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Germline Signaling Mediates the Synergistically Prolonged Longevity Produced by Double Mutations in *daf-2* and *rsks-1* in *C. elegans*

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Summary

Inhibition of DAF-2 (IGF-1 receptor) or RSKS-1 (S6K), key molecules in the insulin/IGF-1 signaling (IIS) and target of rapamycin (TOR) pathways respectively, extends lifespan in *C. elegans*. However it has not been clear how they interact with each other and in which tissues to modulate longevity. Here we demonstrate that mutations in *daf-2* and *rsks-1* when combined produce a nearly five-fold increase in longevity that is much greater than the sum of single mutations. This synergistic lifespan extension requires positive feedback regulation of DAF-16 (FOXO) via the AMP-activated protein kinase (AMPK) complex. We further identify germ line as the key tissue for the synergistic longevity. Moreover, germline-specific inhibition of *rsks-1* activates DAF-16 in the intestine. Together, our findings highlight the importance of the germ line in significantly prolonged longevity by *daf-2 rsks-1*, which provides important implications for interactions between the two major conserved longevity pathways in more complex organisms.

Keywords

C. elegans; *daf-2*; *rsks-1*; *daf-16*; AMPK; germ line; synergistic lifespan extension

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Introduction

Aging can be modulated by both genetic and environmental factors. Alterations in insulin/insulin-like growth factor (IGF-1) signaling (IIS), target of rapamycin (TOR) pathway, signals from the reproductive system, and dietary restriction (DR) significantly affect lifespan (Kenyon, 2005; Kenyon, 2010). The highly conserved TOR kinase serves as a nutrient sensor to promote growth and proliferation via regulation of mRNA translation, ribosomal biogenesis, metabolism, and autophagy (Kapahi et al., 2010; Wullschleger et al., 2006). TOR promotes mRNA translation largely through the downstream ribosomal S6 kinase (S6K) and translation initiation factor eIF-4E-binding protein (4E-BP). Inhibition of TOR or S6K significantly extends lifespan in multiple species (Hansen et al., 2007; Harrison et al., 2009; Jia et al., 2004; Kaeberlein et al., 2005; Kapahi et al., 2004; Pan et al., 2007; Vellai et al., 2003). The mechanisms are overlapping with those by DR, an environmental manipulation that extends lifespan and slows age-related pathologies (Kapahi et al., 2010). *rsks-1* encodes the *C. elegans* ortholog of S6K. In addition to lifespan extension, *rsks-1* mutants also show delayed development and reduced fertility (Hansen et al., 2007; Korta et al., 2012; Pan et al., 2007; Selman et al., 2009). The longevity phenotype of *rsks-1* requires PHA-4, a FOXA transcription factor, and AAK-2, a catalytic subunit of the 5′ adenosine monophosphate-activated protein kinase (AMPK) (Selman et al., 2009; Sheaffer et al., 2008). AMPK is a key cellular energy homeostasis regulator that is also partially required for lifespan extension by reduced IIS (Apfeld et al., 2004).

Inhibition of IIS results in prolonged longevity in worms, flies, mice and probably humans (Clancy et al., 2001; Holzenberger et al., 2003; Kenyon et al., 1993). In *C. elegans*, loss-of-function mutations in *daf-2*, which encodes the insulin/IGF-1 receptor homolog, lead to more than doubled adult lifespan as well as significant changes in development, metabolism and increased stress resistance (Gems et al., 1998; Kenyon et al., 1993; Kimura et al., 1997). The significantly prolonged longevity of *daf-2* is totally dependent upon the downstream DAF-16 (FOXO) transcription factor (Lin et al., 1997; Ogg et al., 1997). Functional genomics studies identified DAF-16 target genes, which are involved in stress response, metabolism and detoxification (Lee et al., 2003; McElwee et al., 2004; Murphy et al., 2003). DAF-16 acts in specific tissues to modulate lifespan. Restoring the DAF-16 activity in the intestine (adipose tissue) substantially increases the lifespan of *daf-16; daf-2* double mutants (Libina et al., 2003). On the other hand, DAF-16 functions through different factors to regulate the expression of downstream genes both cell-autonomously and -non-autonomously (Zhang et al., 2013). These findings suggest that IIS functions in an endocrine-like manner to modulate aging in *C. elegans*.

Signals from the reproductive system regulate lifespan in worms, flies and potentially in mice (Flatt et al., 2008; Hsin and Kenyon, 1999). In *C. elegans*, removal of the germ line significantly extends lifespan through activating DAF-16 in the intestine via a steroid hormone signaling (Berman and Kenyon, 2006; Hsin and Kenyon, 1999). Lifespan extension by germline loss requires DAF-16-mediated regulation of fat metabolism (McCormick et al., 2012; O'Rourke et al., 2009; Wang et al., 2008) and proteasome activity (Vilchez et al., 2012). Interestingly, removal of the germ line in certain *daf-2* mutants synergistically enhances the prolonged longevity phenotype, suggesting there might be regulatory interactions between IIS and signals from the reproductive system (Hsin and Kenyon, 1999).

Despite the well-characterized roles of *daf-2* and *rsks-1* in aging and their apparently overlapping functions, it has not been clear whether and how they might interact with each other to affect longevity. To address this important question, we constructed a *daf-2 rsks-1* double mutant, which displayed a synergistic effect on longevity. This nearly five-fold

lifespan extension is mediated by positive feedback regulation of DAF-16 via AMPK. Further analyses identified germ line as the key tissue for RSKS-1, DAF-16 and AMPK to modulate the synergistically prolonged longevity. Furthermore, inhibition of *rsks-1* in the germ line non-autonomously activates DAF-16 in the intestine. Collectively, our findings demonstrated a novel interaction between IIS and S6K in specific tissues that leads to significantly extended lifespan.

Results

Synergistic lifespan extension by *daf-2 rsks-1* requires DAF-16

To examine the genetic interaction between *daf-2* and *rsks-1*, we constructed a double mutant that carries the *daf-2(e1370)* strong loss-of-function allele and *rsks-1(ok1255)* deletion allele. The double mutant is viable, fertile and does not arrest at the diapause dauer stage under standard culture conditions, which allowed us to characterize the adult lifespan phenotypes. Since the *daf-2* mutation is temperature-sensitive, animals were grown at the permissive temperature (15°C or 20°C) until the late L4 larval stage and then transferred to the restrictive temperature (25°C) during adulthood for survival assays. The *rsks-1* and *daf-2* single mutants increased mean lifespan by 20% and 169%, respectively; whereas the *daf-2 rsks-1* double mutant showed a synergistic lifespan extension by 454% compared to the wild-type N2 (Figure 1A and Table S1). This lifespan extension phenotype was not due to unknown mutations in the background since all mutants were backcrossed with the same wild-type N2 for a minimum of six times. Furthermore, similar lifespan extension phenotypes were observed using another deletion *rsks-1(tm1714)* allele (Figure S1A), another point mutation *daf-2(e1391)* allele (Figure S1B) or when animals were cultured at the intermediate temperature (20°C) throughout life (Figure S1C). We also performed lifespan assays with animals treated with rapamycin to inhibit TOR, the upstream activator of RSKS-1. Rapamycin mildly extended lifespan of N2 by 26%, while it extended lifespan of the *daf-2* mutant by 45% (Figure 1B).

The longevity phenotype of *daf-2* is dependent on the downstream DAF-16 transcription factor (Lin et al., 1997; Ogg et al., 1997). To test the role of DAF-16 in the *daf-2 rsks-1*-mediated synergistic longevity, we constructed a *daf-16; daf-2 rsks-1* triple mutant using the *daf-16(mgDf47)* null allele. The *daf-16* deletion fully suppressed the prolonged longevity phenotype by *daf-2 rsks-1* (Figure 1A and Table S1). We then examined DAF- 16 transcriptional activity by performing quantitative RT-PCR (qRT-PCR) to measure mRNA levels of genes that are regulated by DAF-16. *sod-3* and *hsp-12.3* are activated by DAF-16, while *sams-1* is repressed by DAF-16 at the transcription level. Compared with *daf-2*, the *daf-2 rsks-1* double mutant showed a synergistic enhancement of DAF-16 transcription activity indicated by significantly increased expression of *sod-3* and *hsp- 12.3*, and significantly decreased expression of *sams-1* (Figure 1C). Therefore, deletion of *rsks-1* in *daf-2* leads to synergistically extended lifespan by significantly increasing DAF-16 activity.

