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Heat shock factor 1 mediates the longevity conferred by inhibition of TOR and insulin/IGF-1 signaling pathways in *C. elegans*

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Summary

Target of rapamycin (TOR) signaling is an evolutionarily well-conserved pathway that regulates various physiologic processes, including aging and metabolism. One of the key downstream components of TOR signaling is ribosomal protein S6 kinase (S6K) whose inhibition extends the lifespan of yeast, *Caenorhabditis elegans*, *Drosophila*, and mice. Here, we demonstrate that the activation of heat shock factor 1 (HSF-1), a crucial longevity transcription factor known to act downstream of the insulin/IGF-1 signaling (IIS) pathway, mediates the prolonged lifespan conferred by mutations in *C. elegans S6K* (*rsks-1*). We found that *hsf-1* is required for the longevity caused by down-regulation of components in TOR signaling pathways, including TOR and S6K. The induction of a small heat-shock protein *hsp-16*, a transcriptional target of HSF-1, mediates the long lifespan of *rsks-1* mutants. Moreover, we show that synergistic activation of HSF-1 is required for the further enhanced longevity caused by simultaneous down-regulation of TOR and IIS pathways. Our findings suggest that HSF-1 acts as an essential longevity factor that intersects both IIS and TOR signaling pathways.

Key words: aging; *Caenorhabditis elegans*; HSF-1; insulin/IGF-1 signaling; longevity; mRNA translation; S6 kinase; target of rapamycin.

Introduction

For the last two decades, numerous genes and pathways that influence aging have been discovered using model organisms including yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster*. Many of these genes and pathways have been shown to regulate lifespan in mammals as well (Fontana *et al.*, 2010; Kenyon, 2010). Among them, insulin/IGF-1 signaling (IIS) and target of rapamycin (TOR) pathways are two of the most evolutionarily well-conserved aging-regulatory pathways (Stanfel *et al.*, 2009; Fontana *et al.*, 2010; Kapahi *et al.*, 2010; Kenyon, 2010; Evans *et al.*, 2011). Insulin/IGF-1 signaling and TOR signaling pathways link metabolic status and aging and interact with each other, although the factor(s) mediating the cross talk between these two pathways is incompletely understood.

Target of rapamycin is a protein kinase that acts as a cellular nutrient sensor to regulate various physiologic processes, including metabolism, growth, and aging (Stanfel *et al.*, 2009; Kapahi *et al.*, 2010; Evans *et al.*, 2011; Laplante & Sabatini, 2012). The TOR kinase forms two distinct complexes, TORC1 and TORC2, which have complex-specific adaptors such as raptor and rictor, respectively (Dazert & Hall, 2011; Laplante & Sabatini, 2012). The activity of TORC1 is regulated by upstream signaling proteins such as Rag GTPases in response to changes in the cellular environmental conditions (Kim *et al.*, 2008). When the nutrient supply is sufficient, TORC1 positively regulates cellular growth and proliferation by promoting protein synthesis and/or inhibiting autophagy. To exert its effects on protein synthesis, TORC1 phosphorylates its target proteins, including ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Laplante & Sabatini, 2012). S6K mediates the regulation of mRNA translation via phosphorylation of its substrates, including ribosomal protein subunit 6 (Erikson & Maller, 1985; Laplante & Sabatini, 2012). Recent studies have shown that genetic inhibition of TOR, S6K, or ribosomal protein subunits extends lifespan in many organisms (Fabrizio *et al.*, 2001; Jia *et al.*, 2004; Kapahi *et al.*, 2004; Kaeberlein *et al.*, 2005; Chen *et al.*, 2007; Curran & Ruvkun, 2007; Hansen *et al.*, 2007; Pan *et al.*, 2007; Syntichaki *et al.*, 2007; Selman *et al.*, 2009) and that treatment with the TORC1 inhibitor rapamycin increases the lifespan of yeast, *C. elegans*, *Drosophila*, and mice (Powers *et al.*, 2006; Harrison *et al.*, 2009; Bjedov *et al.*, 2010; Robida-Stubbs *et al.*, 2012). These studies establish the TOR signaling pathway as an evolutionarily conserved aging-regulatory pathway and a promising target for the development of anti-aging medicines.

Reduced IIS confers lifespan extension in many species, potentially including humans (Fontana *et al.*, 2010; Kenyon, 2010). In *C. elegans*, inhibition of *daf-2*, which encodes a homolog of insulin/IGF-1 receptor, doubles lifespan (Kenyon *et al.*, 1993). This long lifespan requires the FOXO transcription factor DAF-16, which induces the expression of many pivotal target genes to promote longevity (Fontana *et al.*, 2010; Kenyon, 2010). Heat shock factor 1 (HSF-1) is another transcription factor crucial for longevity downstream of IIS (Hsu *et al.*, 2003; Morley & Morimoto, 2004). HSF-1 is activated upon reduction in IIS and is required for the long lifespan caused by inhibition of IIS, including *daf-2* mutations (Hsu *et al.*, 2003; Morley & Morimoto, 2004; Chiang *et al.*, 2012). In addition, over-expression of *hsf-1* prolongs the lifespan of *C. elegans*, suggesting that increase in HSF-1 activity is sufficient for longevity (Hsu *et al.*, 2003; Morley & Morimoto, 2004). It has been shown that HSF-1 not only promotes longevity but also enhances stress resistance, innate immunity, and protein homeostasis, and that these effects are achieved mainly through increasing the levels of molecular chaperones at the transcription level (Hsu *et al.*, 2003; Cohen *et al.*, 2006; Singh & Aballay, 2006; Mohri-Shiomi & Garsin, 2008; Steinkraus

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et al., 2008). These studies demonstrated that HSF-1 acts downstream of IIS to extend lifespan; however, whether other longevity signaling pathways, including the TOR pathway, also influence HSF-1 activity is poorly understood.

In this report, we show that HSF-1 mediates the lifespan extension caused by reduction of TOR signaling in *C. elegans*. We found that enhanced lifespan, oxidative stress resistance, and pathogen resistance resulting from inhibition of the TOR signaling pathway are highly dependent on HSF-1. In addition, we demonstrated that mutations in *rsks-1*, which encodes the *C. elegans* ribosomal protein S6 kinase, increase HSF-1 activity, and that *hsp-16*, a small heat-shock protein whose induction is regulated by HSF-1, is required for the longevity of *rsks-1* mutants. Furthermore, we showed that HSF-1 is synergistically activated by the inhibition of both TOR signaling and IIS pathways and is required for the further enhanced longevity of *daf-2 rsks-1* double mutants. Together, our findings establish HSF-1 as a hub protein for the cross talk between TOR signaling and IIS pathways to regulate lifespan.

