear translocation of DAF-16, may constitute a signaling output from the daf-2/IIS pathway.

Loss of asm-3 Promotes the Expression of daf-16 Target Genes

As described above, inactivation of asm-3 triggers nuclear translocation of DAF-16/FOXO and up-regulates endogenous DAF-16 protein levels. However, as previously reported [45–47], nuclear translocation of DAF-16/FOXO is not sufficient for activation of the DAF-16/FOXO transcription activity. We therefore examined whether loss of asm-3 indeed results in an increase of activity of daf-16 target genes [48,49]. The sod-3 gene, encoding superoxide dismutase, is directly regulated by DAF-16; and in partial loss-of-function daf-2 mutants, a GFP transgene under control of the sod-3 promoter (sod-3p::gfp), is highly expressed [11]. We therefore examined transcriptional expression of endogenous sod-3 by using qRT-PCR analysis. We observed that sod-3 mRNA levels were increased by about 3-fold in asm-3(ok1744) mutants over the wild-type (Figure 5A). The expression of another DAF-16-regulated gene, mtl-1 [48], was also modestly increased in asm-3 mutants (Figure 5B). These results indicate that asm-3 regulates expression of daf-16 target genes through controlling DAF-16/FOXO transcriptional activity. In addition, we used a strain carrying the sod-3p::gfp transgene to monitor the daf-16/FOXO transcriptional activity. Inactivation of a single asm gene has only a modest effect on the sod-3p::gfp reporter gene expression (data not shown). However, inactivation of 2 or 3 asm genes, achieved through RNAis of asm-1, asm-2 or asm-1 and asm-2 together in the asm-3(ok1744);rrf-3(pk1426);sod-3p::gfp mutant

Figure 3. Effects of asm-3 on dauer formation regulation in various mutants defective in the daf-2 signaling. (A) Loss of asm-3 enhanced dauer formation of daf-2(e1370) mutants at the semi-permissive temperature 22.5°C. (B) asm-3 mutation greatly enhanced dauer arrest phenotype of age-1(mg305) mutants at 22.5°C. (C) asm-3 mutation did not affect dauer arrest induced by the pdk-1(sa709) mutation at 27°C. The mutant animals carrying sa709 allele formed dauer at 27°C but not at 25°C. (D) asm-3 mutation partially suppressed the dauer arrest phenotype of akt-1(mg306) mutants at 27°C. No dauers at 25°C were observed for the akt-1(mg306) mutant animals with or without the presence of the asm-3(ok1744) allele. (E) asm-3 mutation had no effect on dauer arrest phenotype of daf-7(e1372) mutants at either 22.5°C or 25°C. The asm-3(ok1744) allele by itself did not induce dauer formation at either 22.5°C or 25°C. Error bars indicate standard deviation from triplicates. Details including total worm numbers used in the assay are listed in Table S1.

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background, each led to a marked up-regulation of the \textit{aso-3p::gfp} expression as compared to control vector (L4440) RNAi (Figure 5C). As negative controls, \textit{daf-16} RNAi and \textit{dam-18} RNAi each caused a dramatic reduction of the \textit{aso-3p::gfp} expression when assayed in parallel (Figure 5C). These data indicate that \textit{asm} genes regulate the transcriptional output of \textit{daf-16}.

Chemical Inhibitors of ASM Delay Aging in \textit{C. elegans}

Our genetic analyses suggest that ASM may be a potential target for anti-aging at the organismal level. It has been reported that mammalian ASM activity can be inhibited by the chemicals desipramine and clomipramine [50–52], which are clinically approved drugs for anti-depression. Desipramine and clomipramine have been used for studying the role of human ASM in CD95 death receptor signaling [53,54]. Indeed, we found that these drugs inhibited mammalian ASM activity in cultured cells (data not shown). We then tested whether desipramine and clomipramine had any effects on \textit{C. elegans} lifespan. Remarkably, we found that treatment with desipramine (30 μM) or clomipramine (5 μM) each extended the lifespan of wild-type animals, resulting in a mean lifespan 24% or 14% greater than that of control animals, respectively (Figure 6A, 6B; Table 3, Set #1, #2). Furthermore, the effects of these drugs on lifespan extension were dependent on \textit{daf-16} gene activity, as no lifespan extension was observed in \textit{daf-16}(mgDf47) null mutants (Figure 6C, 6D; Table 3, Set #3, #4). These results are consistent with the data shown in Figure 1. To verify that these drugs indeed target CeASMs, we performed CeASMs activity assays using lysates prepared from drug-treated or vehicle-treated animals. We found that treatment with desipramine or clomipramine each decreased CeASM activity by 78% or 77% as compared to vehicle control, respectively (Figure 6E). This result indicates that these chemicals effectively inhibit CeASMs. Our studies thus demonstrate that inhibition of CeASM activity by chemical compounds extends
Discussion

asm-3 Functions in the daf-2/IIS Pathway to Regulate Animal Lifespan and Dauer Formation