Previous studies have identified transcription factors that are essential for the prolonged longevity by *daf-2* or *rsks-1* single mutant. *hsf-1* encodes the *C. elegans* heat-shock transcription factor ortholog and is required for *daf-2*-medaited lifespan extension (Hsu et al., 2003). Inhibition of *hsf-1* by RNAi decreased lifespan in all four genetic backgrounds tested (Figure S2). *hsf-1* inhibition suppressed the lifespan of *daf-2 rsks-1* at a higher level (73%) than it did in other backgrounds (40% in N2, 57% in *rsks-1*, and 65% in *daf-2*), and the lifespan extension by *daf-2 rsks-1* was almost completely suppressed by the *hsf-1* RNAi treatment (Figure S2). *skn-1* encodes the Nrf transcription factor ortholog that regulates oxidative stress response. Mutations in *skn-1* suppress lifespan extension by certain *daf-2* alleles (Tullet et al., 2008). However, inhibition of *skn-1* did not suppress the synergistic

lifespan extension by *daf-2 rsks-1* (Figure S2D). *pha-4* encodes a FOXA transcription factor that is required for *rsks-1* and DR-mediated lifespan extension (Panowski et al., 2007; Sheaffer et al., 2008). Inhibition of *pha-4* shortened N2 and *rsks-1* lifespan, but had no effect on *daf-2* lifespan (Figure S2A-C). Surprisingly, *pha-4* knock-down did not affect the lifespan of *daf-2 rsks-1* (Figure S2D), supporting the notion that the *daf-2 rsks-1* double mutant extends lifespan by engaging different mechanisms than the single mutants alone.

Effects of *daf-2 rsks-1* on development, reproduction, stress resistance and DR

In order to further investigate the *daf-2 rsks-1* mutant, we examined other phenotypes associated with extended lifespan. In addition to prolonged longevity, mutations in *daf- 2* also affect development, reproduction and stress resistance. Under harsh conditions, *C. elegans* may arrest development entering a diapause stage called dauer for extended survival (Riddle and Albert, 1997). Inhibition of IIS leads to constitutive dauer arrest in a temperature-sensitive manner, with complete penetrance at the restrictive temperature 25°C. Some *daf-2* animals transiently arrest as dauer at the intermediate temperature 22.5°C, which provides a condition to test whether *rsks-1* plays a role in the IIS-dependent dauer formation. We found that *rsks-1* had no significant effect on *daf-2* dauer formation at 22.5°C (Figure 2A), suggesting an uncoupling of mechanisms for the synergistic longevity and dauer formation in *daf-2 rsks-1*.

Previous studies have demonstrated that both *daf-2* and *rsks-1* regulate germline proliferation and reproduction (Korta et al., 2012; Michaelson et al., 2010). We examined the reproduction profiles of N2, *rsks-1*, *daf-2* and *daf-2 rsks-1*. The experiments were performed at 15°C because at higher temperatures, both *daf-2* and *daf-2 rsks-1* mutants show high incidence of embryo retention and thus internal hatching, which leads to death and inaccurate measurement of reproduction. We found that compared to *daf-2*, the *daf-2 rsks-1* mutant showed a delayed and prolonged reproduction period and overall reduced brood size (Figure 2B). This is consistent with the recent studies showing that *daf-2* and *rsks-1* act in parallel to regulate germline stem cell proliferation and differentiation (Korta et al., 2012). The reproductive profile of *daf-2 rsks-1* was similar to that of *rsks-1*, with slightly more severe phenotypes. The lack of correlation between lifespan and reproduction suggests that the trade-off between longevity and fertility is unlikely to be a major cause of the synergistic lifespan extension by *daf-2 rsks-1*.

The extended lifespan of *daf-2* mutants has also been correlated with the activation of stress response genes and increased stress resistance (Murphy et al., 2003; Samuelson et al., 2007). We performed various stress assays to determine whether the synergistic longevity phenotype also correlates with increased stress resistance. Surprisingly, we found that *daf-2 rsks-1* animals were more sensitive to heat stress (35°C), with the mean survival decreased by 34% compared to the *daf-2* single mutant (Figure 2C). However, *daf-2 rsks-1* animals were slightly more resistant to oxidative stress induced by paraquat (Figure 2D) and ultraviolet (UV) stress (Figure 2E). Therefore, increased resistance stress may not be the main causes of the synergistic longevity phenotype.

DR is a robust environmental manipulation that slows down aging. Since both IIS and TOR pathways are regulated by nutrients, we examined whether DR regulates the synergistic longevity by *daf-2 rsks-1* using a modified bacterial food deprivation DR regimen (DR-FD) (Kaeberlein et al., 2006; Lee et al., 2006). Unlike animals growing under *ad libitum* (AL) conditions, the *rsks-1* single mutant under DR-FD did not show lifespan extension (log-rank, $p > 0.05$). However, the *daf-2 rsks-1* double mutant still showed robust lifespan extension by 114% compared to *daf-2* (Figure 2F), suggesting the synergistic longevity phenotype is not dependent on nutrients.

Positive feedback regulation of DAF-16 via AMPK mediates the synergistic longevity by *daf-2 rsks-1*

To characterize the molecular mechanisms of the synergistic longevity by *daf-2 rsks-1*, we compared gene expression profiles in N2, *rsks-1*, *daf-2*, *daf-2 rsks-1* and *daf-16; daf- 2 rsks-1* by microarrays. Genes that are differentially expressed in *daf-2 rsks-1* to a greater extent than in *daf-2* in a DAF-16 dependent manner were chosen for analysis (Figure 3A and Table S2). Gene Ontology (GO) analysis indicated that “aging” and “determination of adult lifespan” are among the most significant GO terms from this group of genes. We chose 42 genes based on their expression patterns and identities to perform a genetic screen by RNAi to identify genes that mediate the synergistic lifespan extension. We found 11 genetic suppressors of *daf-2 rsks-1*, inhibition of which decreases lifespan in *daf-2 rsks-1* to a greater extent than in N2, *rsks-1* and *daf-2* (Table S3).

aakg-4, which encodes the γ regulatory subunit of AMPK, showed the strongest suppression of *daf-2 rsks-1* upon inhibition (Figure 3B and Table S1). In control RNAitreated animals, the *daf-2 rsks-1* double mutant showed an 89% (synergistic) lifespan extension compared to the *daf-2* single mutant (Figure 3B left panel). In *aakg-4* RNAitreated animals, *daf-2 rsks-1* only showed an 18% (additive) lifespan extension compared to *daf-2* (Figure 3B right panel). qRT-PCR experiments confirmed that *aakg-4* mRNA levels were further up-regulated in *daf-2 rsks-1* (Figure S3A), and this regulation requires DAF-16 (Figure S3B). Since chromatin profiling studies by DNA adenine methyltransferase identification (DamID) demonstrated binding of DAF-16 to the *aakg-4* promoter (Schuster et al., 2010), *aakg-4* is likely to be a direct target of DAF-16 that is critical for the synergistic longevity by *daf-2 rsks-1*. Consistently, a deletion in *aak-2*, which encodes the α catalytic subunit of AMPK, also suppressed the synergistic longevity (Figure 3C and Table S1). In the presence of *aak-2*, the *rsks-1* deletion synergistically extended the mean lifespan of *daf-2* by 85% (Figure 3C left panel), whereas without *aak-2, rsks-1* only additively extended the mean lifespan of *daf-2* by 18% (Figure 3C right panel).

