Supplemental Data

An AMPK-FOXO pathway mediates the extension of lifespan induced by a novel method of dietary restriction in *C. elegans*

Eric L. Greer, Dara Dowlatshahi, Max R. Banko, Judit Villen, Kimmi Hoang, Daniel Blanchard, Steven P. Gygi, and Anne Brunet

Supplemental Experimental Procedures

Constructs

GST-DAF-16 bacterial expression construct was constructed by cloning a BamHI fragment into pGEX-4T3 by PCR. DAF-16 mutants (T166A, S202A, S218A, S219A, T312A, S314A, S346A, T366A, S378A, T416A, S454A, T463A, S466A, S499A) in the pGEX-4T3 vector were generated by site directed mutagenesis (Stratagene) using the following sets of primers:

T166A-F: CCAGAGAAACGGTTAGCTCTTGCACAAGTTTACG,

T166A-R: CGTAAACTTGTGCAAGAGCTAACCGTTTCTCTGG,

S202A-F: CGATCCGTCACAATCTGG CTCTTCATTCTCG,

S202A-R: CGAGAATGAAGAGCCAGATTGTGACGGATCG,

S314A-F:

CCATCATCTTTCCGTCCCCGGACTCAAGCGAACCTCTCGATTCCTGG, S314A-R: CCAGGAATCGAGAGGTTCGCTTGAGTCCGGGGACGGAAAGATGATGG,

S321A-F: CGAACCTCTCGATTCCTGGAGCGTCGTCTCGTGTTTCTCC,

S321A-R: GGAGAAACACGAGACGACGCTCCAGGAATCGAGAGGTTCG,

T463A-F: GTAGCTGCACAGCATGCTGTCGCTTCTTCATCG,

T463A-R: CGATGAAGAAGCGACAGCATGCTGTGCAGCTAC,

S466A-F: GCACAGCATACTGTCGCTGCTTCATCGGCTCTTCC,

S466A-R: GGAAGAGCCGATGAAGCAGCGACAGTATGCTGTGC,

The mutations and flanking regions were verified by sequencing. Expression constructs encoding the worm AMPK subunits were created using the Open Biosystem cDNA clones: T01C9.1 (α2, AAK-2), F55F3.1 (β1), and Y41G9A.3 (γ1). Note that the AAK-2 Open Biosystem clone is missing the first 187 bp. The cDNA encoding AAK-2 was cloned into the pECE expression vector in frame with the Flag epitope tag. The worm β1 subunit was cloned into pECE and tagged with the HA epitope. The worm γ1 subunit was cloned into pECE an tagged with the HA epitope**.** Human AMPKβ1 and γ1 expression plasmids were obtained from Origene. Human AMPKα2 wt cDNA (Origene) was sub-cloned into the pECE mammalian expression vector in frame with the Flag epitope.

Oxidative stress resistance in worms

Young adults were synchronized as described above and 5-8 worms were placed in 96 well plates in S medium containing varying concentrations of Methyl viologen dichloride hydrate (Paraquat, Sigma). Animals were placed at 20°C and were probed with a platinum pick once every 45 minutes. Worms were scored as alive as in lifespan assays. At least 40 animals were used for each treatment. Statistical analysis was performed using the Log-ranked tests.

Quantification of bacterial ingestion using DsRed

For each experiment, 30 worms per condition were grown in the same conditions as described for the lifespan assays and were fed dilutions of OP50-1 carrying the plasmid

pFPV25 DsRedT3_S4T under the control of rpsM, a kind gift from Dr. Man-Wah Tan. After two hours, worms were placed on agarose pads on slides with Levamisole (25mM) to paralyze the worms and pictures were taken with a Hamamatsu Orca ER CCD camera. Quantification of the DsRed fluorescence in each worm was performed using ImageJ. The number of worms displaying DsRed in their pharynx and intestinal track was also scored.

DAF-16 localization assays

For each experiment, 30 TJ356 worms (expressing DAF-16:GFP) per condition were grown in the same conditions as described for the lifespan assays. Worms were placed on agarose pads after two, six or twelve hours with Levamisole (25mM) to paralyze the worms and pictures were taken as for DsRed visualization. Representative pictures from six hours of treatment are displayed.

Worm locomotor activity

Worms were treated as described in the lifespan assays and were either placed in *ad libitum* conditions ($5x10^{11}$ bacteria/ml) or in sDR conditions ($5x10^{8}$ bacteria/ml). Locomotor activity was measured at various days of life. For each assay, single worms were hand-picked and placed for one hour on fresh NGM plates with an *ad libitum* concentration of OP50, so that the amount of food was not a variable during the measuring of the worm movements. Pictures were taken of the plates and the tracks that the worms left on the plates were calculated using ImageJ.