In this study, we have shown that asm-3 regulates both animal lifespan and dauer formation in *C. elegans*. Further, several lines of novel evidence suggest that asm-3 functions in the daf-2/IIS pathway. First, the ability of asm-3 deficiency to extend animal lifespan is suppressed by loss-of-function mutations in either daf-16 or daf-18, two well-known negative regulators of the daf-2 pathway (Figure 1C-1F). Second, the asm-3(ok1744) loss-of-function allele does not further extend the lifespan of daf-2(e1370) or pdk-1(sa709) loss-of-function mutants (Figure 2A, 2D), placing asm-3 in the daf-2 and pdk-1 pathway. It is possible that the daf-2(e1370) or pdk-1(sa709) mutation has already sufficiently reduced the gene activity of daf-2 or pdk-1 to below the threshold levels, and thus the presence of the asm-3(ok1744) allele in these mutants cannot further dampen daf-2 signaling output. Third, asm-3 deficiency strongly enhances the lifespan extension phenotype of the age-1(mg305) or aop-1(m889) mutants (Figure 2B, 2C). As age-1(mg305) and aop-1(m889) mutations are partial loss-of-function alleles, it is possible that the daf-2 signaling strength can be further reduced in these strains by the asm-3(ok1744) mutation, resulting in an increase in lifespan. Fourth, inactivation of asm-3, either alone or in combination with inactivation of asm-1 or asm-2 paralog, induces the nuclear translocation of DAF-16::GFP fusion protein, in a result similar to that achieved by inactivation of daf-2, age-1 or akt-1 (Figure 4A, 4B; Figure S5, S6). Overall, these observations suggest that asm-3 normally functions as a positive regulator of daf-2 signaling.

The daf-2/IIS pathway regulates animal development, especially dauer formation, in addition to regulating lifespan. A further line of evidence that asm-3 functions in the daf-2/IIS pathway comes from the observation that the asm-3(ok1744) mutation strongly cooperates with the daf-2(e1370) or age-1(mg305) mutation in inducing dauer formation (Figure 3A, 3B). In addition, loss of asm-3 did not affect dauer formation of daf-7(e1372) mutants.
which are defective in TGF-β-like signaling (Figure 3E). These observations are similar to those reported for the *aap-1* in-
activation, which can enhance the dauer phenotype of *daf-2* or *age-1* mutants, but not of *daf-1*/type I TGF-β receptor-like mutants [28]. These data further support the hypothesis that *asm-3* gene activity normally potentiates *daf-2* and *age-1* signaling.

Of particular interest are the observations that the effects of *asm-3* inactivation in *age-1* or *aap-1* mutant backgrounds are very strong. The *asm-3*(ok1744) allele can further increase the mean lifespan of the *age-1*(mg305) mutants by 67%, and *asm-3*(ok1744);*-age-1*(mg305) double mutants have a mean lifespan 259% greater than that of wild-type animals (Figure 2B). Inactivation of *asm-3* by RNAi also potently extends the lifespan of *aap-1*(m889) mutants (Figure 2C). Consistently, *asm-3*(ok1744) enhanced the dauer formation efficiency of *age-1*(mg305) mutants from 3% to 99% at an intermediate temperature of 22.5°C (Figure 3B). Genetically, these studies suggest that *asm-3* acts in parallel to *age-1* or *aap-1*, in the *daf-2*/IIS pathway (see model in Figure 7A). Since both *age-1*(mg305) and *aap-1*(m889) are partial loss-of-function alleles, it is also possible that *asm-3* normally functions together with *age-1* or *aap-1*, through potentiating *age-1* and *aap-1* gene activities (see model in Figure 7B).
Based on our genetic data on both lifespan and dauer regulation, we propose a model in which *asm-3*, either functioning in parallel or acting together with *age-1/aap-1* (Figure 7A) or working together with *age-1/aap-1* (Figure 7B), leading to activation of *pdk-1* and then suppression of *daf-16* to regulate animal lifespan and dauer arrest.