AMPK serves as an important energy homeostasis regulator by activating catabolic pathways to promote ATP generation upon energy starvation. It is a hetero-trimer that consists of a catalytic α subunit and regulatory β and γ subunits. AMP competes with ATP to bind the γ subunit, which stimulates AMPK by keeping the phosphorylation state of a highly conserved Threonine in the α subunit (Hardie, 2011). Consistent with previous studies (Selman et al., 2009), the *rsks-1* single mutant showed increased AMPK activation as indicated by elevated phosphorylation of AAK-2 (Figure 3D). The *daf-2 rsks- 1* double mutant showed further increase in AAK-2 phosphorylation compared with the *rsks-1* single mutant (Figure 3D). This phenotype is AAKG-4-dependent, as knockingdown *aakg-4* significantly reduced AAK-2 phosphorylation in *daf-2 rsks-1* (Figure 3D). Previous studies demonstrated that AAK-2 directly phosphorylates and activates DAF-16 in *C. elegans* (Greer et al., 2007). We found that inhibition of AMPK either by the *aak-2* deletion or *aakg-4* RNAi blocked the significantly elevated DAF-16 transcriptional activity in *daf-2 rsks-1*, indicated by reduced mRNA levels of *hsp-12.3* and *aakg-4* (Figure 3E and F). The fact that AAK-2 promotes the expression of its own activator AAKG-4 suggests a positive feedback mechanism. In the *daf-2 rsks-1* double mutant, AAK-2 is activated to increase DAF-16 activity, promoting the expression of lifespan determinant genes such as *aakg-4*, which in turn further activates AAK-2 and DAF-16 to form a positive feedback loop that eventually leads to significantly increased DAF-16 activity and lifespan extension (Figure 3G).

Tissue-specific regulation of the synergistic longevity by *daf-2 rsks-1*

In multiple cellular organisms, different tissues coordinately modulate physiology and lifespan at the whole organism level in response to genetic and environmental manipulations. The IIS pathway plays an endocrine role to modulate lifespan in a cellnon-autonomous manner. Genetic mosaics lacking *daf-2* in neuronal precursor cells are long-lived (Apfeld and Kenyon, 1998), and neuronal expression of *daf-2* rescues the *daf- 2* mutant-mediated lifespan extension (Wolkow et al., 2000). However, the essential IIS downstream transcription factor DAF-16 functions mainly in the intestine to modulate longevity (Libina et al., 2003). Although RSKS-1 regulates many cellular processes globally, its tissue-specific role in lifespan determination has not been characterized. To better understand the endocrine functions of *daf-2* and *rsks-1*, we examined tissue-specific involvement of *rsks-1*, *daf-16*, *hsf-1* and *aak-2* in the *daf-2 rsks-1*-mediated synergistic longevity by tissue-specific RNAi. RDE-1 is a member of the PIWI/STING/Argonaute family of proteins that is an essential component of the RNAi machinery (Tabara et al., 1999). Tissue-specific RNAi can be achieved using transgenic *rde-1* mutants complemented with tissue-specific promoters driving *rde-1* cDNA expression (Espelt et al., 2005; Qadota et al., 2007). Additionally, mutation of *rrf-1*, which encodes an RNA-directed RNA polymerase, allows RNAi to be functional exclusively in the germ line but not in somatic tissues (Sijen et al., 2001).

Using these tissue-specific RNAi tools, we identified the tissues in which *rsks-1* RNAi synergistically extended lifespan of *daf-2* animals. Knocking-down *rsks-1* in the *daf-2* single mutant extended the mean lifespan by 54%. Germline- and hypodermis-specific *rsks-1* RNAi extended *daf-2* lifespan by 41% and 39%, respectively. Intestine-specific *rsks-1* RNAi in *daf-2* caused a moderate lifespan extension of 21%. Body wall muscle-specific *rsks-1* RNAi did not extend *daf-2* lifespan significantly (Figure 4A, Figure S4 and Table S1). Thus, *rsks-1* loss-of-function in the germ line and hypodermis are likely to play an important role in the synergistic longevity.

Next, we tested tissue-specific involvement of the key suppressors of *daf-2 rsks-1*, including *daf-16*, *hsf-1*, and *aak-2*. Knocking-down *daf-16*, *hsf-1* and *aak-2* by RNAi in *daf-2 rsks-1* suppressed the mean lifespan by 69%, 72% and 58%, respectively. Upon inhibition of these genes in the germ line, the synergistic longevity was significantly suppressed by *daf-16* RNAi (49%) and *aak-2* RNAi (58%), but only mildly affected by *hsf- 1* RNAi (18%). Intestine-specific knocking-down *daf-16*, *hsf-1* and *aak-2* significantly suppressed *daf-2 rsks-1* lifespan by 50%, 69% and 39%, respectively. Additionally, inhibition of *daf-16* in the hypodermis also significantly decreased *daf-2 rsks-1* lifespan by 63%, whereas knocking-down *aak-2* and *hsf-1* in the hypodermis moderately reduced the lifespan by 35% and 36%, respectively. In the body wall muscle, knocking-down *daf- 16*, *aak-2* and *hsf-1* had little effect on longevity (Figure 4B, Figure S4 and Table S1). In summary, it is likely that DAF-16 functions in the germ line, intestine and hypodermis, AAK-2 functions in the germ line and intestine, while HSF-1 mainly functions in the intestine to modulate the longevity of *daf-2 rsks-1*. These results demonstrated that in addition to the intestine, other tissues especially the germ line also play an important role in aging.

Germline signaling modulates the synergistic longevity by *daf-2 rsks-1*

Previous studies have indicated that signals from the reproductive system modulate aging in multiple species. Signals from the germ line shorten lifespan, whereas signals from the somatic gonad extend lifespan (Hsin and Kenyon, 1999). Prolonged longevity via removal of germline precursor cells requires DAF-16 and DAF-12 (nuclear hormone receptor). Hence, we examined whether germline signaling modulates the synergistic longevity by *daf-2 rsks-1*.

glp-1 encodes a Notch family receptor that is essential for mitotic proliferation of germline cells (Austin and Kimble, 1987; Priess et al., 1987). The long-lived *glp-1* loss-offunction (lf) mutant serves as a genetic mimic of germline removal. Similar to the *rsks-1* mutant, *glp-1(lf)* animals showed significantly increased phosphorylation of AAK-2 (Figure S5A). Consistently, inhibition of *glp-1* by RNAi extended lifespan in N2, but not in the *aak-2* deletion mutant (Figure S5B). *glp-1(ar202)* is a gain-of-function (gf) allele exhibiting germline over-proliferation and shortened adult lifespan. We found that *glp-1(gf)* suppressed the synergistic lifespan extension by *daf-2 rsks-1*. Without *glp-1(gf)*, the *rsks-1* deletion synergistically extended the mean lifespan of *daf-2* by 86% (Figure 5A left panel), whereas with *glp-1(gf)*, *rsks-1* only additively extended the mean lifespan of *daf-2* by 14% (Figure 5A right panel). Consistently, *glp-1(gf)* also significantly decreased AAK-2 phosphorylation (Figure 5B) and DAF-16 transcriptional activity (Figure 5C) in *daf-2 rsks-1*. DAF-12, a nuclear hormone receptor, and KRI-1, an ankyrin repeats containing protein, were identified as important regulators of germline loss-mediated longevity (Berman and Kenyon, 2006; Hsin and Kenyon, 1999). Inhibition of *daf-12* or *kri-1* by RNAi decreased the lifespan of *daf-2 rsks-1* by 24% and 19%, respectively (Figure 5D and Table S1). Together, these results support the idea that germline signals play an important role in *daf-2 rsks-1*-mediated activation of AMPK and DAF-16 and synergistic lifespan extension.

