Supporting Information

Wood et al. 10.1073/pnas.1720673115

SI Materials and Methods

Microscopy and Subcellular Fractionation. The coding DNA sequence of *Drosophila Sirt4* (CG3187-RC) was inserted into either pAWF or pAWG (Drosophila Genomics Resource Center) to generate dSirt4-EGFP (pAWG-dSirt4) and dSirt4-3xFlag (pAWF-dSirt4) expression vectors, and subsequently transfected into S2 cells.

S2 cells were grown in Schneider's *Drosophila* medium supplemented with 10% (vol/vol) FBS and transfected by calcium phosphate precipitation as per standard protocols (1). Transiently transfected S2 cells were plated on acid-washed coverslips treated with Con A (2) and fixed by incubation in 3.7% formaldehyde in complete Schneider's *Drosophila* medium supplemented with 10% (vol/vol) FBS for 30 min at 28 °C. Cells were washed twice in PBS, quenched in 50 mM NH_4Cl in PBS for 5 min, and permeabilized with 0.5% (wt/vol) Triton X-100/PBS for 5 min at room temperature. Cells were washed twice in PBS and incubated with mouse monoclonal M2 anti-Flag (Sigma F-1804; 1:500) and rabbit polyclonal MnSOD (Stressgen Biochemicals SOD-110; 1:250) antibodies in PBS for 1 h at room temperature, followed by washing in PBS and incubation with anti–mouse-Cy2 and anti-rabbit Cy5 antibodies (Jackson ImmunoResearch) in PBS for 1 h at room temperature. Where indicated, mitochondria were labeled by incubation of S2 cells with 200 nM MitoTracker Red CMXRos (Molecular Probes) for 30 min before fixation.

For subcellular fractionation, 4×10^7 S2 cells were homogenized in four packed cell volumes of hypotonic buffer [50 mM Hepes (pH 7.5), 50 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF] using a Dounce homogenizer. Homogenates were adjusted to 0.25 M sucrose and centrifuged at $960 \times g$ for 5 min at 4 °C to sediment nuclei and unbroken cells. The supernatant was subsequently centrifuged at $10{,}000 \times g$ for 10 min at 4 °C to prepare a heavy membrane fraction enriched in mitochondria. The supernatant was then centrifuged at $100{,}000 \times g$ for 60 min at 4 °C to sediment light membranes, with the remaining supernatant representing cytosol. The following antibodies were used for immunoblotting: α-tubulin (Sigma T-5168), Hsp90α (Stressgen Biochemicals SPS-771), Flag (Sigma F-1804), and MnSOD (Stressgen Biochemicals SOD-110).

Bioinformatics. To assess potential mitochondrial targeting sequences, amino acid sequences from the five *Drosophila* sirtuins were analyzed using MitoFates with standard parameters (3). To generate the phylogenetic tree in Fig. S1*B*, the five *Drosophila* and seven mouse sirtuin amino acid sequences were input at www.phylogeny.fr using the "one-click" mode (4). This pipeline runs the following programs in succession: MUSCLE, GBlocks, PhyML, and TreeDyn.

Quantitative PCR. Total RNA from fat bodies dissected from fed and fasted female flies ($n = 25$) was reverse-transcribed, and the resulting cDNA was used as input for measuring *dSirt4* transcript levels by qPCR. The delta delta cycle threshold method was used to quantify *dSirt4* expression, with β-tubulin as an endogenous control. Primer sequences were as follows: β-tubulin–forward (F), ACATCCCGCCCCGTGGTC; β-tubulin–reverse (R), AGAAAGCCTTGCGCCTGAACATAG; Sirt4-F, CCGAAATGTTGTGGAGGTTC; and Sirt4-R, ATTTAGCGACGCCAGTATGC.

Mitochondrial Respiration Assays. Mitochondrial preparations were made from 25 live female flies by gentle homogenization in mitochondrial homogenization buffer [225 mM mannitol, 75 mM sucrose, 10 mM 3-(N-morpholino)propanesulfonic acid, 1 mM EGTA, 0.5% BSA (pH 7.2)]. Samples were centrifuged at $300 \times g$ for 5 min at 4 °C to pellet nuclei and debris. Supernatant was then centrifuged at $6{,}000 \times g$ to pellet mitochondria, which were resuspended in 100 μL of mitochondrial respiration buffer [225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris·HCl, 5 mM KH_2PO_4, 0.1% BSA (pH 7.2)]. Respiration was assayed using a Clark electrode in a 1-mL reaction volume, with 5 μL of 1 M pyruvate and 5 μL of 1 M malate as substrates. Respiratory control ratios were calculated as state III (active)/state IV (ADP-depleted) rates. Three biological replicates were performed for each time point.