Results

While performing a genome-wide RNA interference (RNAi) screen for identification of genes that interact with *rsks-1*, we found that RNAi targeting *hsf-1* was one of the 12 RNAi clones that specifically reduced the body size of *rsks-1* mutants (Fig. S1A). We confirmed this result using *hsf-1(sy441)* and *rsks-1(tm1714)* mutations (Fig. S1B). We were intrigued by this specific genetic interaction and further investigated the relationship between *rsks-1* and *hsf-1*. Because *C. elegans hsf-1* has been established as a crucial life-extending gene (Hsu *et al.*, 2003; Morley & Morimoto, 2004) and *rsks-1* mutations prolong lifespan (Hansen *et al.*, 2007; Pan *et al.*, 2007), we examined the role of *hsf-1* in the longevity of *rsks-1* mutants. We found that *hsf-1* RNAi or mutations completely suppressed the long lifespan of the *rsks-1* mutants (Fig. 1A–C). The effect of *hsf-1* inhibition on the longevity of *rsks-1* mutants was comparable to that of mutations in *daf-16*/FOXO transcription factor or *aak-2*/AMP-activated protein kinase (Fig. 1D,E and Fig. S2; see also Fig. S2 legend for discussion) (Selman *et al.*, 2009). Knockdown of *hsf-1* using RNAi only during adulthood completely suppressed the long lifespan of *rsks-1* mutants as well (Fig. 1F and Fig. S1C). This result excludes the possibility that depletion of HSF-1 during larval development negatively affected the health of the *rsks-1* mutant animals and subsequently reduced the lifespan during adulthood. Together, these data indicate that down-regulation of S6 kinase requires HSF-1 to lengthen lifespan in *C. elegans*.

Because S6K is a key downstream kinase of TORC1 (Laplante & Sabatini, 2012), we asked whether *hsf-1* plays a role in the longevity caused by down-regulation of other components in the TORC1 signaling pathway. We found that *hsf-1* was required for the lifespan extension caused by RNAi knockdown of *daf-15*/raptor or *ragc-1*/Rag GTPase, or by rapamycin treatment (Fig. 2A–C). Down-regulation of TORC1 extends lifespan at least partly through decreasing mRNA translation, which is regulated by phosphorylation of ribosomal protein subunits such as RPS6 by S6K. We therefore examined the effects of *hsf-1* mutations on the longevity caused by reduction in ribosomal protein subunit levels. We found that increased lifespan conferred by knockdown of *rps-6* or *rps-15* was suppressed by *hsf-1* mutations (Fig. 2D,E). These results further support our notion that *hsf-1* is crucial for lifespan extension by inhibition of TORC1 signaling.

Longevity has been shown to correlate well with increased resistance against external stresses and protein aggregation. We therefore determined the role of *rsks-1* and *hsf-1* in the survival rate under various stresses. Among the external stresses, *rsks-1* mutations increased the resistance against oxidative stress but not against heat or osmotic stresses (Fig. 3A and Fig. S3A–C; see also Fig. S3 legend for discussion). We found that *hsf-1* was required for the enhanced resistance of the *rsks-1* mutants to oxidative stress (Fig. 3A). The requirement of *hsf-1* for this increased oxidative stress resistance was comparable to or higher than that of *daf-16*/FOXO whose role in oxidative stress resistance is well known (Fig. 3B). We then examined the role of *hsf-1* in enhanced protein homeostasis in *rsks-1* mutants using polyglutamine-yellow fluorescent protein (Q40::YFP) transgenic animals (Morley *et al.*, 2002), which are an established worm model of polyglutamine diseases. We found that *rsks-1* mutations reduced Q40::YFP (Q40) aggregates and that *hsf-1* was required for the reduction in this aggregate formation (Fig. 3C,D). These data suggest that *rsks-1* mutations enhance oxidative stress resistance and protein homeostasis through HSF-1.

As innate immunity is influenced by various aging-regulatory genes including *hsf-1*, we also tested whether *rsks-1* mutations affected the immunity against *P. aeruginosa* (PA14), a well-established model bacterial pathogen. We found that *rsks-1* mutants were more resistant to PA14 than wild-type and that *hsf-1* was absolutely required for this PA14 resistance (Fig. 4A). In contrast, RNAi targeting several other genes known to be crucial for pathogen resistance, such as *daf-16*/FOXO, *nsy-1*/MAP kinase kinase kinase, or *pmk-1*/MAP kinase (Irazoqui *et al.*, 2010), did not fully suppress the PA14 resistance of *rsks-1* mutants (Fig. 4B–D). Together, these data suggest that *hsf-1* is essential for *rsks-1* mutants to be resistant to oxidative stress, protein aggregation, and PA14. Given the strong correlation between longevity and stress resistance phenotypes in *C. elegans*, this enhanced resistance against various stresses may contribute to the long lifespan of *rsks-1* mutants.

The requirement of *hsf-1* for the longevity, and stress and pathogen resistance caused by *rsks-1* mutations led us to ask whether down-regulation of TOR signaling was sufficient to increase HSF-1 activity. By performing real-time quantitative reverse transcription–PCR (qRT-PCR) analysis, we determined the mRNA expression levels of five known HSF-1 target chaperone genes, *hsp-16.1/2/11* (*hsp-16.1*, *hsp-16.2,* and *hsp-16.11*), *hsp-70*, *hsp-12.6*, *aip-1,* and *sip-1* (Fig. 5A–E and Fig. S4). We found that *hsp-16.1/2/11* and *hsp-70* were significantly induced by *rsks-1* mutations in the presence and the absence of heat shock and that the induction was largely *hsf-1* dependent (Fig. 5A,B and Fig. S4A,B). In contrast, *hsp-12.6* and *aip-1* were induced by *rsks-1* mutations only upon heat-shock treatment (Fig. 5C,D and Fig. S4C,D). We also showed that GFP expression under the control of the *hsp-16.2* promoter (*hsp-16.2::GFP*) (Link *et al.*, 1999; Walker & Lithgow, 2003), a fluorescence reporter for HSF-1 activity, was increased by *rsks-1* mutations, and this up-regulation was suppressed by *hsf-1* RNAi knockdown (Fig. 5F,G). Moreover, we found that *hsp-16.1/2/11* and *hsp-70* were induced by RNAi knockdown of *daf-15* or *ragc-1*, and that *hsf-1* mutations suppressed the induction of these genes (Fig. 5H–K). These data indicate that at least a subset of HSF-1 targets is induced by inhibition of TOR signaling.

Next, we examined the functional significance of the induction of *hsp-16* and *hsp-70* for the longevity of *rsks-1* mutants. We found that RNAi targeting *hsp-16.1/2/11* significantly suppressed the longevity of *rsks-1* mutants but *hsp-70* RNAi did not (Fig. 5L,M). Thus, *rsks-1* mutants may live long, at least in part, through up-regulation of *hsp-16* resulting from the activation of HSF-1.