Cell-non-autonomous activation of DAF-16 by knocking-down of *rsks-1* in the germ line

Since germline signaling has endocrine properties to regulate DAF-16 cell-non-autonomously in the intestine (Berman and Kenyon, 2006), we decided to examine whether inhibition of *rsks-1* in the germ line affects downstream genes cell-autonomously or -non-autonomously. To answer this question, we crossed an integrated *gfp* reporter driven by the *stdh-1* promoter (*Pstdh-1::gfp*) into *daf-2* and *daf- 2; rrf-1* to examine *stdh-1* expression patterns upon *rsks-1* RNAi treatment. *stdh-1* is transcriptionally activated by DAF-16. It is widely expressed in neurons, muscles and intestine, allowing us to monitor DAF-16 activities in various tissues. In the *daf-2* background, *rsks-1* RNAi activated *stdh-1* expression in the intestine. In *daf-2; rrf-1*, which allows RNAi to be functional only in the germ line, *rsks-1* RNAi led to a significant induction of *stdh-1* expression in the intestine (Figure 6A, B). To better quantify the induction of *Pstdh-1::gfp* by *rsks-1* RNAi, we performed Western blotting to measure GFP levels from whole worm lysates since majority of the *Pstdh-1::gfp* expression was from the intestine. Consistent with the imaging results, both regular and germline-specific *rsks-1* RNAi increased *Pstdh-1::gfp* expression (Figure 6C). We then quantified the intensities of GFP bands relative to those of Actin, which serves as the internal control for equal loading. The fold induction of *Pstdh-1::gfp* by *rsks-1* RNAi compared to the control RNAi was calculated. Although the germline-specific RNAi mutant *rrf-1* increased the basal levels of *stdh-1* expression, the induction of *stdh-1* expression by *rsks-1* RNAi is higher in *daf-2* than in *daf-2; rrf-1* (Figure 6D). Thus, inhibition of *rsks-1* in the germ line non-autonomously activates DAF-16 in the intestine.

Discussion

IIS and TOR pathways play conserved roles in modulating lifespan in multiple species. However, it is unclear how they might interactively modulate aging. We set out to address this question by constructing a *daf-2 rsks-1* double mutant, which has reduced function of IIS and an important branch of the TOR pathway. Surprisingly, the *daf-2 rsks- 1* double mutant shows a nearly 5-fold lifespan extension (Figure 1A and Table S1). We defined this phenotype as synergistic lifespan extension based on the observation that longevity of the *daf-2 rsks-1* double mutant is beyond the combined effects of *rsks-1* and *daf-2* single mutants. This synergistic longevity phenotype cannot be explained by the hypothesis that

daf-2 and *rsks-1* function in parallel to modulate lifespan independently since an additive effect will be expected under such an assumption.

The synergistic longevity phenotype is different from what we previously reported that *rsks-1* RNAi further extended *daf-2* lifespan by 24% (Pan et al., 2007). One major change in the experimental procedures was that in the previous study, *daf-2* animals were treated with *rsks-1* RNAi only during adulthood in contrast to this work, in which the double mutant carries the putative null allele of *rsks-1* throughout life. When we treated *daf-2* animals with *rsks-1* RNAi for two generations resulting in a more complete reduction in *rsks-1* mRNA levels, we observed a 54% further lifespan extension (Figure S4A and Table S1). These results suggest that inhibition of *rsks-1* during development is critical for the synergistic longevity phenotype. Consistently, inhibition of the RSKS-1 upstream activator LET-363/CeTOR in *daf-2* during adulthood led to 17% additive lifespan extension (Figure S6). Since *let-363* is an essential gene, inhibition of which during development leads to larval arrest, we used a pharmaceutical approach to inhibit *let-363* by treating animals with rapamycin as previously reported (Robida-Stubbs et al., 2012). Rapamycin treatment throughout life extended lifespan of N2 and *daf-2* animals by 26% and 45%, respectively (Figure 1B). There are multiple possible reasons why rapamycin treatment could not extend lifespan of *daf-2* animals as much as the *rsks-1* deletion mutant does. One possibility is rapamycin treatment did not fully block RSKS-1, which is required for the synergistic longevity. Another possibility is rapamycin treatment at this dosage has been shown to inhibit both TOR complex 1 and complex 2 activities (Robida-Stubbs et al., 2012), and there might be other lifespan determinant genes affected by the drug. Nevertheless, these results are consistent with the idea that inhibiting *rsks-1* in *daf-2* during development leads to synergistic lifespan extension.

Previous studies showed that null mutants of *age-1*, which encodes a catalytic subunit of the phosphatidylinositol-3-kinase (PI3K) in the IIS pathway, exhibit exceptional lifespan extension in a DAF-16-dependent manner (Ayyadevara et al., 2008). Since the *daf-2* mutations we used in this study are not null alleles, one possible explanation for the synergistic longevity by *daf-2 rsks-1* is that the *rsks-1* deletion makes *daf-2* mutant phenotypes more severe. We think this is unlikely to be true because there are many aging-related phenotypes of *daf-2* not enhanced by the *rsks-1* deletion. As shown in Figure 2, *rsks-1* does not affect *daf-2*-mediated dauer arrest, and *rsks-1* has a minor or even opposite effect on most stress resistance. Understanding why these phenotypes are uncoupled from the synergistically prolonged longevity by *daf-2 rsks-1* will help to understand the basic mechanisms of aging.

TOR plays a conserved role in DR-mediated lifespan extension (Kapahi et al., 2010). We tested the effect of nutrients on the synergistic longevity using the DR-FD regimen (Figure 2F). The *rsks-1* single mutant did not show lifespan extension under DR, which is consistent with the idea that DR and reduced TOR signaling function through overlapping mechanisms to extend lifespan. Interestingly, the synergistic longevity by *daf-2 rsks-1* is nutrient independent, suggesting *rsks-1* functions through novel mechanisms to further extend lifespan of *daf-2* animals.

To better understand the molecular mechanisms of the synergistic longevity by *daf-2 rsks-1*, we set out to identify critical mediators by testing known regulators of IIS or *rsks- 1*. The heat-shock factor HSF-1 is critical for *daf-2*-mediated lifespan extension. Inhibition of *hsf-1* almost completely abolished the lifespan extension by *daf-2 rsks-1* (Figure S2). Lifespan extension via genetic or pharmaceutical inhibition of TOR requires the IIS downstream transcription factor SKN-1 (Robida-Stubbs et al., 2012). Surprisingly, inhibition of *skn-1* by RNAi had little effect on the synergistic longevity by *daf-2 rsks-1* (Figure S2). Similarly,

inhibition of PHA-4, a FOXA transcription factor that is required for the *rsks-1* single mutant-mediated lifespan extension, did not affect lifespan of *daf-2 rsks-1* (Figure S2). This is further evidence that the mechanism of the synergistic longevity in the *daf-2 rsks-1* double mutant is distinct from the lifespan extension by the single mutants.

We then performed microarray studies and identified genes that are differentially expressed in *daf-2 rsks-1* (Figure 3A and Table S2). A genetic screen using RNAi helped to identify the AMPK complex as the key mediator of the synergistic longevity by *daf-2 rsks-1* (Figure 3B, C, Table S1 and Table S3). Quantitative analysis of the lifespan data indicated that suppression of *daf-2 rsks-1* lifespan by inhibiting AMPK was not due to general sickness. Instead, inhibition of AMPK suppressed the synergy part of the lifespan extension. Further analysis identified positive feedback regulation of DAF-16 via AMPK in the *daf-2 rsks-1* mutant (Figure 3D-G). AMPK plays important roles in various cellular functions (Hardie, 2011). Under energy-starved conditions, AMPK is activated to promote catabolism and thus ATP production. Further characterization of the role of AMPK in metabolism will aid in the understanding of the synergistic longevity by *daf-2 rsks-1*.