Worm and human AMPK purification

$3.5x10^6$ 293T cells were seeded in 10 cm plates. After 18 hours, the cells were transfected using the calcium phosphate method with 6.66 μg of each of three plasmids encoding human or worm AMPK α, β, and γ. After two days, the cells were serum starved for 2 hours and then treated with 100 mM 2-deoxyglucose (Sigma Ultra) for 5 minutes. The cells were then lysed in lysis buffer (TrisHCl pH 7.5, 50 mM; NaCl, 100 mM; EDTA pH 8.0, 5mM; NaF, 50 mM; β-glycerophosphate, 40 mM; Triton-X100, 0.4%; sodium orthovanadate, 2 mM). Lysates were then incubated with agarose beads coupled to antibodies to the Flag epitope (Sigma) for 2.5 hours. The beads were then washed 3 times in lysis buffer and 1 time in TN (Tris pH 7.5, 50 mM and NaCl, 100 mM). The AMPK kinase complex was eluted from the beads by incubating the beads for 1.5 hours with 40 μl of 1 mg/ml Flag peptide.

Supplemental Table Legends

Table S1. sDR increases worm lifespan and stress resistance

(A) Mean lifespan of dietary restricted versus *ad libitum* fed worms. The mean lifespan values were calculated using a Logrank (Mantel-Cox) statistical test from triplicate samples of 30 worms each. (B) Mean survival time of sDR versus *ad libitum* fed worms. The mean stress resistance values at 200 mM Paraquat were calculated using a Logrank (Mantel-Cox) statistical test from sextuplicate samples of at least 5 worms each (at least 40 worms total). (C) Mean lifespan of WT worms (N2) in response to sDR with UV-irradiated bacteria. The mean lifespan values were calculated as in Table S1A. The

combined p values of the average in mean lifespan values were obtained using Fisher's method of combining independent tests of significance.

Table S2. AAK-2 is necessary for sDR induced longevity

Mean lifespan of *aak-2* mutant worms compared to WT worms (N2) fed Ad libitium or sDR. The mean lifespan and p values were calculated as described for Table S1C.

Table S3. DAF-16 but not DAF-2 are necessary for sDR induced longevity

(A) Mean lifespan of *daf-16* mutant worms compared to WT worms (N2) in response to CR. The mean lifespan and p values were calculated as described for Table S1C. (B) Mean lifespan of *daf-2* and *aak-2* mutant worms compared to WT worms (N2) in response to CR. The mean lifespan and p values were calculated as described for Table S1C.

Table S4. Constitutively Active AMPK increases stress resistance and lifespan in a DAF-16 dependent manner.

(A) Mean stress resistance in worms expressing a constitutively active AMPK compared to control worms. The mean stress resistance values at 300 mM Paraquat were calculated using a Logrank (Mantel-Cox) statistical test from sextuplicate samples of at least 5 worms each (at least 40 worms total). (B) Mean lifespan of transgenic worms expressing the constitutively active (CA) form of AMPK compared to worms expressing an empty vector (EV) in the absence or presence of *daf-16* RNAi. The mean lifespan and p values were calculated as described for Table S1C.

Table S1

A

Bacterial concentration (/ml)	
Mean +/- SD	
$5x10^{12}$	19.89 +/- 0.55
$5x10^{11}$	18.43 +/- 0.50
$5x10^{10}$	21.06 +/- 0.68
$5x10^{9}$	22.31 +/- 0.71
$5x10^{8}$	23.55 +/- 0.74
p values	
$5x10^{12}/5x10^{11}$	0.0510
$5x10^{12}/5x10^{10}$	0.0256
$5x10^{11}/5x10^{10}$	<0.0001
$5x10^{11}/5x10^{8}$	<0.0001
$5x10^{10}/5x10^{9}$	0.0612
$5x10^{10}/5x10^{8}$	0.0027
$5x10^{9}/5x10^{8}$	0.5649

B

Experiment #	1	2
Mean +/- SD		
AL	167.82 +/- 7.70	273.97 +/- 12.12
sDR	228.38 +/- 15.95	347.83 +/- 18.69
p values		
AL/sDR	<0.0001	0.0007

C

Experiment #	1	2	Average
Mean +/- SD			
N2 UV AL	21.78 +/- 0.55	21.79 +/- 0.58	21.79 +/- 0.01
N2 UV sDR	25.24 +/- 0.80	24.76 +/- 0.65	25.00 +/- 0.34
p values			
N2 UV AL/ N2 UV sDR	0.0004	0.0020	<0.001

Table S2

Experiment #	1	2	3	4	5	Average
Mean +/- SD						
N2 Ad Lib	16.55 +/- 0.28	18.42 +/- 0.51	19.04 +/- 0.57	16.61 +/- 0.27	16.94 +/- 0.32	17.51 +/- 1.14
N2 sDR	22.37 +/- 0.43	23.55 +/- 0.75	24.86 +/- 0.71	22.78 +/- 0.41	19.82 +/- 0.45	22.67 +/- 1.86
aak-2 Ad Lib	14.98 +/- 0.19	16.21 +/- 0.48	18.15 +/- 0.48	15.04 +/- 0.20	16.13 +/- 0.36	16.10 +/- 1.29
aak-2 sDR	15.11 +/- 0.20	18.13 +/- 0.49	18.36 +/- 0.52	15.13 +/- 0.21	16.38 +/- 0.37	16.62 +/- 1.57
p values						
N2 Ad Lib/*aak-2* Ad Lib	<0.0001	0.0030	0.0927	<0.0001	0.1102	<0.0001
N2 sDR/*aak-2* sDR	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
N2 sDR/N2 Ad Lib	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
aak-2 sDR/*aak-2* Ad Lib	0.5669	0.0114	0.6962	0.7063	0.7147	>0.25