ATP Assays. ATP assays were performed as described by Tennessen et al. (5). Either five whole animals, or 10 eviscerated female abdomens (containing primarily fat body) were used as inputs for the assay, with five biological replicates for each sample.

1. Schetz JA, Shankar EPN (2004) Protein expression in the Drosophila Schneider 2 cell system. *Curr Protoc Neurosci* Chapter 4:Unit 4.16.
2. Rogers SL, Rogers GC, Sharp DJ, Vale RD (2002) Drosophila EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. *J Cell Biol* 158:873–884.
3. Fukasawa Y, et al. (2015) MitoFates: Improved prediction of mitochondrial targeting sequences and their cleavage sites. *Mol Cell Proteomics* 14:1113–1126.
4. Dereeper A, et al. (2008) Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 36:W465–W469.
5. Tennessen JM, Barry WE, Cox J, Thummel CS (2014) Methods for studying metabolism in Drosophila. *Methods* 68:105–115.

A

Sequence ID	Probability of presequence	Putative cleavage site (processing enzyme)	Net charge
dSirt4	0.798	9 (MPP)	0.222
dSirt2	0.011		0.02
dSirt1	0		-0.2
dSirt6	0		-0.077
dSirt7	0		-0.2

B

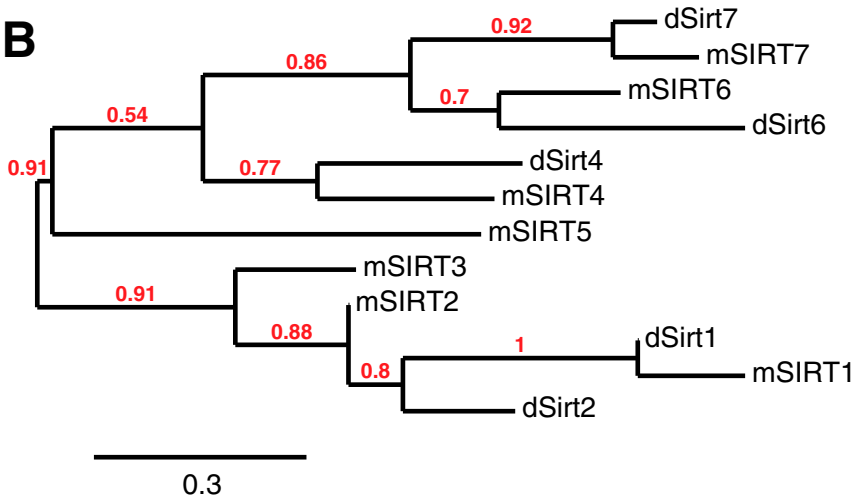

Fig. S1. (*A*) dSirt4 is predicted to be localized to mitochondria. The five *Drosophila* sirtuin orthologs were processed with MitoFates to analyze potential N-terminal mitochondrial targeting sequences and predict mitochondrial localization. Only dSirt4 has a high probability (green) of mitochondrial localization. In addition to probability, the potential cleavage site [mitochondrial processing peptidase (MPP)] and net charge (mitochondrial targeting sequences are positively charged) are shown. (*B*) Phylogenetic relationship between sirtuins of mouse (mSIRT1–mSIRT7) and *Drosophila* (dSirt1, dSirt2, dSirt4, dSirt6, and dSirt7). The tree was generated using the phylogeny.fr suite of tools. The scale bar represents substitutions per site, and branch support values (from PhyML) are shown in red.

