Supplemental materials

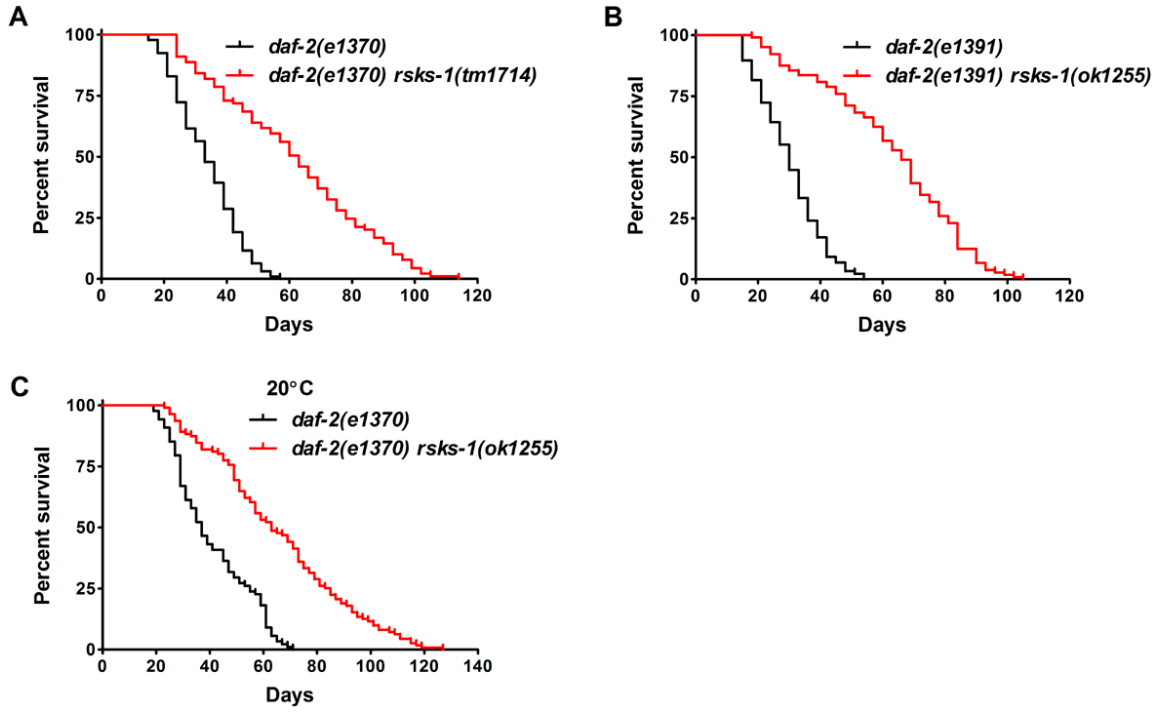

Figure S1. Synergistic longevity by *daf-2 rsks-1* under different genetic and environmental conditions. (A) The *rsks-1(tm1714)* deletion further extended the mean lifespan of *daf-2(e1370)* by 82% at 25°C. (B) The *rsks-1(ok1255)* deletion further extended the mean lifespan of *daf-2(e1391)* by 108% at 25°C. (C) The *daf-2(e1370) rsks-1(ok1255)* double mutant showed synergistic lifespan extension at 20°C. Mean lifespan extension compared to the wild-type N2: 12% by *rsks-1*; 231% by *daf-2*; and 371% by *daf-2 rsks-1*.