HSF-1 is a well-known longevity transcription factor downstream of the IIS pathway (Hsu *et al.*, 2003; Morley & Morimoto, 2004; Chiang *et al.*, 2012). We therefore explored the possibility that HSF-1 acts as a

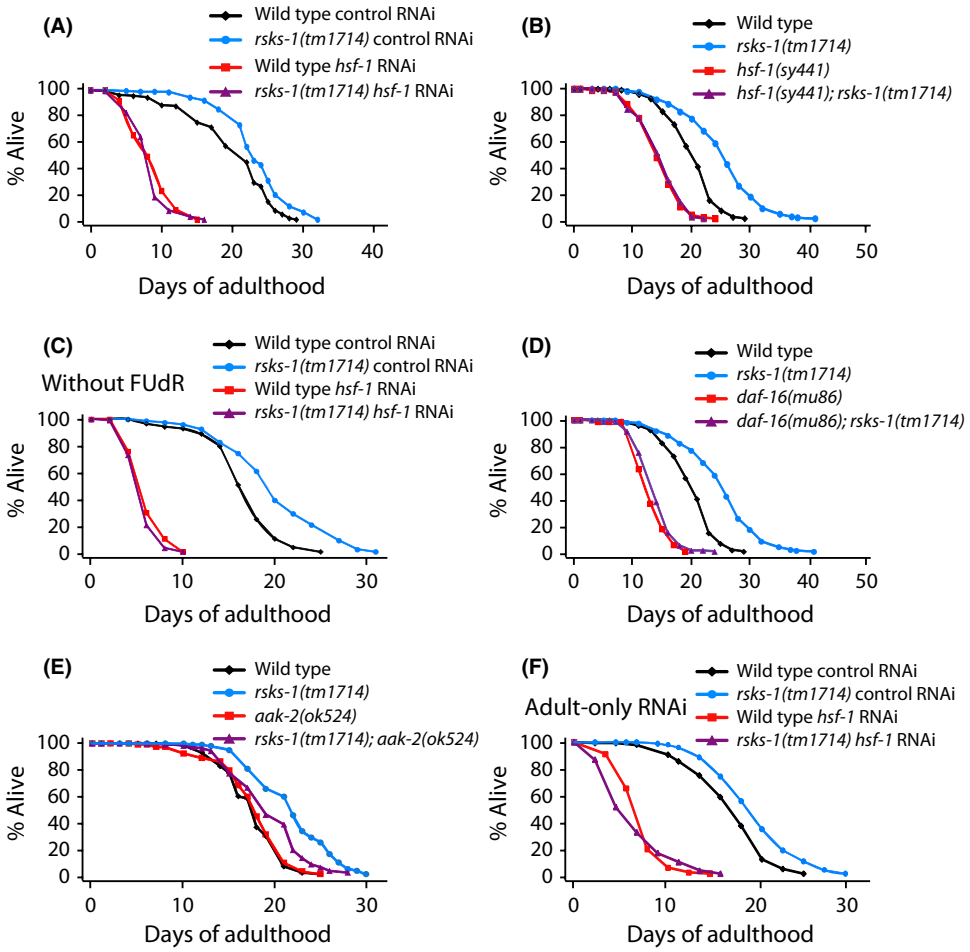

Fig. 1 HSF-1 is required for the longevity caused by the inhibition of S6 kinase (*rsks-1*). (A, B) The long lifespan of *rsks-1 (tm1714)* mutants was completely suppressed by *hsf-1* RNAi (A) or *hsf-1 (sy441)* mutations (B). Because *hsf-1(sy441)* mutants display larval arrest phenotype at 25 °C (Hajdu-Cronin *et al.*, 2004), we tested whether the development of *hsf-1 (sy441)* mutants was affected at 22.5 °C. We found that *hsf-1(sy441)* mutants did not display the larval arrest phenotype at 20 °C or 22.5 °C (Fig. S1D), the temperatures for the lifespan assays (A) and (B), respectively. (C) *hsf-1* is required for the longevity of *rsks-1* mutants without FUdR treatment. (D, E) The long lifespan of *rsks-1 (tm1714)* mutants was suppressed by *daf-16(mu86)* (D) or *aak-2(ok524)* (E) mutations. (F) *hsf-1* RNAi treatment only during adulthood (adult-only RNAi) suppressed the longevity of *rsks-1(tm1714)* mutants. See also Fig. S1C for the lifespan results of *rsks-1(tm1714)* mutants treated with *hsf-1* RNAi only during adulthood without FUdR. A summary of the lifespan data presented in this and other Figures and additional repeats are included in Table S1. Lifespan experiments were carried out at 20 °C for (A) and at 22.5 °C for (B–F).

hub transcription factor for the cross talk between insulin/IGF-1 and TOR signaling pathways. Consistent with this idea, we found that the synergistic lifespan-extending effect by *rsks-1* and *daf-2* mutations was largely abolished by *hsf-1* RNAi (Fig. 6A). In addition, *daf-16* mutations fully suppressed the longevity of *daf-2 rsks-1* double mutants (Fig. 6B). In contrast, enhanced dauer formation caused by *rsks-1* mutations in *daf-2* mutants, which requires *daf-16*, was not suppressed by *hsf-1* RNAi (Fig. 6C). These data suggest that HSF-1 requirement for the enhanced longevity of the *daf-2 rsks-1* double mutant is distinct compared with that of the dauer phenotype of the double mutant. We also found that *hsp-16.1/2/11* and *hsp-70* were further induced by *rsks-1* mutations in *daf-2*/insulin/IGF-1 receptor mutants and that these highly induced mRNA levels were largely suppressed by *hsf-1* RNAi (Fig. 6D,E). Together, these data indicate that HSF-1 as well as DAF-16 acts as a key transcription factor mediating the synergistic longevity conferred by simultaneous down-regulation of both IIS and TOR pathways.

Discussion

In this study, we show that HSF-1 is a crucial longevity transcription factor acting downstream of the TOR signaling pathway. We found that genetic inhibition of *hsf-1* completely abrogated the long lifespan caused by inhibition of TOR signaling components. We also demonstrated that inhibition of *C. elegans* S6 kinase (*rsks-1*), a key downstream kinase of TOR, increased the level of a subset of HSF-1 targets. In addition, *hsf-1* was required for the resistance of *rsks-1* mutants to various internal and external stresses, which has been shown to strongly correlate with longevity. Furthermore, we showed that HSF-1 was required for the longevity caused by simultaneous inhibition of both IIS and TOR signaling. Together, our findings suggest that HSF-1 may act as a converging transcription factor downstream of these two most evolutionarily well-conserved longevity pathways (Fig. 6F).