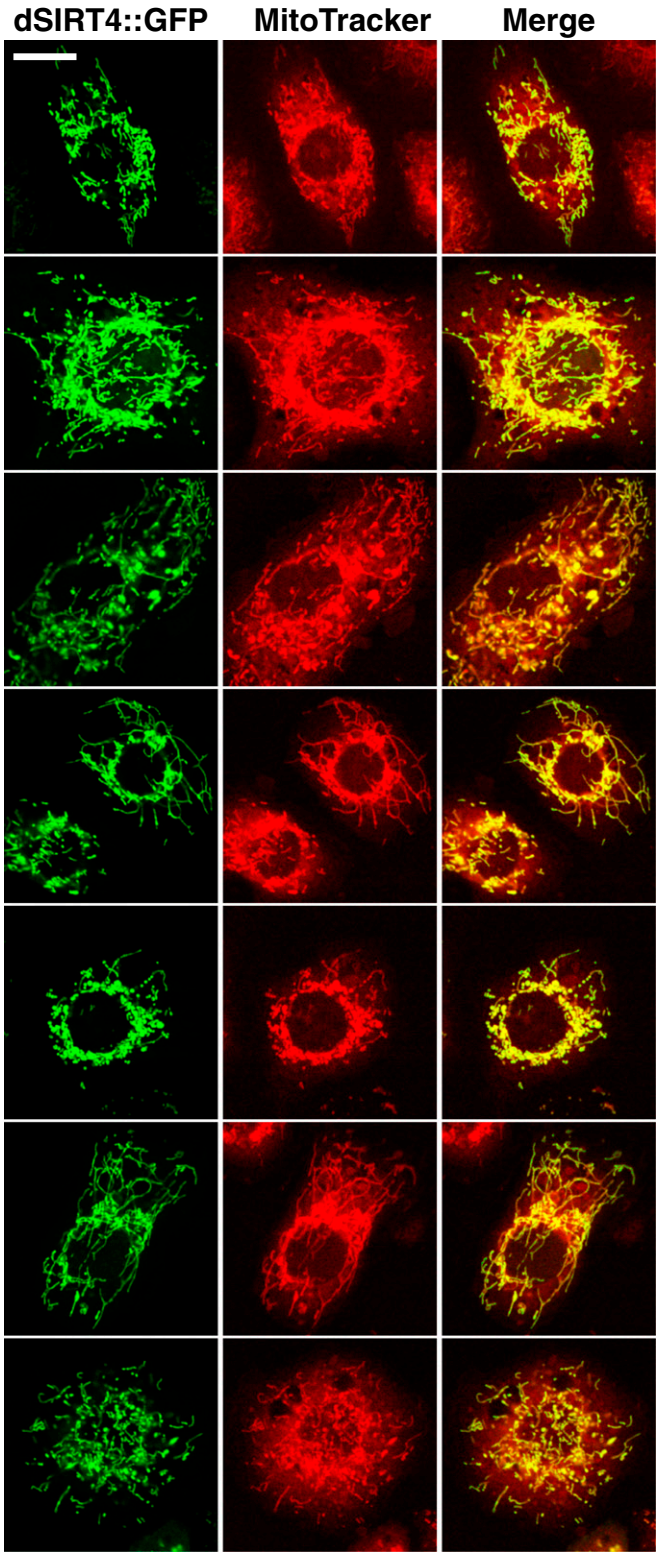

Fig. S2. Additional images showing dSirt4::GFP localizes to mitochondria in S2 cells. (Scale bar, 5 μm.)