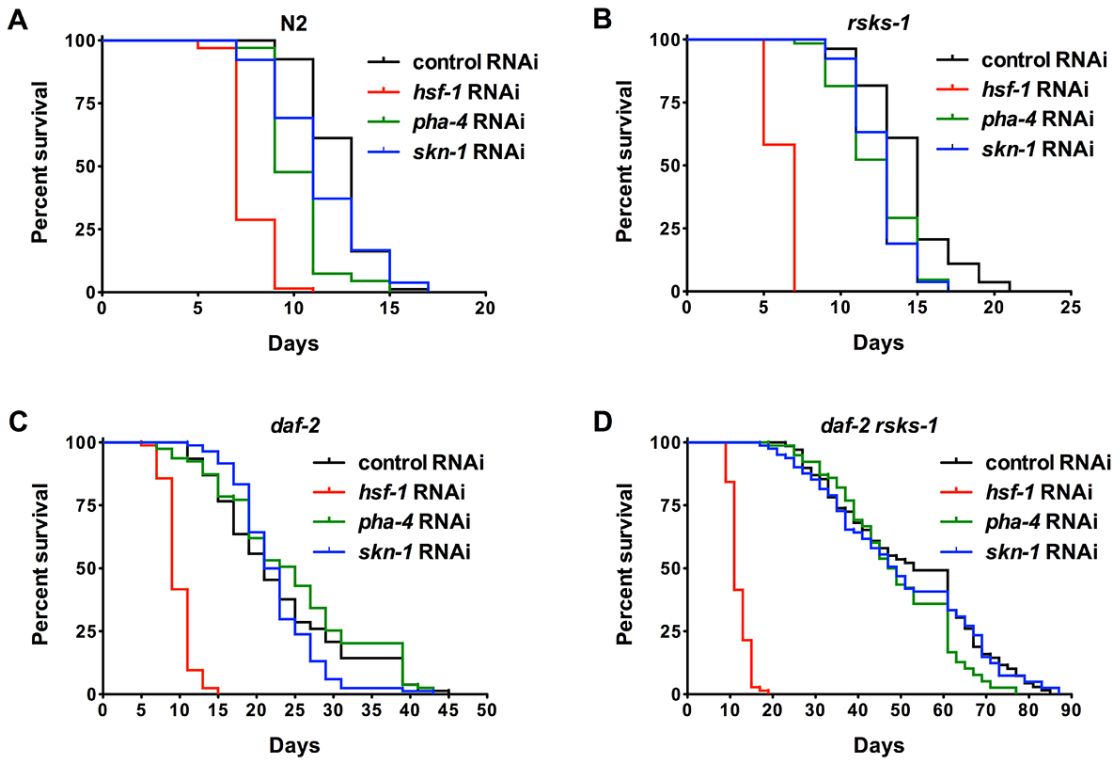

Figure S2. The synergistic longevity of *daf-2 rsks-1* can be suppressed by inhibition of *hsf-1*, but not *pha-4* or *skn-1*. (A) Inhibition of *hsf-1*, *pha-4* and *skn-1* decreased N2 lifespan by 40%, 19% and 9%, respectively (log-rank, $p < 0.05$). (B) Inhibition of *hsf-1*, *pha-4* and *skn-1* decreased *rsks-1* lifespan by 57%, 15% and 14%, respectively (log-rank, $p < 0.0001$). (C) Inhibition of *hsf-1* decreased *daf-2* lifespan by 65% (log-rank, $p < 0.0001$), whereas *pha-4* or *skn-1* RNAi knocking-down did not affect *daf-2* lifespan (long-rank, $p > 0.05$). (D) Inhibition of *hsf-1* decreased *daf-2 rsks-1* lifespan by 73% (log-rank, $p < 0.0001$), whereas *pha-4* or *skn-1* RNAi knocking-down did not affect *daf-2 rsks-1* lifespan (long-rank, $p > 0.05$).