### Table 3. Effects of drug treatment on animal lifespan.

| Genotype             | Drug Treatment | Mean Lifespan ± SEM (Days) | Relative Mean Lifespan (%) | P value |
|----------------------|----------------|-----------------------------|-----------------------------|---------|
| Set #1               |                |                             |                             |         |
| N2 (wild-type)       | 0 μM (control) | 13.1±0.40                   | 100                         | –       |
|                      | 30 μM (desipramine) | 16.3±0.51                   | 124                         | <0.0001 |
| Set #2               |                |                             |                             |         |
| N2 (wild-type)       | 0 μM (control) | 14.0±0.41                   | 100                         | –       |
|                      | 5 μM (clomipramine) | 15.9±0.40                   | 114                         | 0.0012  |
| Set #3               |                |                             |                             |         |
| daf-16(mgDf47)       | 0 μM (control) | 14.0±0.45                   | 100                         | –       |
|                      | 30 μM (desipramine) | 14.3±0.39                   | 102                         | 0.713   |
| Set #4               |                |                             |                             |         |
| daf-16(mgDf47)       | 0 μM (control) | 14.1±0.50                   | 100                         | –       |
|                      | 5 μM (clomipramine) | 14.3±0.45                   | 101                         | 0.8707  |

N2 (wild-type) or daf-16(mgDf47) mutant animals were assayed on plates either containing desipramine (30 μM), clomipramine (5 μM), or no drug control (0 μM). All the lifespan assays were carried out at 20 °C. Mean lifespan, relative mean lifespan and statistical analyses (P values) for each assay were listed. Standard error of the mean, SEM, is included in parenthesis. Each set of the lifespan experiments was repeated at least three independent times and similar results were obtained. Data from representative sets of experiments are shown. Greater than 50 worms were counted for each condition in each experiment.

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**Figure 7. A model of *asm-3* function.** *asm-3* functions downstream of *daf-2*, acting either in parallel to *age-1/aap-1* (A) or working together with *age-1/aap-1* (B), leading to activation of *pdk-1* and *akt-1*, and then suppression of *daf-16*.

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process also involves ASM-3. Up-regulation of DAF-16 protein levels has been previously observed in 

tel-1 mutants and eak-7 mutants, both known to have a longer animal lifespan [12,40]. Future studies are needed to determine how DAF-2 and ASM-3 regulate DAF-16 protein levels.

A Potential Mechanism of ASM-3 in the Regulation of Trans-membrane Receptor Signaling

The predicted C. elegans ASM-3 peptide shares a strong homology with the human ASM in the presumed catalytic domain of the ASM enzyme (Figure 1S). In cultured human cells, ASM-dependent ceramide production in lipid rafts has been shown to be essential for CD95 death receptor mediated signaling to apoptosis [18,19,53]. However, the endogenous CD95 receptors have recently been found to have a pro-survival and pro-proliferation function in the mouse knockout system [20,21]. It appears that the biological consequence of CD95 receptor signaling is dependent on cell context and stimulus used. It remains to be investigated whether the pro-proliferation and pro-survival signaling by the CD95 receptors requires the participation of ASM.

Our studies have provided strong genetic evidence that ASM-3 is a positive regulator of signaling through the DAF-2 receptors. This is the first report that ASM functions in vivo as a positive regulator for a receptor tyrosine kinase. By analogy with human ASM in the formation of ceramide-enriched lipid rafts, it is possible that similar lipid rafts may be involved in facilitating the DAF-2 receptor signaling in C. elegans. In light of the fact that the strongest phenotypes of asm-3 inactivation are observed in age-1 mutant backgrounds, it can be further speculated that transducing the signals from DAF-2 receptor to the AGE-1 PI 3-kinase homolog may involve ASM-3-dependent lipid rafts. In support of this hypothesis, we have observed that, in mammalian systems, human ASM regulates the lipid raft localization of the IGF-1 receptors [P. Ghosh, Y. Kim, X. Xiong and H. Sun, unpublished observations]. Taken together, our studies indicate that ASM-3 proteins, along with their products ceramides, play a positive role in facilitating DAF-2 receptor signaling.

asm-3 Serves as a Molecular Target for Anti-aging

Our genetic studies have shown that asm-3 regulates animal lifespan through modulation of the daf-2/ins pathway. These results suggest the potential of using ASM as a molecular target for anti-aging at organismal level. Indeed, we have shown that two chemical compounds, desipramine and clomipramine, which are known to inhibit mammalian ASM activity [50–52], can effectively extend wild-type animal lifespan by 24% or 14%, respectively (Figure 6A, 6B). Such lifespan extension phenotypes require the presence of daf-16, as no lifespan extension was observed in the daf-16(mg47) null mutant background (Figure 6C, 6D). The effects of these compounds on lifespan are very similar to the lifespan extension resulting from genetic inactivation of the asm gene family (Figure 1). Consistently, our biochemical studies have demonstrated that these compounds indeed greatly decrease the CeASM activities (Figure 6E). Thus, these data suggest that these two drugs mediate their effects through inhibition of CeASMs. Together, these results further underscore the importance of ASM in the regulation of animal lifespan and its role as a potential target for anti-aging. Moreover, since mammalian ASM is known to localize to the outer leaflet of the plasma membrane [16], ASM presents itself as an even more attractive target for anti-aging drug development.