We noted the specific genetic interaction between *rsks-1* and *hsf-1* initially through an unbiased modifier RNAi screen using the slow-growth phenotype of *rsks-1* mutants. We then found the requirement of HSF-1 in other physiological phenotypes of *rsks-1* mutants, including lifespan extension, stress resistance, and pathogen resistance. Because *hsf-1* RNAi influenced the growth of *rsks-1* mutants, HSF-1 might be considered a limiting factor for *rsks-1* mutants to normally develop to adulthood and to live long. However, we prefer our model proposing that HSF-1 acts as an instructive factor for the longevity and the stress resistance of *rsks-1* mutants for the following reasons. First, we showed that *hsf-1* RNAi only during adulthood was sufficient for suppressing the longevity of *rsks-1* mutants, excluding the possibility that *hsf-1* RNAi results in general sickness during growth. Second, we found that *rsks-1* mutations increased a subset of HSF-1 target gene expression and that one HSF-1 target gene, *hsp-16*, was at least partly required for the long lifespan. Third, we showed that TOR signaling and IIS synergistically increased the level of HSF-1 target genes. These data are consistent with our model that the reduction in TOR signaling activates HSF-1, which in turn extends lifespan.

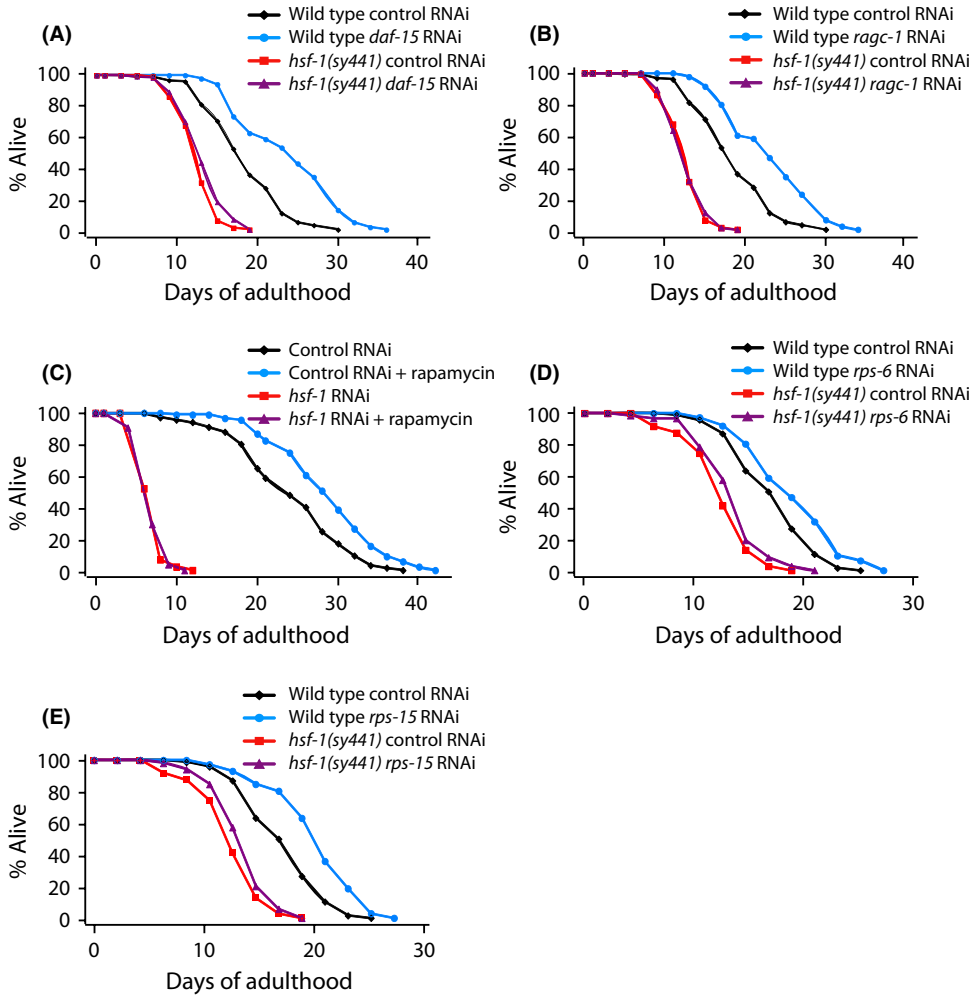

Fig. 2 HSF-1 is required for the long lifespan resulting from the inhibition of various components of TOR signaling. (A, B) Lifespan extension caused by inhibiting one of the components in TORC1 required *hsf-1*. *hsf-1(sy441)* mutation suppressed the prolonged lifespan caused by RNAi targeting *daf-15*/raptor (A) or *ragc-1*/Rag GTPase (B). (C) The longevity conferred by treating wild type with rapamycin (100 μM) was suppressed by RNAi knockdown of *hsf-1*. (D, E) Long lifespan conferred by knockdown of *rps-6* (D) or *rps-15* (E) was dependent on *hsf-1*. Note that we carried out lifespan assays by treating animals with RNAi only during adulthood, because *daf-15* homozygous mutations or RNAi clones targeting ribosomal protein subunits result in larval arrest phenotypes (Jia *et al.*, 2004; Hansen *et al.*, 2007). All lifespan experiments in this figure were carried out at 22.5 °C.