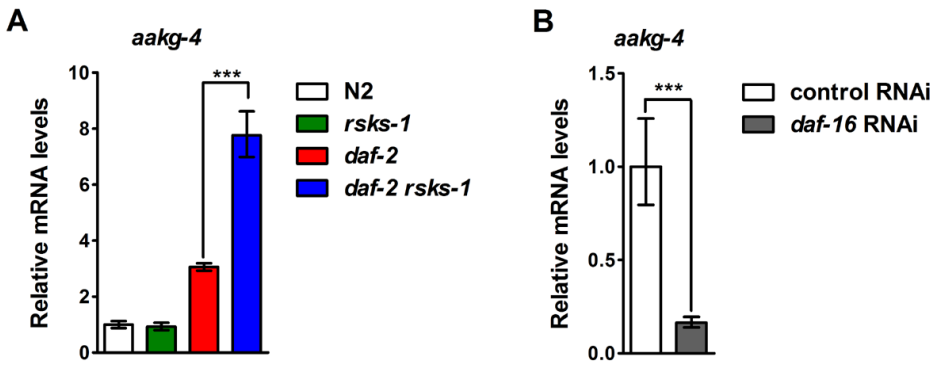

Figure S3. *aakg-4* is activated by DAF-16 in the *daf-2 rsks-1* double mutant. mRNA levels of *aakg-4* were quantified using qRT-PCR. (A) *aakg-4* was transcriptionally further up-regulated in *daf-2 rsks-1* compared to *daf-2*. (B) Inhibition of *daf-16* by RNAi significantly decreased *aakg-4* transcription in *daf-2 rsks-1*. Asterisks indicate statistical differences using two-tailed t tests: ***, $p < 0.001$.