Materials and Methods

C. elegans Strains

We used N2 Bristol as the wild-type strain. In addition, strains carrying the following mutant alleles were used: asm-3(ok1744), daf-2(e1370), age-1(mg305), akt-1(mg306), daf-16(mg26), daf-16(mg47), daf-18(m2037), rrf-3(pk1426), asm-3(ok1744);daf-2(e1370), asm-3(ok1744);age-1(mg305), asm-3(ok1744);akt-1(mg306), asm-3(ok1744);daf-16(mg47), asm-3(ok1744);daf-17(m272), asm-3(ok1744);rfy-3(pk1426), daf-18(m2037);rfy-3(pk1426), rfy-3(pk1426);jz356;daf-16;gfp rol-6(su1006), asm-3(ok1744);rfy-3(pk1426);jz356;daf-16;gfp rol-6(su1006), rfy-3(pk1426);mu84[pAD76(sod-3::gfp)], asm-3(ok1744);rfy-3(pk1426);mu84 [pAD76(sod-3::gfp)]. The asm-3(ok1744) strain was obtained from C. elegans Genetic Center (CGC), and backcrossed to the wild-type N2 strain for 3 times. The daf-18(m2037) strain was described previously [15]. All other strains were also obtained from CGC except age-1(mg305), akt-1(mg306) and pdk-1(sa709) which are kindly provided by Dr. Patrick Hu (University of Michigan, Ann Arbor). All the double mutant strains were constructed by standard genetic techniques and the presence of homozygous mutant alleles were confirmed by PCR genotyping, restriction fragment length polymorphisms or strong dauer phenotype at 25°C or 27°C. All strains used in this study were maintained at 15°C using standard techniques for the control of C. elegans [55]. E. coli OP50 was used as the food source on NGM plates.

Construction of asm-1 and asm-2 RNAi Clones

The constructs were based on PCR strategy from Worm Base. asm-1 and asm-2 amplicons, sjj_B0252.2(1141bp) and sjj_ZK455.4(1107bp), respectively, were amplified from genomic DNA of N2 wild-type worms by PCR and subcloned to TOPO TA cloning vector (pCR2.1-TOPO, Invitrogen). The following primers were used for asm-1 RNAi construct: Forward: 5'-ttctgggcaagagatcgttg-3'; Reverse: 5'-cctctgtttctttgctgcttg-3'. The primers used for asm-2 RNAi construct are as follows: Forward: 5'-tggacaatgacagacc-3'; Reverse: 5'-ctggactaatctttcctgcgg-3'. Unique restriction sites from the subcloned vector were used for cloning into the L4440 vector (a kind gift from Dr. A. Fire). The derived RNAi constructs were confirmed by restriction mapping and DNA sequencing. The constructs were transformed into E. coli HT115 (DE3) strain [56]. The asm-3, daf-16 and daf-18 RNAi constructs were recovered from the RNAi feeding library [57,58] and confirmed by DNA sequencing. The daf-2 RNAi construct has been described previously [25].

Lifespan Assay on NGM Agar Plates

Lifespan assays were carried out on NGM plates at 20°C, and plates were seeded with E. coli OP50. In order to synchronize worms, eggs were first isolated from gravid adults and then hatched overnight in the S1 basal media. The synchronized L1 larva were then placed on OP50-seeded NGM agar plates and allowed to grow to adult. During the egg-laying period, adult worms were daily transferred to new OP50-seeded agar plates. Worms were then examined every other day for survival and scored. Where indicated, FuDR was also included in the plates. FuDR, 5-Fluoro-2'-deoxyuridine, is used to inhibit DNA synthesis and thus to prevent the production of progeny. Animals hatched from egg preparation were grown on regular OP50-seeded NGM agar plates until L4 stage and then transferred to plates containing 50 μg/ml FuDR. Animal survival was scored every other day and transferred to new plates containing 50 μg/ml FuDR every 2–3
days. Animals were considered dead when failed to respond to gentle touches by a platinum wire. Adult lifespan were counted using the L4 stage as Day 0. For each set of experiments, animals from different strains or conditions were assayed in parallel. Each set of experiments was carried out at least 2 times. In all the lifespan assays performed on regular NGM plates, RNAi-inducing plates or drug-containing plates, at least 50 worms were counted per strain per experiment; while the animals that were bagged, exploded or crawled off the plate were censored from the counts.