Both IIS and signals from the reproductive system have endocrine functions. Modulation of these pathways in one tissue leads to non-autonomous activation of DAF-16 in the intestine (Berman and Kenyon, 2006; Libina et al., 2003). To better understand how aging is coordinately modulated across multiple tissues, we tested the involvement of key regulators of the *daf-2 rsks-1*-mediated synergistic longevity by tissue-specific RNAi. We found that *rsks-1*, *daf-16* and *aak-2* function in the germ line to regulate the synergistic lifespan extension (Figure 4), which can also be suppressed by a genetic mutation that causes germ line over-proliferation and by inhibiting key mediators of the germline signaling (Figure 5). In addition, inhibiting *rsks-1* in the germ line leads to non-autonomous activation of DAF-16 in the intestine (Figure 6). Previous studies on the tissue-specific requirements of key longevity determinants, including DAF-16, mainly employed transgenic rescue approaches. However, the traditional microinjection method creates transgenic lines with high copy number of transgenes, which will be silenced in the germ line. Our results indicate the germ line as an important tissue to integrate signals from the IIS pathway and S6K for lifespan determination.

Similar to the *rsks-1* single mutant, *daf-2 rsks-1* animals showed significantly delayed, prolonged and overall reduced reproduction (Figure 2B). This is consistent with a recent study showing that RSKS-1 acts in parallel with the IIS pathway to play an essential role in the establishment of the germline stem cell/progenitor pool (Korta et al., 2012). Interestingly, RSKS-1 functions cell-autonomously to regulate the germline progenitor establishment. This effect is independent of its known suppressors in the regulation of lifespan (Korta et al., 2012). These findings suggest that the synergistic longevity of *daf-2 rsks-1* cannot simply be linked with its functions in germline development and reproduction.

In *C. elegans*, the intestine carries out multiple nutrient-related functions, and it is the site for food digestion and absorption, fat storage, and immune response. DAF-16 is one of the essential transcription factors that function in the intestine to modulate lifespan. We found that intestinal-specific inhibition of *daf-16*, *aak-2* or *hsf-1* largely abolishes the synergistic lifespan extension of *daf-2 rsks-1* (Figure 4B). However, knocking-down of *rsks-1* in the intestine only has an additive effect on *daf-2* lifespan (Figure 4A), suggesting that *rsks-1* may function through non-autonomous mechanisms to activate DAF-16.

The hypodermis is considered as part of the epithelial system in *C. elegans*. It is involved in basic body plan establishment, cell fate specification, axon migration, apoptotic cells removal, and fat storage. We found that hypodermis-specific knocking-down of *rsks-1* in

daf-2 also leads to synergistic lifespan extension, and that hypodermis-specific knocking-down of *daf-16* significantly reduces the synergistic lifespan extension (Figure 4). Our results provide evidence for the important role of the hypodermis in lifespan determination. In future studies, it will be interesting to examine which biological functions of the hypodermis are involved in regulating the synergistic longevity by *daf-2 rsks-1*.

Previous studies showed that muscle decline is one of the major physiological causes of aging in *C. elegans* (Herndon et al., 2002). Neither *rsks-1* nor the downstream regulators *daf-16*, *hsf-1* and *aak-2* seem to function in the muscle to modulate the synergistic lifespan extension (Figure 4). However, we cannot rule out the possibility that these regulators may function in other tissues to non-autonomously regulate muscle functions in *daf-2 rsks-1*. Characterization of age-dependent muscle decline in *daf-2 rsks-1* will help to understand whether muscle functions are important for the synergistic lifespan extension.

There are limitations for assessing tissue-specific involvement of key regulators in lifespan determination by RNAi such as uncertainty of knock-down efficiency and potential leakiness. It has been reported that in *rrf-1* mutants, RNAi can be processed in certain somatic tissues including the intestine at least for the genes tested (Kumsta and Hansen, 2012). However, the critical function of *rsks-1* in the germ line is unlikely to be an artifact as *rsks-1* knock-down in the intestine of *daf-2* animals did not lead to synergistic lifespan extension. Moreover, inhibition of certain strong suppressors of *daf-2 rsks-1* such as *hsf-1* in the intestine but not in the germ line significantly decreased the synergistic lifespan extension by *daf-2 rsks-1*. Further analyses by single-copied, isoform-specific transgenic rescue will help to quantitatively determine the tissue-specific involvement of key regulators in the synergistic lifespan extension by *daf-2 rsks-1*.

It has not been clear whether DAF-16 is quantitatively more active or it is uniquely activated in certain tissues such as the germ line of *daf-2 rsks-1*. Though we identified the AMPK-mediated positive feedback regulation of DAF-16 based on genes that are expressed to a greater extent in *daf-2 rsks-1* animals, we speculate that the double mutant has some unique properties as shown in dauer formation and various stress tolerance assays. Our data with the phenotypic analysis of the double mutant and epistasis analysis of tissue requirement of DAF-16 suggests that with the *rsks-1* deletion, DAF-16 plays a more important role in certain tissues like the germ line to further extend lifespan of *daf-2*. Characterization of the genes that are uniquely up-regulated in *daf-2 rsks-1* or those that are regulated independently of DAF-16 will help distinguish these models.

In conclusion, we found that the *daf-2 rsks-1* double mutant shows synergistic lifespan extension, which is achieved through positive feedback regulation of DAF-16 by AMPK. Tissue-specific epistasis analysis suggests that this enhanced activation of DAF-16 is initiated by signals from the germ line and that the germ line tissue may play a key role in integrating the interactions between *daf-2* and *rsks-1* to cause synergistic lifespan extension. Since DAF-2, RSKS-1, AMPK and DAF-16 are highly conserved molecules, similar regulation may also exist in mammals. Further characterization of the *daf-2 rsks- 1*-mediated synergistic longevity will contribute to a better understanding of the molecular mechanisms of aging and age-related diseases.

Experimental Procedures

Lifespan assays

Animals were maintained at 15°C or 20°C until late L4 stages and then transferred to 25°C. The first day of adulthood is Day 1 in survival curves. Animals were scored as alive, dead or lost every 2–3 days.

qRT-PCR assays

The SYBR Green dye (Quanta) was used for qRT-PCR reactions performed on an LC480 machine (Roche). Relative-fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). qRT-PCR experiments were performed three times with consistent results using three independent RNA preparations.

Microarray analysis

Microarray hybridization was performed at the Buck Institute Genomics Core using the NimbleGen 12-Plex Gene Expression Arrays and arrays were quantified using the NimbleScan2 software.

Western blotting

Western blotting for phosphorylated AAK-2 (Cell Signaling, 1:300), Actin (Cell Signaling, 1:1,000) and GFP (UC Davis/NIH NeuroMab Facility, 1:1,000) were performed using the LICOR system. Band intensities were quantified using the Odyssey V3.0 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1. *daf-2 rsks-1* double mutant shows synergistic lifespan extension in *C. elegans*
2. AMPK mediates positive feedback regulation of DAF-16 in *daf-2 rsks-1*
3. Germ line is a key tissue in modulating the synergistic longevity of *daf-2 rsks-1*
4. Inhibiting *rsks-1* in the germ line leads to cell-non-autonomous activation of DAF-16