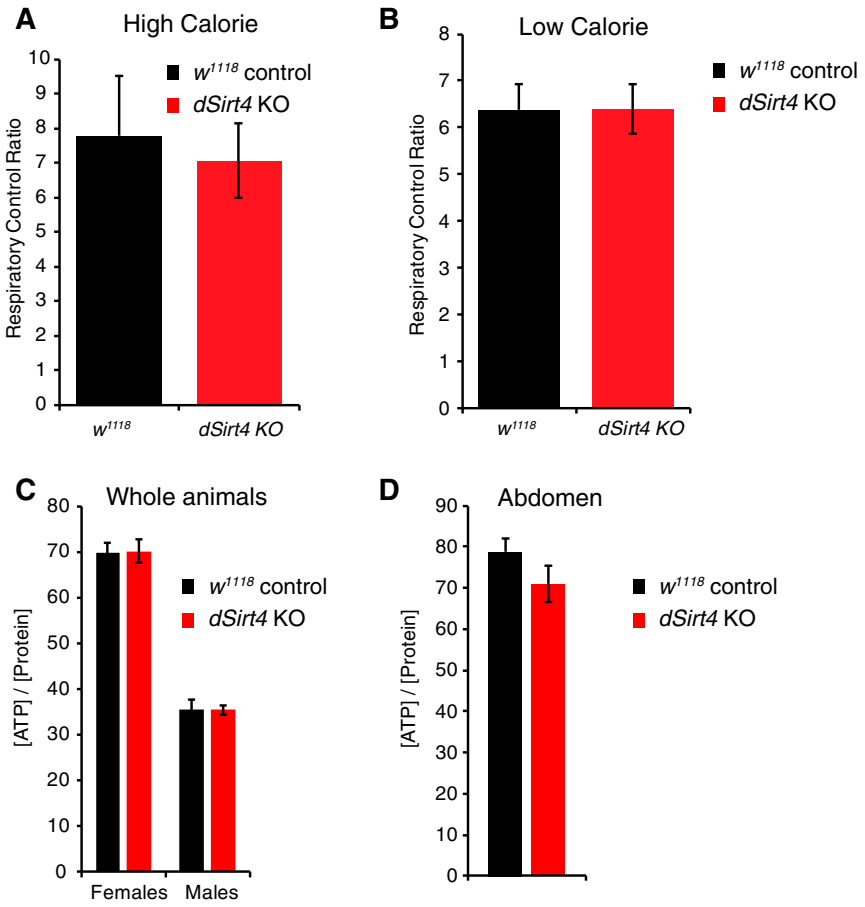

Fig. S3. Mitochondria of *dSirt4* knockout (KO) flies are not grossly impaired. Respiratory control ratios (state III/state IV) of live mitochondria extracted from female flies maintained on high-calorie (*A*, 15% SY) or low-calorie (*B*, 5% SY) food are shown. Data represent averages of three independent biological replicates; differences between samples are not significant. (*C*) ATP levels are normal in *dSirt4* KO flies. ATP levels in whole flies (micromolar) normalized to total protein content (milligrams per milliliter) are shown for both female and male w^{1118} control (black) and *dSirt4* KO (red) flies. (*D*) ATP levels measured as in *C* from eviscerated abdomens of female flies. Eviscerated abdomens contain primarily fat body tissue. Data in *C* and *D* represent the average of five independent biological replicates.