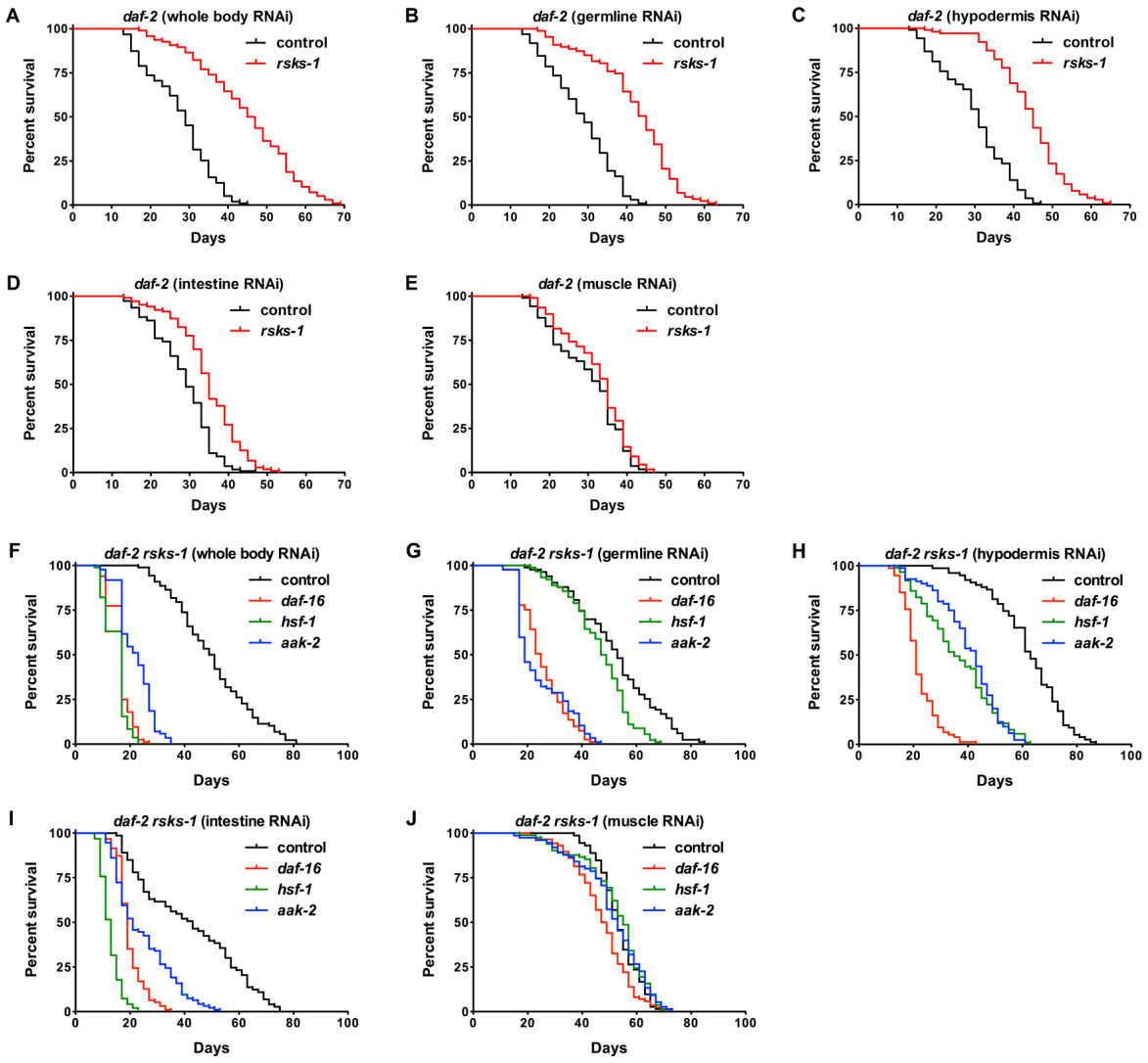

Figure S4. Tissue-specific involvement of RSKS-1, DAF-16, HSF-1 and AAK-2 in the synergistically prolonged longevity by *daf-2 rsks-1*. (A-E) Effects of tissue-specific RNAi knocking-down of *rsks-1* on *daf-2* lifespan. (A-C) Synergistically prolonged longevity could be achieved by *rsks-1* RNAi knocking-down in the whole body (A), germ line (B) or hypodermis (C) of *daf-2* animals. (D) *rsks-1* RNAi knocking-down in the intestine of *daf-2* caused additive lifespan extension. (E) *rsks-1* RNAi knocking-down in the muscle of *daf-2* did not affect lifespan significantly. (F-J) Effects of tissue-specific RNAi knocking-down of *daf-16*, *hsf-1* and *aak-2* on *daf-2 rsks-1* lifespan. (F) Inhibition of *daf-16*, *hsf-1* or *aak-2*

by RNAi significantly suppressed the synergistic lifespan extension by *daf-2 rsks-1*. (G) In the germ line, RNAi knocking-down of *daf-16* or *aak-2* significantly reduced lifespan of *daf-2 rsks-1* animals, whereas *hsf-1* RNAi had a minor effect on *daf-2 rsks-1* lifespan. (H) In the hypodermis, RNAi knocking-down of *daf-16* significantly reduced lifespan of *daf-2 rsks-1*, whereas *hsf-1* or *aak-2* RNAi had an intermediate effect on *daf-2 rsks-1* lifespan. (I) In the intestine, RNAi knocking-down of *daf-16* or *hsf-1* significantly reduced lifespan of *daf-2 rsks-1*, whereas *aak-2* RNAi had an intermediate effect on *daf-2 rsks-1* lifespan. (J) In the muscle, RNAi knocking-down of *daf-16*, *hsf-1* or *aak-2* did not significantly affect lifespan of *daf-2 rsks-1*. Quantitative data and statistical analyses are included in Table S1.