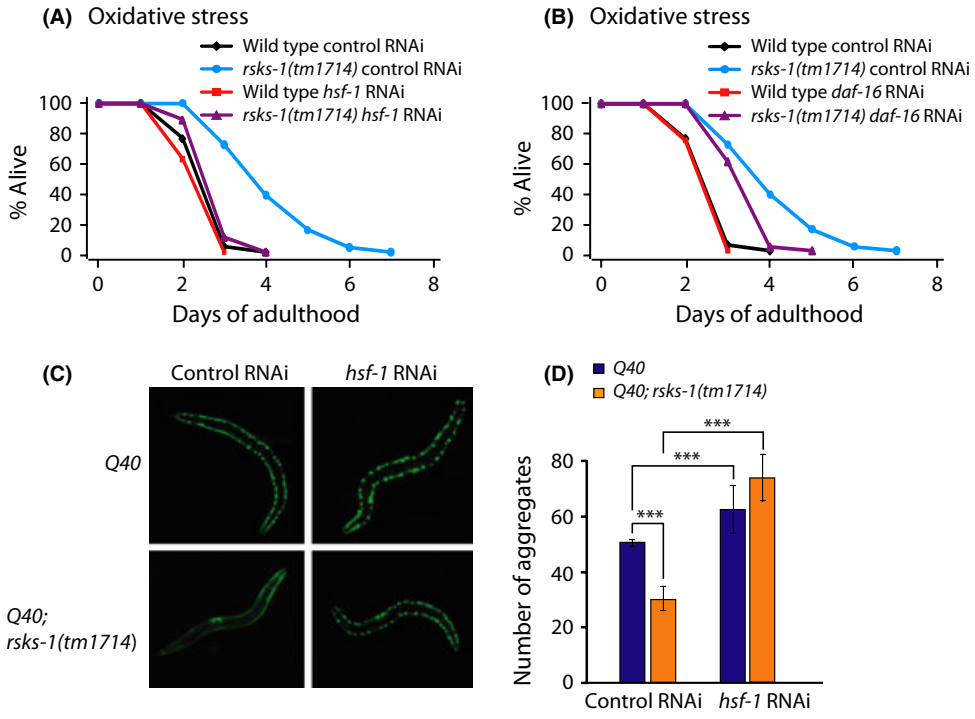

Fig. 3 Enhanced resistance of *rsks-1* mutants against oxidative stress and polyglutamine aggregation requires *hsf-1*. (A, B) Increased resistance of *rsks-1 (tm1714)* mutants against oxidative stress caused by paraquat (20 mM) treatment was completely suppressed by *hsf-1* RNAi (A) and partially suppressed by *daf-16*/FOXO RNAi (B). (C, D) *rsks-1(tm1714)* mutation decreased the level of aggregated YFP-fused polyglutamine repeats (Q40). (C) RNAi knockdown of *hsf-1* reversed the reduced polyglutamine aggregation in *rsks-1(tm1714)* mutant animals. (D) Quantification of the number of Q40::YFP (Q40) aggregates. Error bars represent the standard error of the mean (s.e.m.) ($n > 30$, ***$P < 0.001$, two-tailed Student's *t*-test). Statistical analysis of the data presented in this figure and additional repeats are described in Table S2.

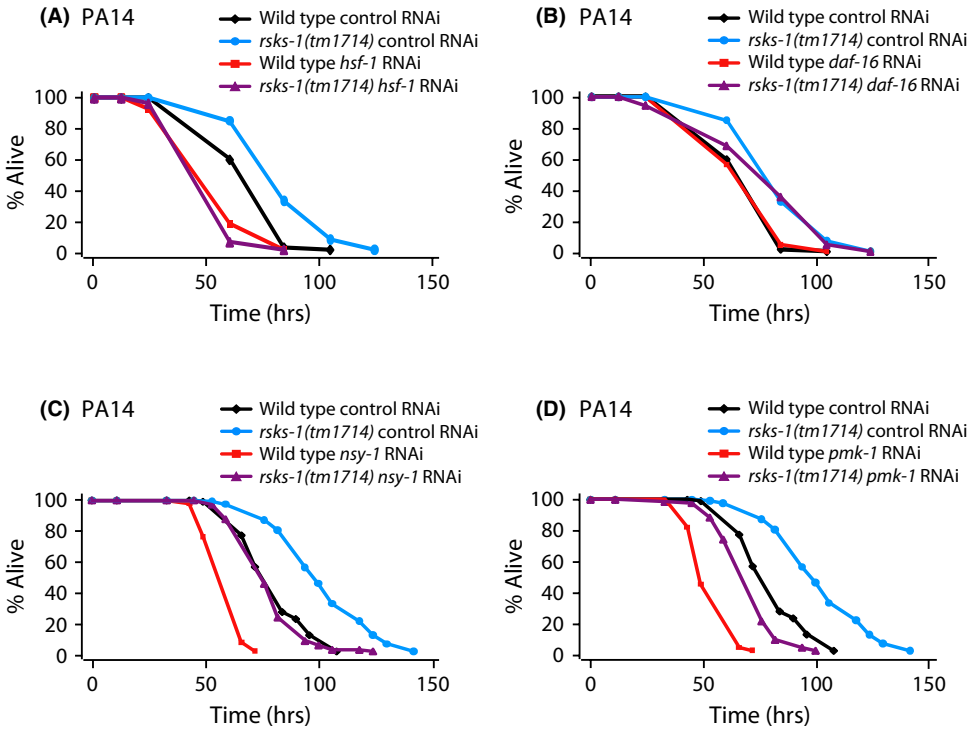

Fig. 4 Resistance to PA14 conferred by *rsks-1* mutations requires *hsf-1*. (A, B) *hsf-1* was required for the increased survival of *rsks-1(tm1714)* mutants feeding on the pathogen *P. aeruginosa* (PA14) (A), whereas *daf-16*/FOXO RNAi (B) had little effects on the survival. (C, D) RNAi targeting *nsy-1*/MAP kinase kinase kinase (C) or *pmk-1*/MAP kinase (D) did not fully suppress the pathogen resistance caused by *rsks-1(tm1714)* mutations. A summary of the data presented in this figure and additional repeats are described in Table S2.

How does TOR signaling influence the activity of HSF-1? Although a recent study using cultured mammalian cells showed that mammalian TOR (mTOR) phosphorylates and activates HSF-1 (Chou *et al.*, 2012), this may not be directly translated into our study. First, the serine residue in HSF-1 that is phosphorylated by mTOR (Chou *et al.*, 2012) is not conserved across organisms, including yeast, *C. elegans*, *Drosophila,* and zebrafish. Thus, this regulatory mechanism of HSF-1 by TOR may have evolved relatively recently. Moreover, in contrast to Chou *et al.*'s paper suggesting the activation of HSF-1 by TOR, we found that reduction in TOR signaling activates HSF-1. Thus, the role of phosphorylation of HSF-1 by TOR in lifespan regulation remains to be determined in future studies.

What will be the functional significance of up-regulation of HSF-1 activity by reduced TOR signaling? A previous report showed that dietary restriction increases lifespan and enhances resistance to polyglutamine aggregation in an *hsf-1*-dependent manner (Steinkraus *et al.*, 2008). As dietary restriction reduces TOR signaling (Kaeberlein & Kennedy, 2011), it seems likely that dietary restriction promotes longevity and stress resistance via inhibiting TOR signaling and subsequently increasing HSF-1 activity. Inhibition of TOR signaling is also well known to decrease mRNA translation, which leads to reduction in the overall protein level. Several studies have indicated that reduced translation by TOR signaling increases lifespan and stress resistance (Stanfel *et al.*, 2009; Kapahi *et al.*, 2010; Evans *et al.*, 2011; Laplante & Sabatini, 2012). Under these conditions, organisms may need to maintain biological functions using available proteins that were already synthesized. Therefore, the thrifty usage of the available proteins would be very critical for survival. Our data imply that reduced TOR signaling results in the activation of HSF-1 and subsequent up-regulation of molecular chaperones, which helps to improve overall protein quality control. This enhanced protein quality control in general may in turn increase lifespan and stress resistance. Alternatively, activated HSF-1 may induce specific chaperones, which facilitate maintaining or activating a subset of client proteins that are important for longevity and stress resistance. Consistent with this idea, only a subset of known HSF-1 target genes is induced by *rsks-1* mutations. Moreover, a small heat-shock protein that has been shown to be necessary and sufficient for the longevity caused by reduced IIS (Hsu *et al.*, 2003; Walker & Lithgow, 2003; Morley & Morimoto, 2004) was required for the long lifespan of *rsks-1* mutants. This indicates that the client proteins of HSP-16 are pivotal longevity proteins. It will be interesting to investigate these two alternative but not mutually exclusive possibilities to further understand the molecular basis of lifespan regulation by TOR signaling. Future studies using biochemical approaches to determine how the activity of HSF-1 is increased by reduced TOR signaling in animals will shed light on this issue.