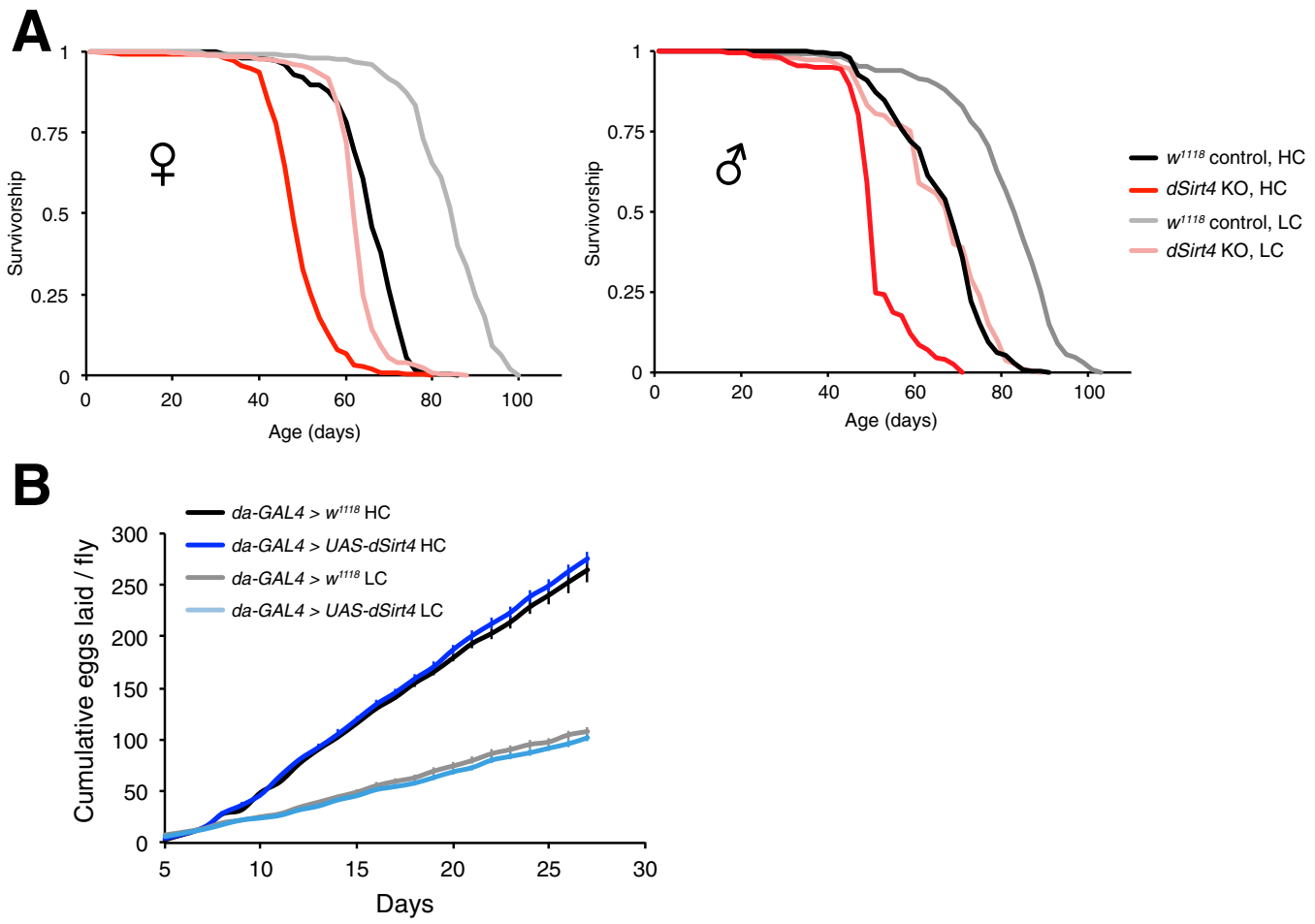

Fig. S4. (*A*) *dSirt4* knockout (KO) flies maintain a normal response to dietary restriction. Survivorship of female (*Left*) and male (*Right*) flies is shown on both high-calorie (HC, 15% SY food) and low-calorie (LC, 5% SY food) diets for both w^{1118} control and *dSirt4* KO flies. (*Left*) For females, w^{1118} control flies show a 30% lifespan extension on a LC vs. HC diet, and *dSirt4* KO flies show a 29% extension. (*Right*) For males, w^{1118} control flies show a 20% lifespan extension on a LC vs. HC diet, and *dSirt4* KO flies show a 35% extension. Log-rank $P < 10^{-10}$ for all lifespans shown. Full lifespan statistics, including *n* (number of individuals assayed), median, mean, and maximum lifespans, are presented in Table S1. (*B*) Overexpression of *dSirt4* does not change fertility significantly. Flies overexpressing *dSirt4* ubiquitously (*da-GAL4* > *UAS-dSirt4*) have similar or slightly elevated fertility relative to matched controls (*da-GAL4* > w^{1118}). Cumulative eggs laid are shown for both genotypes on high- and low-calorie diets. A total of 10 vials, five flies per vial, were analyzed for each condition. Error bars represent SEM.