**Lifespan Assay on RNAi-inducing Plates**

RNAi bacteria culture was grown in Luria broth media with 100 μg/ml ampicillin at 37°C for 16–18 hours and then seeded onto NGM plates containing 5 mM IPTG and 50 μg/ml carbanicillin. In case of double knockdown, RNAi bacteria cultures of same density were mixed at 1:1 ratio and were then seeded on RNAi-inducing plates. The plates were kept at room temperature overnight to induce the expression of the RNAi molecules. Lifespan assay was previously described [25]. Briefly, the synchronized L1 larval animals prepared by egg preparation were plated on the RNAi-inducing plates and allowed to grow to adults. The adult animals were transferred to new plates every day until adult animals stop laying eggs and after that the animals were scored every other day and transferred to new plates every 2–3 days. All the RNAi experiments were carried out in the *rf-3(pk1426)* background, a strain known to be hypersensitive to RNAi, with the exception of RNAi experiment in the *aop-1(m409)* background carrying the *rf-3(+)* allele. Lifespan assays were carried out at 20°C. Each set of experiments were conducted at least 2 times.

**Lifespan Assay on Drug-containing NGM Plates**

To prepare NGM plates containing desipramine or clomipramine, drugs were first dissolved in water as 100 mM stock solutions and sterilized by filtration. Drugs were then properly diluted to the indicated final concentration during the solidification step of the NGM agar plate preparation. The synchronized L1 larvae prepared by egg preparation were placed on NGM agar plates and allowed to develop to the L4 larva. Synchronized L4 animals were then transferred to regular NGM (control) plates or plates containing drug desipramine (30 μM) or clomipramine (5 μM). Animals were then transferred to drug-containing plates or regular NGM plates every day until animals stop laying eggs. The populations were scored every other day and transferred to new drug-containing plates or regular NGM plates every 2–3 days. Adult lifespan was counted using the L4 stage as Day 0. Lifespan assays were carried out at 20°C. Drug inhibition experiments have been carried out >3 times and consistent results have been obtained from independent experiments.

**Statistical Analysis**

Statistical analyses of all the survival curves were performed as described [11] using the software GraphPad 5.0. Survival curve of each population was compared with control using Log-rank (Mantel-Cox) test and an experimental data set with a P value <0.05 was considered to be significantly different from the control data set.

**Oxidative Stress Response Assay using Paraquat**

The synchronized L1 animals after standard egg preparation were placed on NGM agar plates seeded with *E. coli* OP50 and allowed to develop to the L4 stage larva at 20°C. A 1-mL 80 mM paraquat solution was then treated on the 6-cm plates at the L4 stage and after soaking for about 20 minutes, the plates were air-dried in a chemical hood till plates were dried. The dried plates were shifted to 20°C incubator and survivor of the animals was scored every day. The experiment was repeated at least two independent times. Total worms counted per plate were greater than 100.

**Heat Stress Response Assay at 35°C**

The synchronized L1 animals after egg preparation were put on NGM agar plates seeded with *E. coli* OP50 and allowed to grow to Day 1 stage worms (young adult worms) at 20°C. At Day 1, the plates were shifted to 35°C and then scored after 6, 9, 12 or 15 hours afterwards. The plates used for counting in each time point were discarded to avoid the complication of recovery at room temperature on scoring plates. Experiments were conducted for two times, each in triplicates, and the standard deviations were calculated. The number of worms counted per strain per plate was greater than 200.

**Dauer Assay**

All the strains used for dauer assay were synchronized by egg preparation and the synchronized L1 larval animals were placed on NGM agar plates seeded with *E. coli* OP50. The plates were incubated at 22.5°C, 25°C or 27°C. Numbers of dauer and nondauer in the population were visually scored after 72, 48 and 45 hours later for the assays carried out at 22.5°C, 25°C or 27°C, respectively. All assays were performed in triplicates and experiments were conducted at least 2 times.

**DAF-16::GFP Localization and sod-3p::GFP Expression Assay**

We crossed the *rf-3(pk1426)* or the *asm-3(ok1744);rf-3(pk1426)* allele into wild-type strain carrying the daf-16::gfp or sod-3p::gfp transgene and derived the following GFP-expressing transgenic strains: 1) *rf-3(pk1426)*; *zIs356[daf-16::daf-16:16::gfp, rol-6(sa1006)]; 2) *rf-3(pk1426)*; *asm-3(ok1744)*; *zIs356[daf-16::daf-16:16::gfp, rol-6(sa1006)]; 3) *rf-3(pk1426);* *mbl84[pAD76(sod-3::gfp)]; 4) *asm-3(ok1744);rf-3(pk1426);muIs04 [pAD76(sod-3::gfp)]. The GFP-expressing transgenic strains were synchronized at L1 larval stage in the S1 basal media after standard egg preparation and placed onto RNAi-inducing bacteria plates at 20°C. All the worms were transferred to new RNAi-inducing bacteria plates every day thereafter. For fluorescence microscopy, worms were transferred to 2% agarose pads and examined under Olympus CKX41 fluorescence microscope with 4x objective. Worms were photographed with the attached digital camera QICAM FAST1394. For image comparison of individual conditions in each set of experiments, identical exposure times were used for image capture. Animals were examined on adult Day 1, Day 3 or Day 4 as described in the Figure legends, with the L4 stage counted as adult Day 0.