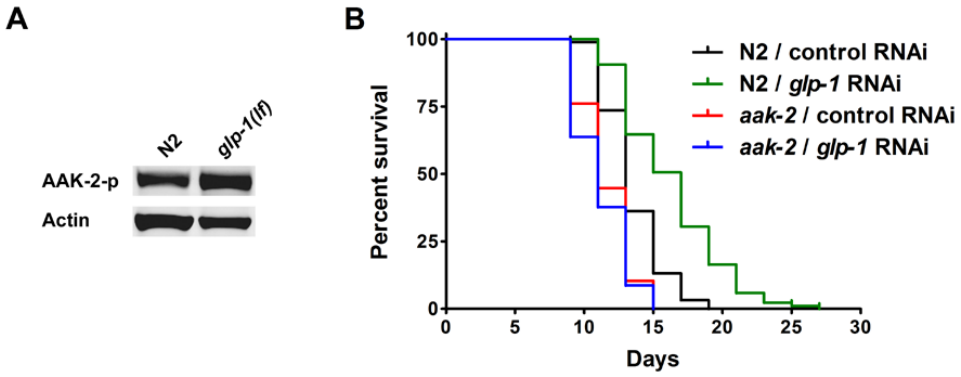

Figure S5. Prolonged longevity by a *glp-1(lf)* mutation is mediated by AMPK. (A) The germline-less *glp-1(lf)* mutant showed significantly increased phosphorylation of AAK-2. (B) Lifespan of the *aak-2* deletion mutant could not be extended by *glp-1* RNAi (log-rank, $p > 0.05$).

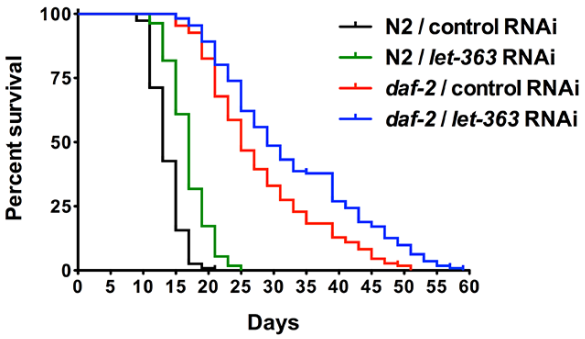

Figure S6. Adulthood inhibition of *let-363*/*CeTOR* does not synergistically extend lifespan of *daf-2* animals. Inhibition of *let-363* during adulthood increased lifespan of N2 and *daf-2* by 24% and 17%, respectively (log-rank, $p < 0.0001$).

Table S1 Statistical analyses of lifespan experiments

a, tissues in which RNAi is effective;

b, average lifespan extension;

c, numbers of animals scored;

d, log-rank tests.

Table S2 Gene expression data from microarray studies

Table S3 Identification of suppressors of *daf-2 rsks-1*-mediated lifespan extension

a, numbers of animals scored;

b, log-rank test

Extended Experimental Procedures

Strains

Mutations and transgenics used in this work include *daf-16(mgDf47) I*, *rrf-1(pk1417) I*, *daf-2(e1370) III*, *daf-2(e1391) III*, *rsks-1(ok1255) III*, *rsks-1(tm1714) III*, *glp-1(e2144) III*, *glp-1(ar202) III*, *rde-1(ne219) V*, *aak-2(ok524) X*, *kbIs[Pnhx-2::rde-1 + rol-6]*, *neIs9[myo-3::HA::RDE-1 + pRF4(rol-6(su1006))]*, *kzIs9[pKK1260(lin-12p::nls::GFP) + pKK1253(lin-26p::rde-1) + pRF6(rol-6(su1006)]*, *kzIs20[pDM#715(hlh-1p::rde-1) + pTG95(sur-5p::nls::GFP)]*, and *sIs10314[rCesC06B3.4::GFP + pCeh361]*.

Lifespan assays

Animals were maintained at 15°C or 20°C until late L4 stages and then transferred to fresh NGM plates with 5-fluoro-2′-deoxyuridine (FUdR, 10 μg / ml) at 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. Animals that failed to display touch-provoked movement were scored as dead. Animals that died from causes other than aging were scored as lost. Animals were transferred to fresh plates every 3–6 days. Survival curves were plotted and compared for log-rank tests using the Prism 4 software (Graphpad Software, Inc., San Diego, CA, USA).