Table S3

A

Experiment #	1	2	3	Average
Mean +/- SD				
N2 Ad Lib	19.04 +/- 0.57	21.65 +/- 0.38	15.08 +/- 0.29	18.59 +/- 3.31
N2 sDR	24.86 +/- 0.71	25.59 +/- 0.42	18.60 +/- 0.50	23.02 +/- 3.84
daf-16 Ad Lib	16.23 +/- 0.32	16.28 +/- 0.24	12.99 +/- 0.32	15.17 +/- 1.89
daf-16 sDR	16.70 +/- 0.32	16.99 +/- 0.35	13.45 +/- 0.39	15.71 +/- 1.97
p values				
N2 Ad Lib/*daf-16* Ad Lib	<0.0001	<0.0001	<0.0001	<0.0001
N2 sDR/*daf-16* sDR	<0.0001	<0.0001	<0.0001	<0.0001
N2 sDR/N2 Ad Lib	<0.0001	<0.0001	<0.0001	<0.0001
daf-16 sDR/*daf-16* Ad Lib	0.3591	0.0299	0.2808	>0.05

B

Experiment #	1	2	Average
Mean +/- SD			
N2 AL	16.55 +/- 0..28	16.61 +/- 0.28	16.58 +/- 0.04
N2 sDR	22.37 +/- 0.43	22.78 +/- 0.42	22.58 +/- 0.29
aak-2 AL	14.98 +/- .19	15.04 +/- 0.20	15.01 +/- 0.04
aak-2 sDR	15.11 +/- 0.20	15.13 +/- 0.21	15.12 +/- 0.01
daf-2 AL	29.53 +/- 0.83	31.57 +/- 0.83	30.55 +/- 1.44
daf-2 sDR	38.69 +/- 0.99	37.82 +/- 0.95	38.26 +/- 0.62
p values			
N2 AL/N2 sDR	<0.0001	<0.0001	<0.0001
aak-2 AL/*aak-2* sDR	0.5669	0.7063	>0.75
daf-2 AL/*daf-2* sDR	<0.0001	<0.0001	<0.0001
N2 AL/*aak-2* AL	<0.0001	<0.0001	<0.0001
N2 AL/*daf-2* AL	<0.0001	<0.0001	<0.0001
N2 sDR/*aak-2* sDR	<0.0001	<0.0001	<0.0001
N2 sD*R/daf-2* sDR	<0.0001	<0.0001	<0.0001

Table S4

A

Experiment #	1	2
Mean +/- SD		
EV1	145.16 +/- 13.15	120.00 +/- 11.88
CA3	270.00 +/- 15.95	225.00 +/- 16.70
EV1 ctl	127.12 +/- 10.43	126.00 +/- 15.17
CA3 ctl	189.23 +/- 13.55	221.79 +/- 19.71
EV1 daf-16	101.79 +/- 8.41	98.57 +/- 11.46
CA3 daf-16	118.13 +/- 10.05	115.31 +/- 12.97
p values		
EV1/CA3	<0.0001	<0.0001
EV1 ctl/CA3 ctl	0.0050	0.0013
EV1 ctl/EV1 *daf-16*	0.0610	0.1326
CA3 ctl/CA3 *daf-16*	<0.0001	0.0002
CA3 *daf-16*/EV1 *daf-16*	0.2461	0.3916

B

Experiment #	1	2	3	Average
Mean +/- SD				
CA2 ctl			17.75 +/- 0.74	
CA3 ctl	19.02 +/- 0.45	16.91 +/- 0.41	19.07 +/- 0.72	18.33 +/- 1.23
EV1 ctl	17.13 +/- 0.36	16.02 +/- 0.36	16.21 +/- 0.58	16.45 +/- 0.60
CA3 *daf-16*	14.92 +/- 0.35	11.47 +/- 0.22		13.20 +/- 2.44
EV1 *daf-16*	14.98 +/- 0.31	11.83 +/- 0.23		13.41 +/- 2.23
p values				
EV1 ctl/CA2 ctl			0.0384	
EV1 ctl/CA3 ctl	0.0006	0.0867	0.0006	<0.005
EV1 ctl/EV1 *daf-16*	<0.0001	<0.0001		<0.0001
CA3 ctl/CA3 *daf-16*	<0.0001	<0.0001		<0.0001
CA3 *daf-16*/EV1 *daf-16*	0.8557	0.2478		>0.5