**RNA Isolation and qRT-PCR**

Total RNA was isolated from synchronized adult Day 1 worms using Trizol reagent (Invitrogen). RNA purity was checked by UV absorbance (260/280 ratio). cDNA was synthesized with primer Oligo(dT)20 by using SuperScript III First-Strand Kit (Invitrogen) according to the manufacturer’s protocol. RT-PCR reactions were performed with a 20 μl of Power SYBR PCR Master Mix (Applied Biosystems) using triplicates for each sample. PCR reaction was carried out on Real Time PCR Machine 7500 Fast (Applied Biosystems) under the condition of 95°C for 5 minutes for denaturation, followed by 40 cycles of 95°C for 15 seconds and
60°C for 1 hour. The reaction products were analyzed using software provided by the onboard software from the Real Time PCR machine. The primers used for qRT-PCR are as follows: Primer for act-1 (133 bp in product size) [34,39]; Forward primer: 5'-ccaggaattgtagcctagcgaaga-3'; Reverse primer: 5'-tgaggagggggtagctagacctagaa-3'; Reverse primer: 5'-ccaggaattgtagcctagcgaaga-3'; Primer for mtl-1 (98 bp in product size) [34,39]; Forward primer: 5'-ctgaggagggggtagctagacctagaa-3'; Reverse primer: 5'-ccaggaattgtagcctagcgaaga-3'; Primer for ml-1 (111 bp in product size); Forward primer: 5'-atggcttgcaagtgtgactg-3'; Reverse primer: 5'-ttctcaggtccttacgc-3'. act-1 was used as an internal control for normalization of input RNA levels. All experiments have been repeated >2 times and consistent results have been obtained from independent experiments.

Preparation of Worm lysate and Western Blot Analysis

Synchronized young adult worms (at adult Day 1) were rinsed three times with M9 buffer to remove bacteria and then washed one time with ddH2O to remove salt. 0.4 volume of 5X SDS-PAGE sample buffer (312.5 mM Tris-HCl, pH 6.8, 50% Glycerol and 10% SDS) was added to each tube containing the packed worms. Tubes were subjected to 3 cycles of freezing/heating (CO2/ethanol bath and boiling at 95°C for 10 minutes). Samples were then sonicated using water bath sonicator (VWR B1500AMT) for 3 cycles of 2 minutes per cycle. Lysates were clarified by centrifugation at 12000 rpm for 1 minute and supernatants were transferred to new tubes, and protein concentrations were quantified using BCA protein assay reagent (Thermo Scientific). For experiments involving RNAi-treated samples, adult Day 2 worms from plates seeded with RNAi-inducing bacteria were used. Equal amounts of protein lysates were analyzed by SDS-PAGE analysis, and the proteins were transferred to nitrocellulose membrane. Membranes were blocked with 1X TBST (150 mM NaCl, 20 mM Tris-base, pH 7.4 and 0.05% Tween-20) containing 5% non-fat dry milk for 1 hour at room temperature. Primary antibody was applied and incubated at 4°C overnight. Filters were extensively washed, and then incubated with a properly washed secondary antibody for 1 hour at room temperature. Following washes, filters were developed with Western lightning Plus-ECL (Perkin Elmer) and exposed to X-ray films. The intensity of DAF-16 was normalized against beta-actin, and calculated from the western blots using the ImageJ software.

Quantification of DAF-16 Protein Levels

The relative abundance of DAF-16 protein levels were calculated from the western blots using the ImageJ software. The intensity of DAF-16 was normalized against beta-actin, and the numbers were presented as a percentage relative to the N2 or the vector control. The controls were set as 100%.

Supporting Information

Figure S1 Multiple alignment of C. elegans ASM-3, H. sapiens ASM and D. melanogaster ASM. The C-terminal catalytic domain of CeASM-3 was aligned with that of HsASM and DmASM. Sequences used in the alignment correspond to amino acids 117 to 464 of CeASM-3, 186 to 523 of HsASM and 254 to 596 of DmASM. Identical residues are shaded black and similar residues are shaded gray. CeASM-3 shares 42% identity with HsASM and 39% identity with DmASM. Alignment was performed using Clustal W2 and BioEdit v7.0.5. Consensus symbol “*” is used for conserved substitutions or semi-conserved substitutions, respectively.

Figure S2 Genomic structures of asm-3 and ok1744 allele. The genomic arrangement of asm-3 is illustrated according to the Worm database (www.wormbase.org). The ok1744 allele contains a deletion of 1558 bp which removes exon VII through exon XIII of asm-3, followed by a 7 bp insertion. As a consequence, such changes shall lead to production of a peptide that is truncated at the amino acid 272 (Pro272).