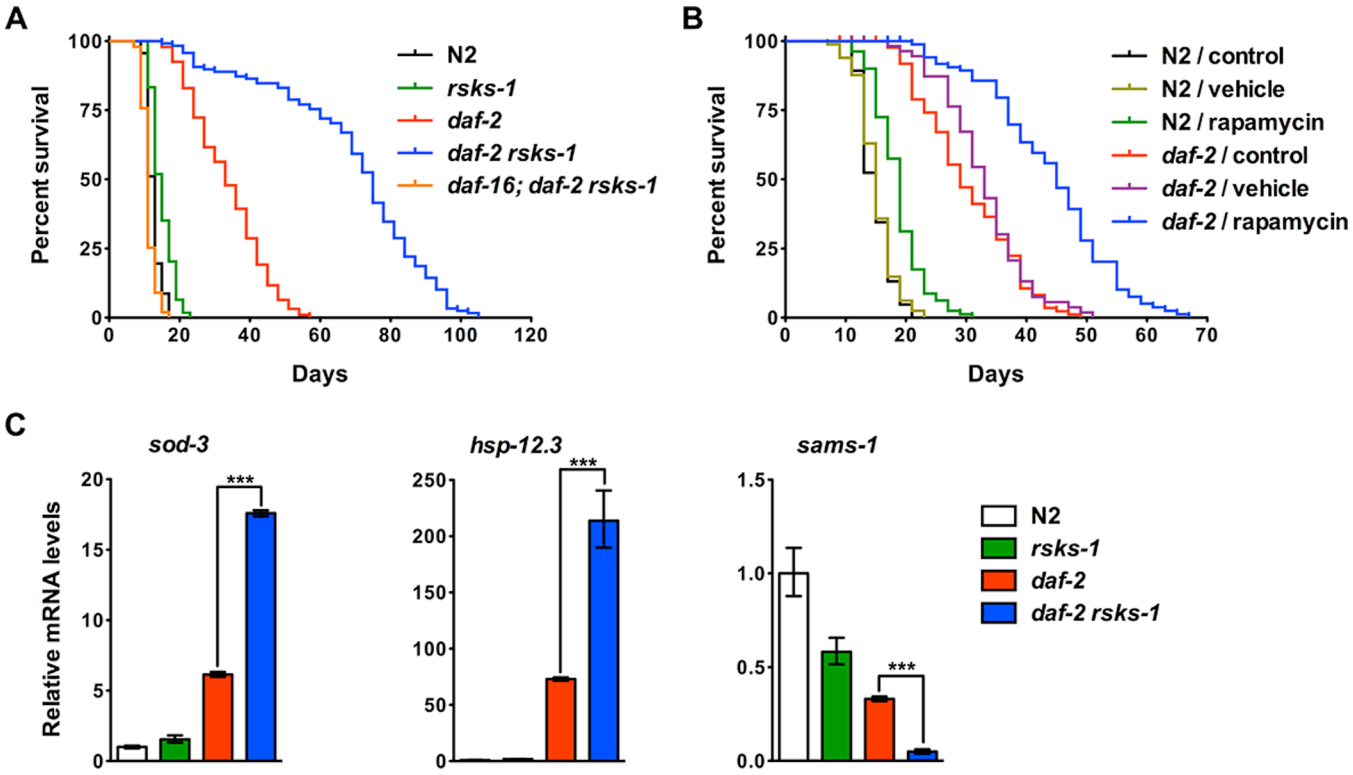

Figure 1. Double mutations in *daf-2* and *rsks-1* lead to synergistically prolonged longevity that requires DAF-16

(A) The *daf-2 rsks-1* double mutant showed synergistically prolonged longevity (454% extension compared to N2) that is dependent on DAF-16. (B) Inhibition of TOR by rapamycin led to increased lifespan extension in *daf-2* compared to N2. Rapamycin (100 μM) extended N2 and *daf-2* lifespan by 26% and 45%, respectively (log-rank, $p < 0.0001$). Animals treated with the vehicle (DMSO) alone did not show significantly affected lifespan (log-rank, $p > 0.05$). Quantitative data and statistical analyses are included in Table S1. (C) *daf-2 rsks-1* animals showed significantly increased DAF-16 transcriptional activity. mRNA levels of DAF-16 targets that are either activated (*sod-3* and *hsp-12.3*) or inhibited (*sams-1*) by DAF-16 were quantified using qRT-PCR. Asterisks indicate statistical differences using two-tailed t tests: ***, $p < 0.001$.

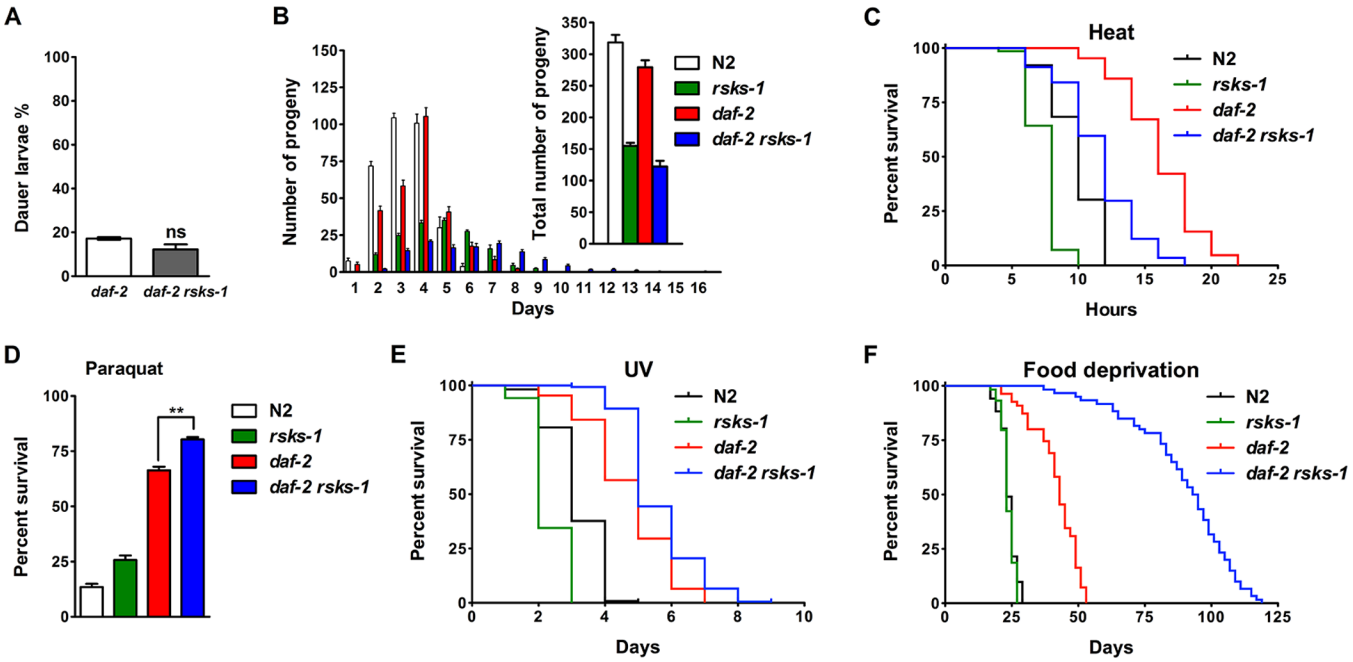

Figure 2. Effects of *daf-2 rsks-1* on development, reproduction, stress resistance and dietary restriction

(A) *rsks-1* did not affect *daf-2* dauer formation at 22.5°C. ns, not significant. (B) *daf-2 rsks-1* animals showed delayed, prolonged and overall reduced reproduction. (C) *daf-2 rsks-1* animals were more sensitive to heat stress (35°C) than *daf-2* (log-rank, $p < 0.0001$). (D) *daf-2 rsks-1* animals were more resistant to oxidative stress by paraquat compared to *daf-2* (**, $p < 0.01$, t - test). (E) *daf-2 rsks-1* animals were more resistant to UV stress (2,000 J/m^2) than *daf-2* (log-rank, $p < 0.0001$). (F) *daf-2 rsks-1* animals showed significantly increased survival under DR by bacterial food deprivation compared to *daf-2* (log-rank, $p < 0.0001$).