Several previous studies have revealed that TOR signaling and IIS pathways employ common transcription factors, including SKN-1/NRF-2 and DAF-16/FOXO to regulate the lifespan of *C. elegans* (Sheaffer *et al.*, 2008; Tullet *et al.*, 2008; Wang *et al.*, 2010; Robida-Stubbs *et al.*, 2012). Here, we show that HSF-1 functions as an intersecting point or downstream of the intersecting point of TOR and IIS pathways. Future studies to elucidate the mechanisms by which these transcription factors interact with one another upon reduction in TOR signaling and IIS pathways will be important for providing insights into solving the secrets of human aging, particularly because TOR signaling and IIS pathways are two of the most crucial longevity pathways.

Experimental procedures

Strains

The following strains were analyzed in this study.

N2 wild-type, VB633 *rsks-1(sv31) III,* FX01714 *rsks-1(tm1714) III,* IJ109 *rsks-1(tm1714) III* outcrossed 4 times to Lee-lab N2, which is the same as Kenyon-lab N2, CF2725 *aak-2(ok524) X* outcrossed 3 times to Kenyon-lab N2, CF2495 *hsf-1(sy441) I* outcrossed three times to

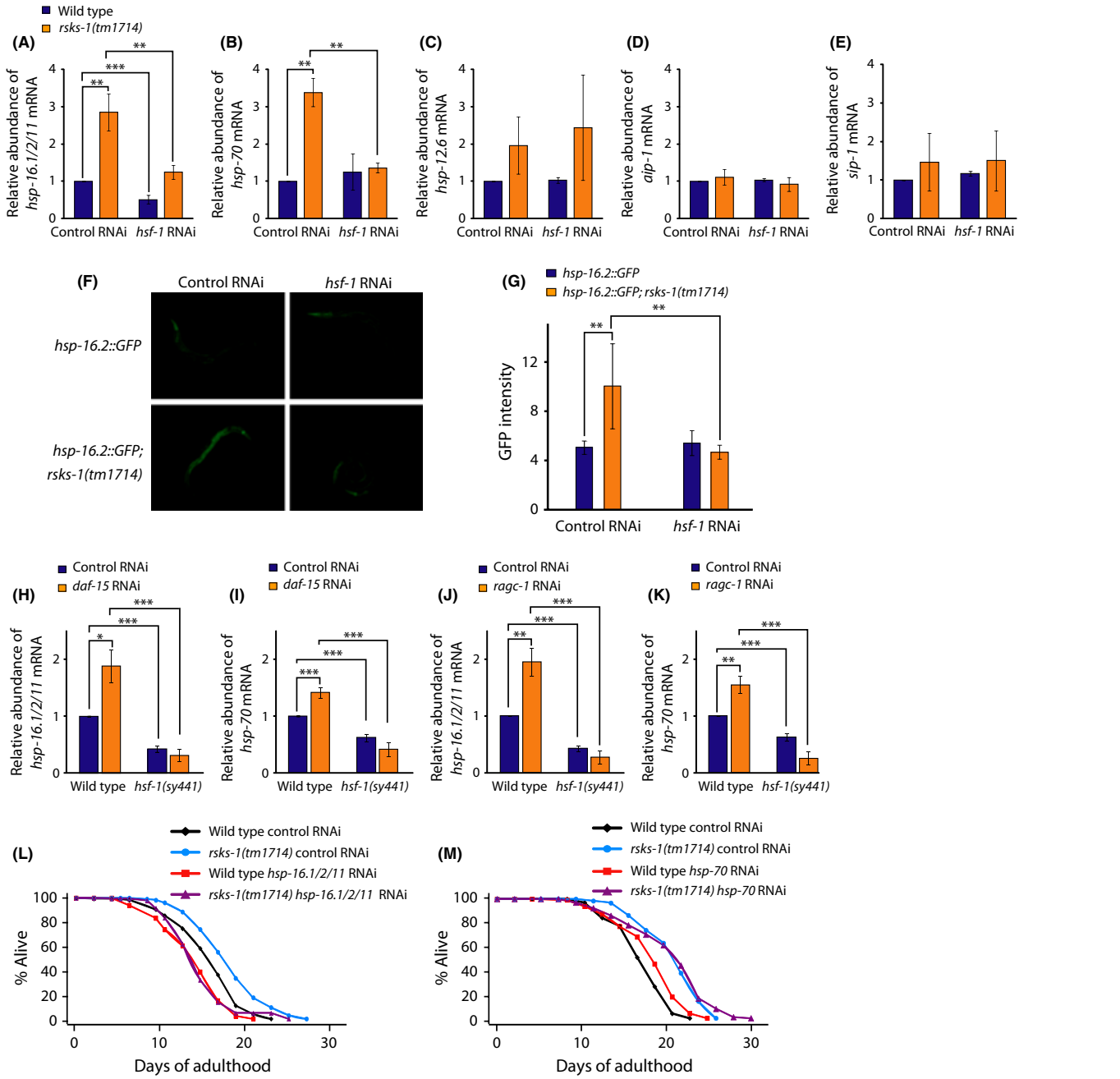

Fig. 5 *rsks-1* mutations extend lifespan through increasing the level of small heat-shock proteins in an HSF-1-dependent manner. (A–E) Of five HSF-1 target genes that we tested, mRNA levels of *hsp-16.1/2/11* (A) and *hsp-70* (C12C8.1) (B) were highly increased by the *rsks-1(tm1714)* mutations in an HSF-1-dependent manner in the absence of heat shock. mRNA expression of the other three tested HSF-1 target chaperon genes, *hsp-12.6* (C), *aip-1* (D), and *sip-1* (E), was not significantly affected by *rsks-1* mutations or *hsf-1* RNAi. Error bars represent the standard error of the mean (s.e.m) ($n = 4$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t*-test). (F) After incubating animals for 1 h at 32 °C, the level of GFP expression under the control of an *hsp-16.2* promoter (*hsp-16.2::GFP*) was significantly increased by *rsks-1(tm1714)* mutations in an HSF-1-dependent manner. (G) Quantification of GFP pixel intensity. Experiments were performed three times independently, and error bars represent the standard error of the mean (s.e.m) ($n > 30$, $^{**}P < 0.01$, two-tailed Student's *t*-test). (H, I) *daf-15* RNAi increased the mRNA levels of *hsp-16.1/2/11* (H) and *hsp-70* (I) in an *hsf-1*-dependent manner. (J, K) Likewise, *ragc-1* RNAi induced the mRNA expression of *hsp-16.1/2/11* (J) and *hsp-70* (K), and *hsf-1* mutations suppressed the induction of these genes. Error bars represent the standard error of the mean (s.e.m) ($n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t*-test). (L) *hsp-16.1/2/11* RNAi, which targets *hsp-16.1*, *hsp-16.2,* and *hsp-16.11*, suppressed the longevity conferred by *rsks-1(tm1714)* mutations in three of four trials (two of two trials at 20 °C and one of two trials at 22.5 °C.). We speculate that the inconsistency might originate from the differences in temperatures (20 °C vs. 22.5 °C). In addition, we cannot exclude the possibility that *hsp-16.1/2/11* RNAi did not work for one trial that did not display the longevity suppression or the RNAi exhibited variations among trials. Note that *hsp-16.1/2/11* RNAi was designed to target *hsp-16.1*, but because of over 88% sequence identity among *hsp-16.1, hsp-16.2,* and *hsp-16.11* genes, the RNAi clone is expected to target *hsp-16.1, hsp-16.2,* and *hsp-16.11* genes. (M) RNAi knockdown of *hsp-70* did not decrease the lifespan of *rsks-1(tm1714)* mutants or the wild-type. Note that *hsp-70* RNAi increased the lifespan of wild-type at 22.5 °C, while having no effects at 20 °C (Table S1). It was previously reported that *hsp-70* RNAi did not influence the lifespan of wild-type *C. elegans* at 25 °C (Morley & Morimoto, 2004). It is possible that *hsp-70* RNAi may increase the lifespan of worms at an intermediate temperature (22.5 °C) via compensatory induction of life-extending genes, while not affecting lifespan at 20 °C or 25 °C. Currently, we do not have mechanistic explanation for this result. All lifespan experiments in this figure were carried out at 22.5 °C.