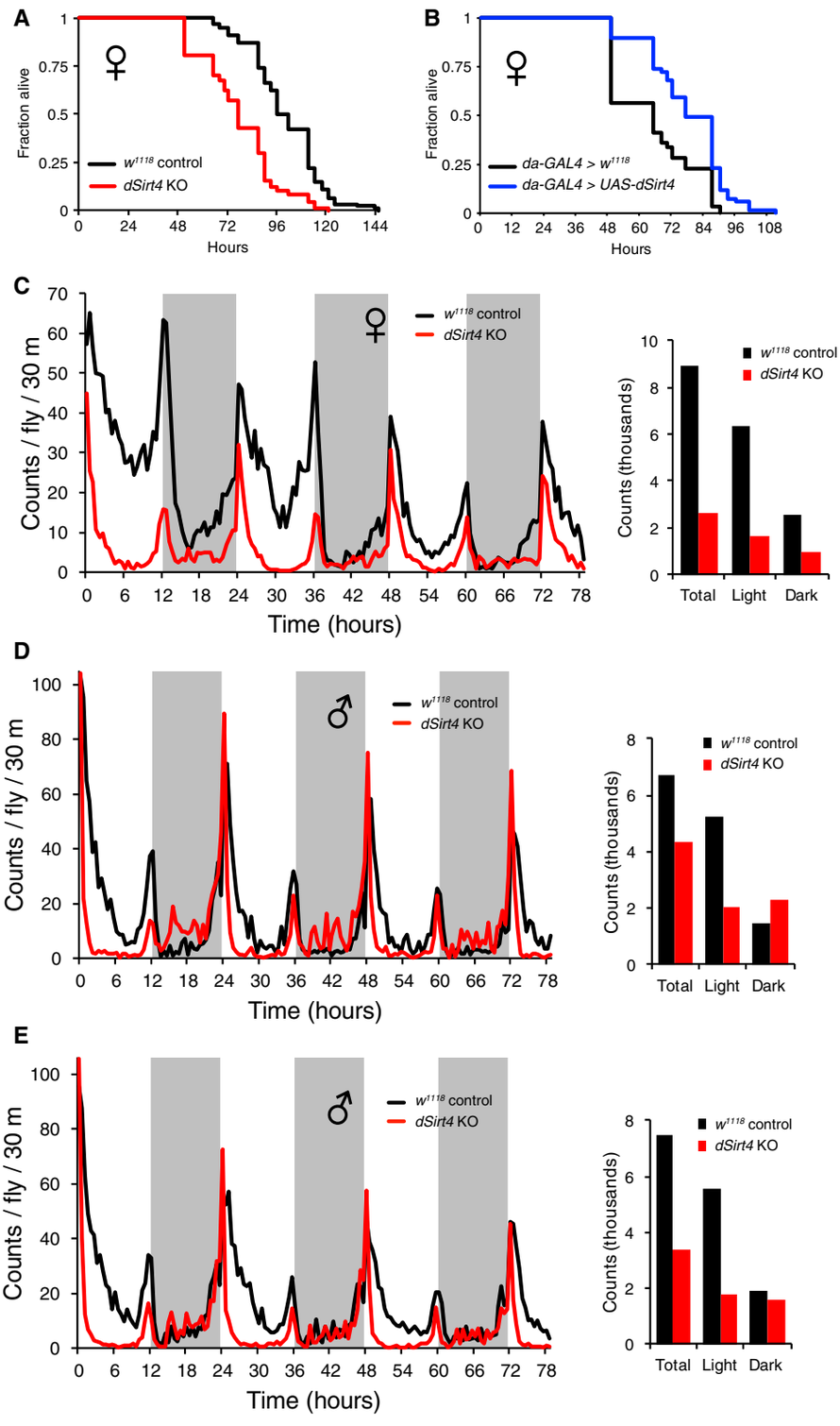

Fig. S5. Additional starvation and activity assays. (*A*) *dSirt4* knockout (KO) flies are starvation-sensitive relative to genetically matched controls. Survivorship of w^{1118} control (median survival = 96 h, mean = 100 h, $n = 100$) and *dSirt4* KO (median survival = 77 h, mean = 77.4 h, $n = 98$) female flies are shown. Log-rank $P < 10^{-10}$. (*B*) *dSirt4* transgenic flies (*da-GAL4* > *UAS-dSirt4*) are starvation-resistant relative to controls (*da-GAL4* > w^{1118}). Survivorship of *da-GAL4* > w^{1118} control (median survival = 65 h, mean = 64.5 h, $n = 92$) and *da-GAL4* > *UAS-dSirt4* transgenic (median survival = 77 h, mean = 78 h, $n = 69$) female flies are shown. Log-rank $P < 10^{-6}$. (*C*–*E*) *dSirt4* KO flies have decreased spontaneous activity compared with controls. Activity profiles are shown for female flies on low-calorie (5% SY) food (*C*), males on high-calorie (15% SY) food (*D*), and males on low-calorie (5% SY) food (*E*). Total integrated counts shown in the activity plot, as well as light period and dark period counts, are displayed in bar graphs. Gray bars indicate dark periods (12-h cycle). Decreased activity is especially apparent during daylight hours and at natural peaks around dusk/dawn transitions. Data represent the average of three replicate vials, with 20 flies per vial, and are presented as the number of counts per 30-min bin, normalized for the number of flies.