Figure S3 Multiple alignment of C. elegans ASM-3, ASM-1 and ASM-2. The predicted C-terminal catalytic domain of CeASM-3 was aligned with that of CeASM-1 and CeASM-2. Sequences used in the alignment correspond to amino acids 117 to 464 of CeASM-3, 138 to 478 of CeASM-1 and 167 to 526 of CeASM-2. Identical or similar residues are shaded black or gray,
treated animals, no nuclear translocation of DAF-16::GFP was observed.

(TIF)

Figure S6 Synergistic effect of DAF-16::GFP nuclear localization. DAF-16::GFP cellular distributions were examined by fluorescence microscopy. asm-3(ok1744);rrf-3(pk1426);daf-16::gfp mutants were examined on adult day 4. In the vector alone treated animals, DAF-16::GFP proteins were partially nuclear-localized due to the presence of asm-3(ok1744) allele. Further inactivation of asm-1, asm-2, or asm-3 together with asm-2 (asm-1/ asm-2) by RNAi induced more pronounced nuclear localization of DAF-16::GFP. RNAi inactivation of daf-16, a negative control, markedly reduced DAF-16::GFP expression. The daf-16 RNAi prevented the nuclear localization of DAF-16::GFP proteins caused by loss of asm-3.

(TIF)

Table S1 Effects of asm-3 on dauer formation. Dauer formation experiments were carried out for the wild-type animals and various mutants at the indicated temperatures. Dauer formation assays were conducted in triplicates and experiments were repeated at least two times. Data from representative experiments are shown. The average percentages of nondauer and dauer, as well total numbers of animals used for each assay, were listed.

(PDF)

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Author Contributions

Conceived and designed the experiments: YK HS. Performed the experiments: YK. Analyzed the data: YK HS. Contributed reagents/materials/analysis tools: YK HS. Wrote the paper: YK HS.

References

1. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A C. elegans mutant that lives twice as long as wild type. Nature 366: 665–667.
2. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G (1997) daf-2, an insulin-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science 277: 942–946.
3. Russell SJ, Kahn CR (2007) Endocrine regulation of ageing. Nat Rev Mol Cell Biol 8: 681–691.
4. Kenyon CJ (2010) The genetics of aging. Nature 464: 504–512.
5. Tatar M, Bartke A, Antebi A (2003) The endocrine regulation of aging by insulin-like signals. Science 299: 1345–1351.
6. Landis JN, Murphy CT (2010) Integration of diverse inputs in the regulation of Caenorhabditis elegans DAF-16/FOXO. Dev Dyn 239: 1405–1412.
7. Ogg S, Partridge L, Goffeau A, Partridge J, et al. (1997) The fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature 389: 994–999.
8. Lin K, Dworkin JB, Rodan A, Kenyon C (1997) daf-16::GFP, a novel marker that functions to double the life-span of C. elegans. Science 278: 1319–1322.
9. Henderson ST, Johnson TE (2001) daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr. Biol. 11: 1975–1980.
10. Finch CE, Ruvkun G (2001) The genetics of aging. Annu Rev Genomics Hum Genet 2: 433–462.
11. Libina N, Berman JR, Kenyon C (2003) Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. Cell 115: 498–502.
12. Li W, Gao B, Lee SM, Bennett K, Fang D (2007) RLE-1, an E3 ubiquitin ligase, regulates C. elegans aging by catalyzing DAF-16 polyubiquitination. Dev Cell 12: 235–246.
13. Kwon ES, Narasimhan SD, Yen K, Tissenbaum HA (2010) A new DAF-16 isoform regulates longevity. Nature 466: 498–502.
14. Ogg S, Ruvkun G (1998) The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. Mol Cell 2: 387–393.
15. Mihaylova VT, Berland CZ, Manjarrez L, Stern MJ, Sun H (1999) The PTEN tumor suppressor homolog in Caenorhabditis elegans regulates longevity and dauer formation in an insulin receptor-like signaling pathway. Proc Natl Acad Sci 96: 7472–7473.
16. Smith EL, Schuchman EH (2008) The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. Faseb J 22: 3419–3431.
17. Simons K, Tooneur D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1: 31–41.
18. Grassme H, Jäde A, Richle A, Schwarz H, Berger J, et al. (2001) CD95 Signaling via Ceramide-rich Membrane Rafts. J Biol Chem 276: 20589–20596.
19. Cremesti A, Paris F, Grassme H, Heller N, Tschopp J, et al. (2001) Ceramide enables Fas to cap and kill. J Biol Chem 276: 23954–23961.
20. Chen L, Park SM, Tumanov AV, Has A, Sawada K, et al. (2010) CD95 promotes tumour growth. Nature 463: 492–496.
21. Ikizani K, Ikdowski-Baldis J, Hannun YA (2008) The sphingolipid salvage pathway in ceramide metabolism and signaling. Cell Signal 20: 1010–1018.
22. Zeitlin YH, Hannun YA (2007) Translational aspects of sphingolipid metabolism. Trends Mol Med 13: 327–336.
23. Deng X, Yin X, Allan R, Lu DD, Maurer CW, et al. (2008) Ceramide biogenesis is required for radiation-induced apoptosis in the germ line of C. elegans. Science 322: 110–115.
24. Lin X, Hengartner MO, Kolesnick R (1998) Caenorhabditis elegans contains two distinct acid sphingomyelinases. J Biol Chem 273: 14574–14579.
25. Kim Y, Sun H (2007) Functional genomic approach to identify novel genes involved in the regulation of oxidative stress resistance and animal lifespan. Aging Cell 6: 489–503.
26. Morris JZ, Tissenbaum HA, Ruvkun G (1996) A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. Nature 382: 536–539.
27. Iser WB, Gami MS, Wolkow CA (2007) Insulin signaling in Caenorhabditis elegans is used for conserved substitutions or semi-conserved substitutions, respectively.
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Wolkow CA, Munoz MJ, Riddle DL, Ruvkun G (2002) Insulin receptor substrate and p35 orthodox adaptor proteins function in the Caenorhabditis elegans daf-2/Insulin-like signaling pathway. J Biol Chem 277: 49591–49597.