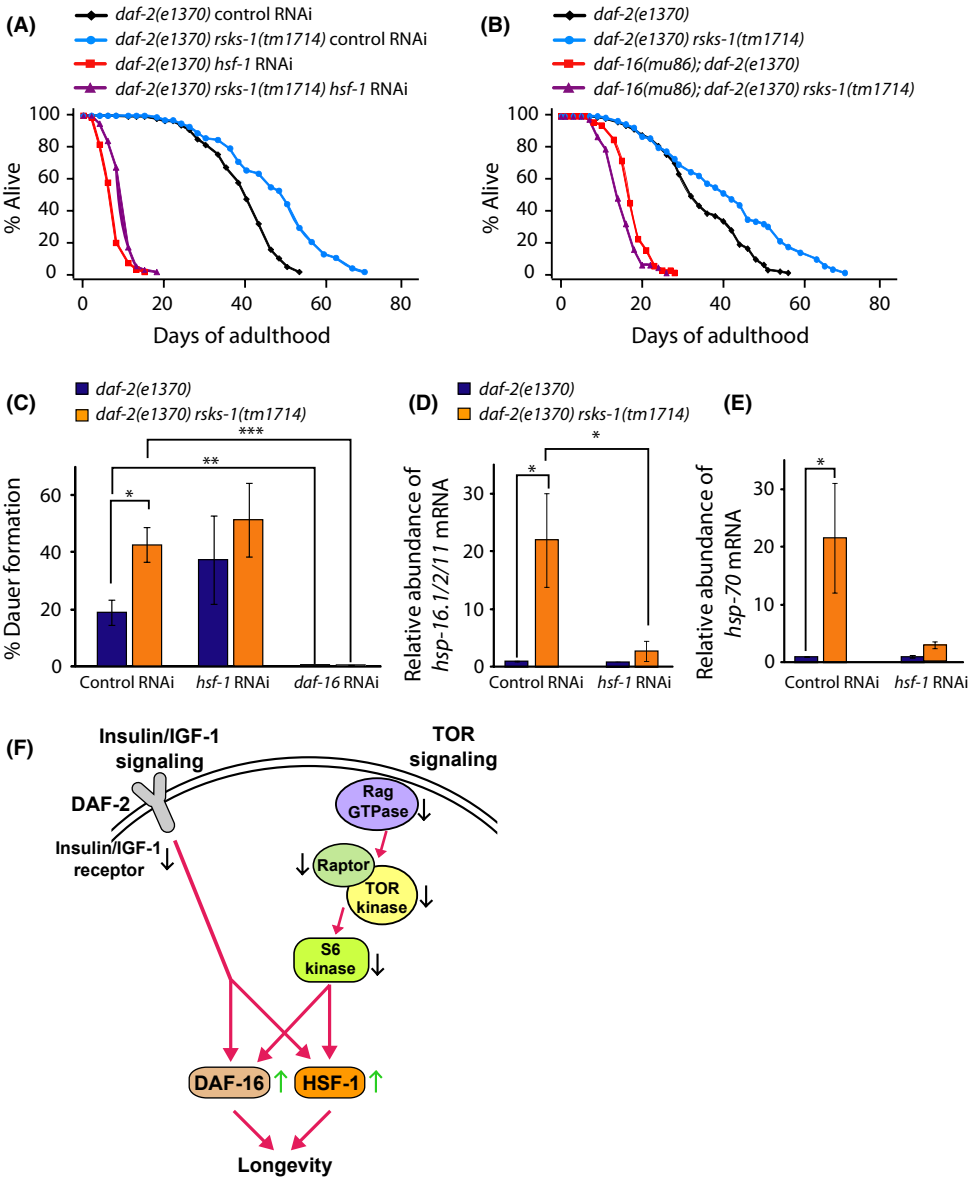

Fig. 6 HSF-1 is synergistically activated and is required for the extended longevity caused by simultaneous down-regulation of *daf-2* and *rsks-1*. (A, B) *rsks-1(tm1714)* mutations further increased the long lifespan of *daf-2(e1370)* mutants. This very long lifespan was suppressed by *hsf-1* RNAi (A) or *daf-16* mutations (B). (C) *rsks-1* mutations enhanced dauer formation in *daf-2* mutants. This increased dauer formation was not affected by *hsf-1* RNAi in either *daf-2(e1370)* or *daf-2(e1370) rsks-1(tm1714)* mutants. In contrast, *daf-16* RNAi suppressed dauer formation in *daf-2 (e1370)* and *daf-2(e1370) rsks-1(tm1714)* mutants. Likewise, *daf-16* mutations suppressed the dauer formation of *rsks-1* single mutants at 25 °C but *hsf-1* RNAi did not (Fig. S5A,B). Error bars represent the standard error of the mean (s.e.m) ($n = 4$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t*-test). (D, E) *hsp-16.1/2/11* (D) and *hsp-70* (E) mRNA levels were further increased by *rsks-1(tm1714)* mutations in *daf-2(e1370)* mutants, and *hsf-1* RNAi nearly reversed the induction of these chaperone genes in *daf-2(e1370) rsks-1(tm1714)* double mutants. Error bars represent the standard error of the mean (s.e.m) ($n = 4$, $^*P < 0.05$, two-tailed Student's *t*-test). (F) Schematic representation of the longevity pathways by which TOR signaling and IIS regulate HSF-1. Inhibition of DAF-2/insulin/IGF-1 receptor in the IIS or positive regulators of TOR signaling, including Rag GTPase, raptor, TOR kinase, and S6 kinase, increases the activities of DAF-16 and HSF-1, which appears to lead to longevity. All lifespan experiments in this figure were carried out at 20 °C.