Table S1. Statistics for all lifespan experiments described in the current study

Genotype	Replicate	Sex	Food	*n*	Median lifespan	Mean lifespan	Maximum lifespan	Log-rank *P* value	Change in median lifespan vs. control, %	Change in maximal lifespan vs. control, %
w^{1118} control	1	F	5% SY	244	86	83.93	97.5			
dSirt4 KO	1	F	5% SY	246	62	62.44	74	1.00E-10	−27.9	−24.1
w^{1118} control	1	F	15% SY	243	66	65.23	75.8			
dSirt4 KO	1	F	15% SY	251	48	49.16	66.5	1.00E-10	−27.3	−12.3
w^{1118} control	2	F	5% SY	233	89	87.04	99.5			
dSirt4 KO	2	F	5% SY	242	53	58.02	75.8	1.00E-10	−40.4	−23.8
w^{1118} control	2	F	15% SY	226	71	69.5	78			
dSirt4 KO	2	F	15% SY	242	51	52.3	66.7	1.00E-10	−28.2	−14.5
da-GAL4> w^{1118} control	1	F	5% SY	238	65	60.1	81.8			
da-GAL4 > UAS-dSirt4	1	F	5% SY	240	78	72.55	89.6	1.00E-10	20.0	9.5
da-GAL4 > w^{1118} control	2	F	5% SY	243	57	55.53	71.2			
da-GAL4 > UAS-dSirt4	2	F	5% SY	222	67	66.62	98	1.00E-10	17.5	37.6
tub-GAL4 > w^{1118} control	1	F	5% SY	236	63	62.82	85.2			
tub-GAL4 > UAS-dSirt4	1	F	5% SY	224	71	71.59	93.9	1.00E-10	12.7	10.2
tub-GAL4 > w^{1118} control	2	F	5% SY	240	69	67.83	88.3			
tub-GAL4 > UAS-dSirt4	2	F	5% SY	227	79	73.96	97.9	1.00E-10	14.5	10.9
ppl-GAL4 > w^{1118} control	1	F	15% SY	245	57	53.6	76.4			
ppl-GAL4 > UAS-dSIrt4	1	F	15% SY	246	65	65.8	87.2	1.00E-10	14.0	14.1
ppl-GAL4 > w^{1118} control	2	F	15% SY	72	64	62.61	84.9			
ppl-GAL4 > UAS-dSIrt4	2	F	15% SY	75	72	66.64	86.8	0.0493	12.5	2.2
ppl-GAL4 > w^{1118} control	3	F	5% SY	234	74	70.52	91.3			
ppl-GAL4 > UAS-dSIrt4	3	F	5% SY	238	86	77.25	93.6	3.90E-09	16.2	2.5
w^{1118} control	1	M	5% SY	233	83	81.13	97.7			
dSirt4 KO	1	M	5% SY	185	69	65.32	82.6	1.00E-10	−16.9	−15.5
w^{1118} control	1	M	15% SY	243	69	66.31	82.2			
dSirt4 KO	1	M	15% SY	198	51	51.23	66.3	1.00E-10	−26.1	−19.3
w^{1118} control	2	M	5% SY	243	65	61.23	84.4			
dSirt4 KO	2	M	5% SY	241	52	49.27	72.6	1.00E-10	−20.0	−14.0
w^{1118} control	2	M	15% SY	245	52	45.25	71.9			
dSirt4 KO	2	M	15% SY	245	31	36.58	65.4	1.00E-10	−40.4	−9.0
da-GAL4 > w^{1118} control	1	M	5% SY	244	61	61.18	82.6			
da-GAL4 > UAS-dSirt4	1	M	5% SY	235	73	71.68	95.6	1.00E-10	19.7	15.7
da-GAL4 > w^{1118} control	2	M	5% SY	245	57	57.82	79.1			
da-GAL4 > UAS-dSirt4	2	M	5% SY	171	61	62.54	89.8	0.000009	7.0	13.5
tub-GAL4 > w^{1118} control	1	M	5% SY	215	71	70.58	93.3			
tub-GAL4 > UAS-dSirt4	1	M	5% SY	219	73	72.91	92.6	0.3387	2.8	−0.8
tub-GAL4 > w^{1118} control	2	M	5% SY	226	81	77.56	94.2			
tub-GAL4 > UAS-dSirt4	2	M	5% SY	233	75	73.41	89.3	0.0000014	−7.4	−5.2
ppl-GAL4 > w^{1118} control	1	M	15% SY	238	49	49.42	64.3			
ppl-GAL4 > UAS-dSirt4	1	M	15% SY	248	59	56.43	70.6	1.00E-10	20.4	9.8
ppl-GAL4 > w^{1118} control	2	M	15% SY	93	62	61.1	77.3			
ppl-GAL4 > UAS-dSirt4	2	M	15% SY	92	76	71.98	84.9	1.00E-10	22.6	9.8
ppl-GAL4 > w^{1118} control	3	M	5% SY	234	64	60.79	77.2			
ppl-GAL4 > UAS-dSirt4	3	M	5% SY	240	70	66.76	82.2	1.00E-10	9.4	−27.9

For each experiment, *n* values (number of individuals assayed), mean lifespan, median lifespan, and maximal lifespan, calculated as the mean lifespan of the latest surviving 10% of the cohort, as well as the *P* value for the log-rank statistical test, are reported. Percent change in median lifespan compared with controls is also presented. Each lifespan experiment was repeated at least twice with similar results, and representative experiments are shown in the figures, with the presented lifespans labeled as replicate 1 in this table. F, female; M, male.