Paradis S, Alion M, Toker A, Thomas JM, Ruvkun G (1999) A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. Genes Dev 13: 1438–1452.

Hu P, Xu J, Ruvkun G (2006) Two membrane-associated tyrosine phosphatase homologs potentiate C. elegans AKT-1/PKB signaling. PLoS Genet 2: 930–943.

Honda Y, Honda S (1999) The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. Fauch J 13: 1385–1393.

Yanae S, Yasuda K, Ishii N (2002) Adaptive responses to oxidative damage in three mutants of Caenorhabditis elegans (age-1, me-1 and daf-16) that affect lifespan. Mech Ageing Dev 123: 1579–1587.

Ayyadevara S, Alla R, Thaden JJ, Shmookler Reis RJ (2008) Remarkable longevity and stress resistance of nematode PDIk-null mutants. Aging Cell 7: 13–22.

Li J, Ebata A, Dong Y, Rizki G, Iwata T, et al. (2008) Caenorhabditis elegans HCF-1 functions in longevity maintenance as a DAF-16 regulator. PLoS Biol 6: 1870–1886.

Masse I, Molin L, Mouchiroud L, Vanheems P, Palladino F, et al. (2008) A novel role for the SMG-1 kinase in lifespan and oxidative stress resistance in Caenorhabditis elegans. PLoS ONE 3: e3354.

Lithgow GJ, White TM, Melov S, Johnson TE. (1995) Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc Natl Acad Sci 92: 7540–7544.

Houthoofd K, Fidalgo MA, Hoogewijs D, Braeckman BP, Lenaerts I, et al. (2005) Metabolism, physiology and stress defense in three aging Ins/IGF-1 stress. Proc Natl Acad Sci 92: 7540–7544.

Masse I, Molin L, Mouchiroud L, Vanheems P, Palladino F, et al. (2008) A novel role for the SMG-1 kinase in lifespan and oxidative stress resistance in Caenorhabditis elegans. PLoS ONE 3: e3354.

Ayyadevara S, Alla R, Thaden JJ, Shmookler Reis RJ (2008) Remarkable longevity and stress resistance of nematode PDIk-null mutants. Aging Cell 7: 13–22.

Li J, Ebata A, Dong Y, Rizki G, Iwata T, et al. (2008) Caenorhabditis elegans HCF-1 functions in longevity maintenance as a DAF-16 regulator. PLoS Biol 6: 1870–1886.

Masse I, Molin L, Mouchiroud L, Vanheems P, Palladino F, et al. (2008) A novel role for the SMG-1 kinase in lifespan and oxidative stress resistance in Caenorhabditis elegans. PLoS ONE 3: e3354.

Lithgow GJ, White TM, Melov S, Johnson TE. (1995) Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc Natl Acad Sci 92: 7540–7544.

Houthoofd K, Fidalgo MA, Hoogewijs D, Braeckman BP, Lenaerts I, et al. (2005) Metabolism, physiology and stress defense in three aging Ins/IGF-1 stress. Proc Natl Acad Sci 92: 7540–7544.

Li J, Ebata A, Dong Y, Rizki G, Iwata T, et al. (2008) Caenorhabditis elegans HCF-1 functions in longevity maintenance as a DAF-16 regulator. PLoS Biol 6: 1870–1886.

Masse I, Molin L, Mouchiroud L, Vanheems P, Palladino F, et al. (2008) A novel role for the SMG-1 kinase in lifespan and oxidative stress resistance in Caenorhabditis elegans. PLoS ONE 3: e3354.