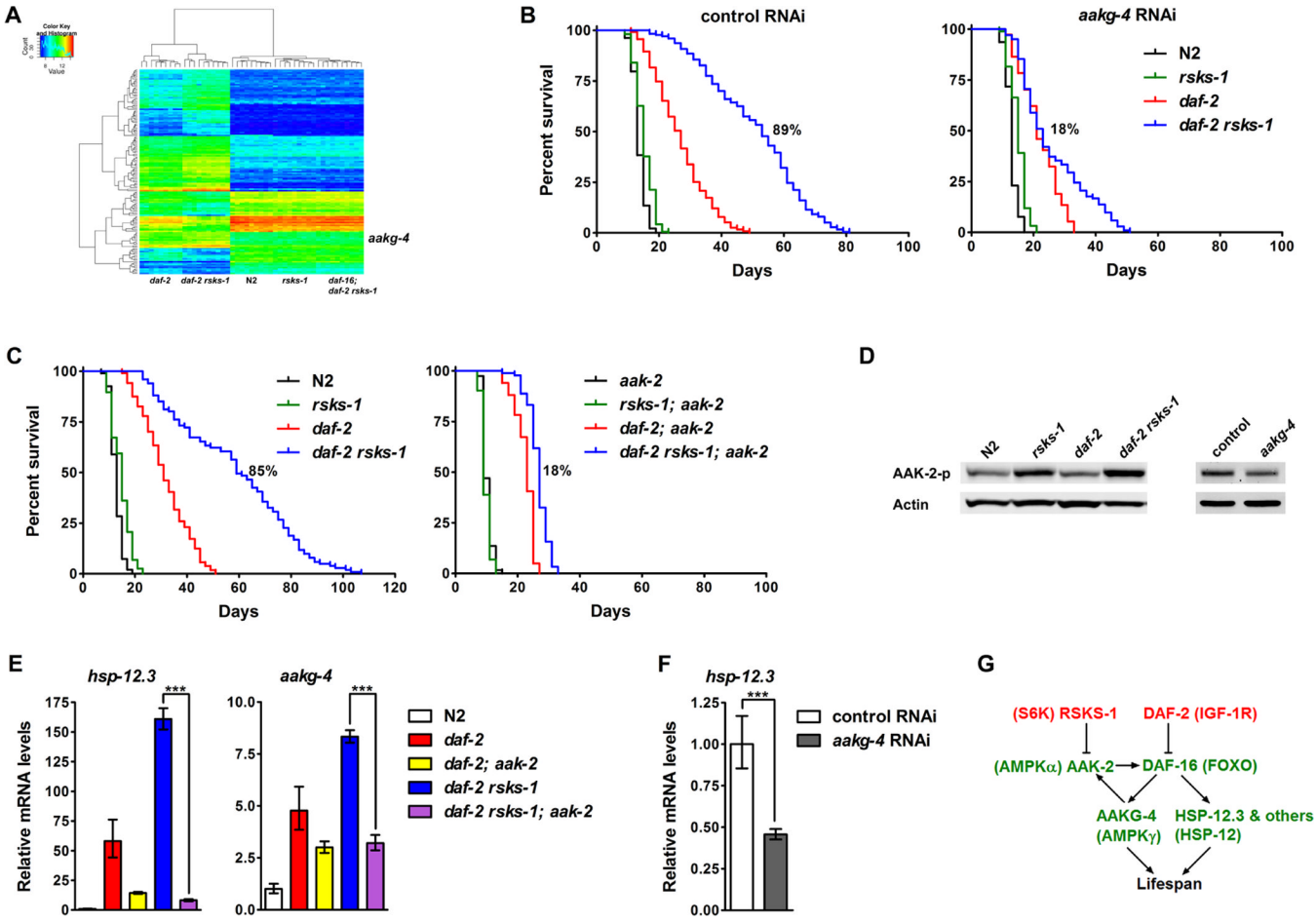

Figure 3. The synergistic longevity by *daf-2 rsks-1* is mediated by positive feedback regulation of DAF-16 via AMPK

(A) Genes that are differentially expressed in *daf-2 rsks-1* were identified by microarrays analyses. (B) Identification of *aakg-4* as a strong suppressor of *daf-2 rsks-1*. *rsks-1*-mediated lifespan extension in *daf-2* (*daf-2* vs. *daf-2 rsks-1*): 89% (control RNAi), 18% (*aakg-4* RNAi). (C) A deletion in *aak-2* suppressed the synergistic longevity by *daf-2 rsks-1*. *rsks-1*-mediated lifespan extension in *daf-2* (*daf-2* vs. *daf-2 rsks-1*): 85% (with *aak-2*), 18% (without *aak-2*). Quantitative data and statistical analyses are included in Table S1. (D) The *daf-2 rsks-1* double mutant showed further increased phosphorylation of AAK-2. Inhibition of *aakg-4* significantly decreased AAK-2 phosphorylation in *daf-2 rsks-1*. (E) The *aak-2* deletion suppressed the significantly increased DAF-16 transcriptional activity in *daf-2 rsks-1*. ***, $p < 0.001$. (F) Inhibition of *aakg-4* in *daf-2 rsks-1* reduced DAF-16 transcriptional activity. ***, $p < 0.001$. (G) A model depicting the synergistic lifespan extension through positive feedback regulation of DAF-16 via AMPK.

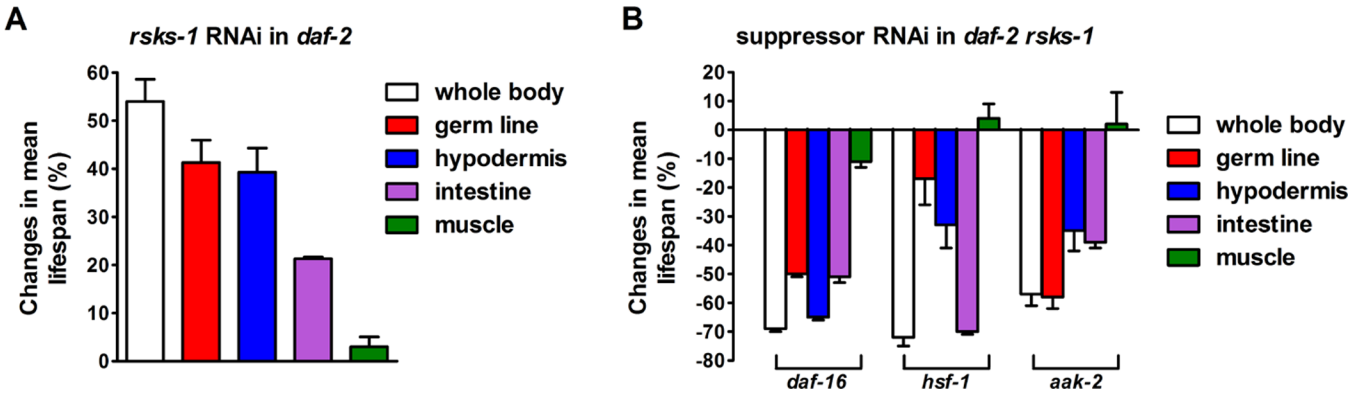

Figure 4. Tissue-specific regulation of the synergistically prolonged longevity by *daf-2 rsks-1*

(A) Mean lifespan extension by *rsks-1* RNAi knocking-down in different tissues of *daf-2* animals. (B) Changes in mean lifespan relative to the control RNAi-treated animals by tissue-specific RNAi knocking-down of *daf-16*, *hsf-1* and *aak-2* in *daf-2 rsks-1*. Data from three independent experiments are shown. Survival curves are included in Figure S4. Quantitative data and statistical analyses are included in Table S1.