Dauer, reproduction and stress resistance assays

Dauer: synchronized eggs were collected and incubated at 22.5°C for 3 days before scoring for dauer and other stages of development. Reproduction: L4 larvae growing at

15°C were transferred daily to fresh plates and progeny produced during that 24-hour period were counted. Stress resistance: synchronized Day 1 adults were collected for stress assays. Animals were treated with 35°C for heat stress, paraquat (100 mM for 2 hours) for oxidative stress, UV radiation (2,000 J / m^2) for UV stress and food deprivation for starvation stress. The dietary restriction by bacterial food deprivation assay was modified from the previously published method (Kaeberlein et al., 2006; Lee et al., 2006). Peptone was excluded from the NGM plates to create a more nutrient-free condition. Animals not responding to touch were scored as dead. Survival was plotted and analyzed using the Prism 4 software.

qRT-PCR assays

Day 2 adult animals were collected for total RNA extractions using the Trizol reagent (Invitrogen). The first strand cDNA was synthesized using the reverse transcription system (Qiagen). SYBR Green dye (Quanta) was used for qRT-PCR. Reactions were performed in triplicates 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. Primer sequences are available upon request.

Microarray experiments

Microarray hybridization was performed at the Buck Institute Genomics Core using the NimbleGen 12-Plex Gene Expression Arrays. Day 2 adult animals were collected for total

RNA extraction (Qiagen miRneasy Mini Kit). Each strain was examined in 9-10 biological replicates. The concentration and quality of total RNA samples were assessed using a Nanodrop and Agilent's Bioanalyzer. 200 ng of total RNA was amplified for each experimental sample (Sigma TransPlex Complete Whole Transcriptome Amplification Kit). 1 μg of ds-cDNA was labeled with Cy3 for each experimental sample (NimbleGen One-Color DNA Labeling Kit). Total RNA extraction, amplification and labeling were randomized across experimental groups. Sample hybridization was done according to the manufacturers' protocol for the 12-plex Gene Expression array platform. 4 μg of each Cy3-labeled sample was loaded onto an array on the 12-plex chip. Hybridization was done overnight on a NimbleGen hybridization system 4 unit at 42°C for 16 hours. The 12-plex chip was washed, dried and scanned on a GenePix 4200A scanner at 300PMT, 100POW. Arrays were then quantified using the NimbleScan2 software.

Microarray data analyses

Lowess normalization using the limma package in R (Smyth, 2005) was used to normalize the expression data. The primary question of interest was whether the expression of a gene is significantly amplified in the *daf-2 rsks-1* double mutant than in the *daf-2* and *rsks-1* single mutants. To test this hypothesis, a linear model was estimated for each gene. Specifically, the expression for gene *g* was modeled as:

$$E(Y_g|Experiment) = \beta_{1g}\, I(baseline) + \beta_{2g}\, I(daf\text{-}2) + \beta_{3g}\, I(rsks\text{-}1) + \beta_{4g}\, I(daf\text{-}2) \times I(rsks\text{-}1)$$

where each indicator designates a different experimental condition. The primary parameter of interest is β_{4g} which represents the interaction between the *daf-2* and

rsks-1 mutants and a rejection of the null hypothesis that $\beta_{4g} = 0$, indicating that in the double mutant there is a departure from additivity in the expression of that gene. To estimate the standard errors, an empirical Bayes procedure was performed through the limma package to calculate a moderated t-statistic for each contrast of interest (Smyth, 2004). The p-values were adjusted to control the false discovery rate (FDR) using the Benjamini-Hochberg (BH) procedure. A standard significance level 0.05 on β_{4g} was used. Since the focus was on identifying genes that had amplified expression in the double mutant, only those genes where either (a) $\beta_{2g} = \beta_{3g} = 0$, $\beta_{4g} \neq 0$ or (b) $\beta_{4g} \neq 0$ & $\beta_{2g} \mid \beta_{3g} \neq 0$ & $\text{sign}(\beta_{4g}) = \text{sign}(\beta_{2g}) \mid \text{sign}(\beta_{3g})$ (i.e. the amplification of expression was in the same direction) were considered. The program Ontologizer was used to perform GO analyses (Grossmann et al., 2007) . Significant terms were discovered using a parent-child analysis and an FDR cutoff of 0.10.

References

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