Kenyon-lab N2, CF1042 *daf-16(mu86) I* outcrossed 11 times to Kenyon-lab N2, IJ184 *rmIs133[unc-54p::Q40::YFP]* outcrossed 4 times to Kenyon-lab N2, CL2070 *dvIs70[hsp-16.2p::GFP; rol-6D]*, IJ120 *rsks-1 (tm1714) III; aak-2(ok524) X*, IJ121 *hsf-1(sy441) I; rsks-1(tm1714) III*, IJ123 *daf-16(mu86) I; rsks-1(tm1714) III*, IJ185 *rsks-1(tm1714) III; rmIs133[unc-54p::Q40::YFP]*, IJ186 *rsks-1(tm1714) III; dvIs70[hsp-16.2p::GFP; rol-6D],* CF1041 *daf-2(e1370) III* outcrossed 6 times to Kenyon-lab N2, CF1085 *daf-16(mu86) I; daf-2(e1370) III,* IJ124 *daf-2 (e1370) rsks-1(tm1714) III*, IJ413 *daf-16(mu86) I; daf-2(e1370) rsks-1 (tm1714) III.*

Lifespan assays

Lifespan assays were performed as described previously with some modifications (Lee *et al.*, 2010). Worms were synchronized by allowing gravid adults to lay eggs for 12 h, and the progeny were allowed to develop. When worms were at young adult stage, approximately 30 worms were transferred onto each new NGM plate containing 5 μM 2′ fluoro-5′deoxyuridine (FUdR, Sigma). For the experiment shown in Fig. 1C and Fig. S1C, approximately 30 worms were synchronized at young adult stage and worms were transferred to fresh plates without FUdR every 2–3 days. We carried out lifespan assays at 20 °C or 22.5 °C for the following reasons. When we measured the lifespan of *rsks-1* mutants at 20 °C, the animals tend to display a vulval extrusion phenotype, which required censoring of many animals. To circumvent this problem, we performed the lifespan assays mostly at 22.5 °C after maintaining the worms at 22.5 °C for at least two generations. We carried out the lifespan experiments for *daf-2(e1370)* or *daf-2(e1370) rsks-1(tm1714)* mutants at 20 °C, because many of these animals arrested as dauer larvae at 22.5 °C. We specified the temperature at which each lifespan was measured in the Figure Legends and the Supporting Tables. Animals that ruptured, exhibited internal hatching of progeny, burrowed, or crawled off the plates were censored but included in the lifespan analysis as censored animals, as described

previously (Lee *et al.*, 2010). OASIS (online application of survival analysis, http://sbi.postech.ac.kr/oasis) was used for statistical analysis (Yang *et al.*, 2011). *P* values were calculated using the log-rank (Mantel–Cox method) test.

Rapamycin treatment

Rapamycin (LC Laboratories, Woburn, MA, USA) was dissolved in DMSO to obtain a 50 mg mL^{-1} stock solution and added onto plates to achieve the final concentration of 100 μM. The control plates were prepared to contain the same amount of DMSO without rapamycin.

Quantitative RT–PCR assays

Synchronized worms at young (day 1) adult stage were prepared for quantitative RT–PCR assays. RNA isoplus (Takara, Shiga, Japan) reagent was used for the extraction of total RNA from *C. elegans*. Total cDNA was synthesized using the reverse transcription system (Promega, Madison, WI, USA) and used as templates for qPCR with the SYBR green dye (Takara, Shiga, Japan). The PCR reaction was carried out in a StepOne Real-Time PCR system (Applied Biosystems, USA). mRNA of *hsp-16.1/2/11*, *hsp-70*, *aip-1*, *sip-1*, or *hsp-12.6* was amplified with gene-specific primers and normalized to *ama-1* mRNA level. The sequences of the gene-specific primers are described in the Supporting Information.

Oxidative stress resistance assays

Oxidative stress resistance assays were performed using paraquat as described previously with some modifications (Lee *et al.*, 2010). Gravid worms were transferred onto NGM plates seeded with *Escherichia coli* to lay eggs for 12 h. Progeny were grown on the plates to reach adulthood, and approximately 80 young (day 1) adults were transferred onto bacteria-containing plates treated with paraquat (methyl viologen; Sigma, St. Louis, MO, USA) at a final concentration of 20 mM and with 5 μM FUdR. Worms were incubated at 20 °C and scored as dead when they did not respond to prodding with a platinum pick. The survival assays were repeated at least twice. OASIS was used for statistical analysis, and *P* values were calculated using the log-rank (Mantel–Cox method) test.

PA14 slow-killing assays

PA14 slow-killing assays were carried out as described previously with minor modifications (Tan *et al.*, 1999). *P. aeruginosa* (PA14) was cultured in LB media at 37 °C overnight, and 5 μL of the culture was seeded onto high-peptone (0.35%) agar plates with 5 μM FUdR. The bacteria were then incubated at 37 °C for 24 h followed by incubation at 25 °C for 8–16 h for slow-killing assay. Approximately 100 young adult worms were transferred onto two PA14-seeded plates for each experiment. Worms were scored alive if they responded to prodding. OASIS was used for statistical analysis, and *P* values were calculated using the log-rank (Mantel–Cox method) test.

Fluorescence microscopy analysis

The number of polyglutamine (Q40::YFP) aggregates defined as discrete structures with edges recognizable from surrounding fluorescence was counted as described previously using Axio Scope A1 compound microscope (Zeiss, Oberkochen, Germany) (Hsu *et al.*, 2003). For measuring the level of *hsp-16.2::GFP* expression, young (day 1) adult worms were subjected to heat shock for 1 h at 32 °C, then recovered at 20 °C for 6 h, and monitored for GFP level using the Axio Scope A1 compound microscope. The fluorescence pixel intensity was quantified using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/).

Dauer assays

Dauer assay was performed as described previously with some modifications (Gaglia *et al.*, 2012). At least 150 worms were synchronized by allowing 15–30 adult worms to lay eggs at 22.5 °C for 6 h. Because the *rsks-1(tm1714)* mutations caused developmental delay in *daf-2* mutants, dauers were scored after 2 days for *daf-2(e1370)* mutants and 3 days for *daf-2(e1370) rsks-1(tm1714)* mutants. The assay was independently repeated four times.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Genetic interaction between *hsf-1* and *rsks-1* for growth during development and adult lifespan phenotypes.

Fig. S2 Decrease in the lifespan of *rsks-1* mutants by *daf-16* RNAi treatment.

Fig. S3 The effects of *rsks-1* mutations on the survival responses to heat and osmotic stresses.

Fig. S4 *rsks-1* mutations induce a subset of HSF-1 target genes in an *hsf-1*-dependent manner upon treatment with heat shock.

Fig. S5 Increased dauer formation by *rsks-1* mutation requires *daf-16* but not *hsf-1*.

Table S1 Lifespan analysis.

Table S2 Survival analysis other than lifespan analysis.

Data S1 Experimental procedures.