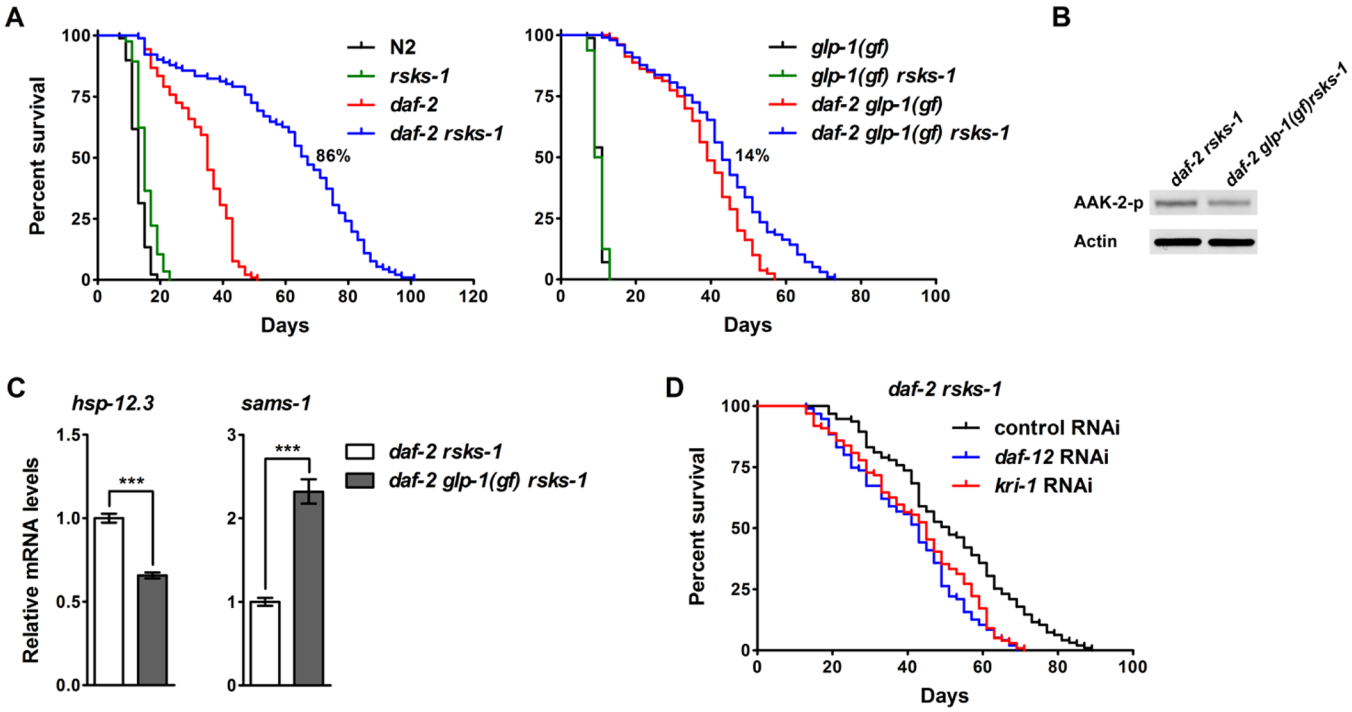

Figure 5. Germline signaling modulates the *daf-2 rsks-1*-mediated synergistic lifespan extension through AMPK and DAF-16

(A) The *glp-1(gf)* mutation suppressed the synergistic longevity by *daf-2 rsks-1*. *rsks-1*-mediated lifespan extension in *daf-2* (*daf-2* vs. *daf-2 rsks-1*): 86% [without *glp-1(gf)*], 14% [with *glp-1(gf)*]. (B) The *glp-1(gf)* mutation decreased phosphorylation of AAK-2 in *daf-2 rsks-1*. (C) The *glp-1(gf)* mutation suppressed the significantly increased DAF-16 transcriptional activity in *daf-2 rsks-1*. ***, $p < 0.001$. (D) Inhibition of DAF-12 or KRI-1, essential mediators of the germline signaling, significantly suppressed the synergistic longevity by *daf-2 rsks-1* (log-rank, $p < 0.0001$). Quantitative data and statistical analyses are included in Table S1.

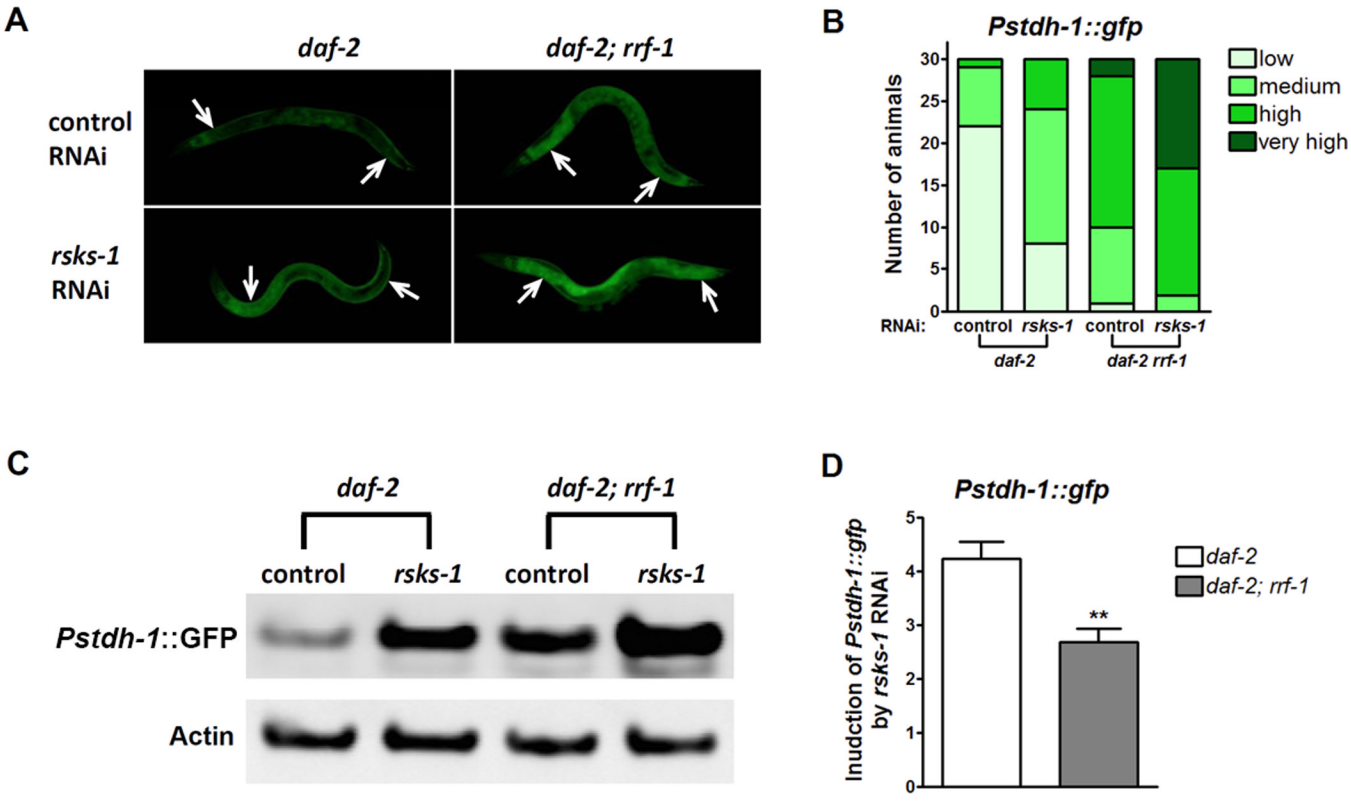

Figure 6. Cell-non-autonomous activation of DAF-16 by *rsks-1* RNAi knocking-down in the germ line

(A) Activation of the DAF-16 target *stdh-1* by *rsks-1* RNAi in *daf-2* and *daf- 2; rrf-1*. *rsks-1* RNAi knocking-down in the germ line of *daf-2* animals (*daf-2; rrf-1*) significantly increased the intestinal expression of GFP driven by the *stdh-1* promoter. Arrows indicate the anterior and posterior intestine. (B) Quantification of GFP expression driven by the *stdh-1* promoter. Thirty animals were examined for each treatment. (C) Measurement of *Pstdh-1::gfp* expression by Western blots using anti-GFP antibodies. (D) Quantification of GFP expression from Western blots. Relative GFP levels were calculated by normalizing the intensities of GFP bands to Actin. Fold increase in *Pstdh-1::gfp* expression induced by *rsks-1* RNAi was calculated through dividing the relative GFP levels in *rsks-1* RNAi-treated animals by those in control RNAi-treated animals. **, $p < 0.01$. The quantification was performed with four biological replicates.