Table S2. *P* values for all pairwise comparisons presented in Fig. 5 (unpaired two-tailed *t* test)

Metabolite	Control fed vs. control fasted	Control fed vs. KO fed	Control fed vs. KO fasted	Control fasted vs. KO fed	Control fasted vs. KO fasted	KO fed vs. KO fasted
Lactic acid	0.402	0.051	0.032	0.954	0.586	0.173
Pyruvic acid	0.471	0.057	0.128	0.031	0.062	0.187
DHAP	0.294	0.002	0.691	9.52E-04	0.351	2.88E-05
Phosphoenolpyruvate	0.701	0.014	0.877	0.013	0.751	0.008
Ribose	0.848	0.015	0.002	0.008	8.02E-05	0.583
D-ribose-5-phosphate	0.029	3.22E-05	0.051	6.87E-06	9.97E-04	1.39E-04
Glucose-1-phosphate	0.029	2.18E-06	1.20E-05	1.84E-07	3.21E-04	2.64E-09
Glucose-6-phosphate	0.068	1.52E-04	7.37E-06	1.41E-05	1.77E-04	3.11E-08
Valine	9.96E-04	0.003	5.68E-05	0.117	3.47E-04	0.004
Leucine	0.002	0.009	1.86E-05	0.103	4.01E-04	0.201
Isoleucine	1.52E-04	0.003	1.01E-06	0.180	6.78E-05	0.049
Citric acid	0.002	0.749	0.308	0.002	0.003	0.212
Isocitric acid	0.225	0.055	0.152	0.840	0.823	0.557
Succinic acid	0.080	0.002	0.022	0.175	0.251	0.844
Fumaric acid	0.143	0.259	0.001	0.859	4.90E-04	0.079
Malic acid	0.633	0.654	0.009	0.875	9.78E-04	0.089
Lauric acid	0.002	0.042	1.32E-06	3.48E-04	0.154	3.26E-06
Myristic acid	0.183	0.103	0.016	0.050	0.779	0.036
Palmitic acid	0.203	0.178	0.010	0.669	0.342	0.838
Stearic acid	0.276	0.951	0.540	0.309	0.578	0.542
Linoleic acid	0.154	0.115	9.40E-07	0.312	1.23E-05	0.018
Oleic acid	0.425	0.092	2.41E-04	0.137	1.92E-04	0.132
Laurate C12:0	0.007	0.039	1.47E-06	0.727	0.006	0.010
Myristate C14:0	0.177	0.058	0.013	0.649	0.487	0.823
Myristoleate C14:1	3.22E-04	0.109	2.20E-05	0.006	2.48E-04	1.03E-04
Pentadecanoate C15:0	0.302	0.504	0.335	0.584	0.770	0.708
Palmitate C16:0	0.445	0.152	0.045	0.317	0.161	0.986
Palmitoleate C16:1n-7	0.058	0.097	0.036	0.888	0.369	0.484
Stearate C18:0	0.144	0.737	4.13E-04	0.110	0.018	4.30E-04
Oleate C18:1n-9	0.953	0.232	0.159	0.322	0.192	0.040
Linolelaidate C18:2n-6	0.944	0.605	0.017	0.675	0.024	0.021
Linoleate C18:2n-6	0.950	0.607	0.017	0.674	0.024	0.021
Arachidate C20:0	0.471	0.312	0.069	0.808	0.046	0.028
Behenate C22:0	0.289	0.281	7.06E-04	0.774	0.020	0.002
Docosadienoate C22:2n-6	0.727	0.890	0.008	0.695	0.016	0.029
Docosahexaenoate C22:6n-3	0.161	0.019	2.19E-04	0.876	0.007	0.002
Lignocerate C24:0	0.443	0.598	0.017	0.689	0.102	0.016

Nonsignificant *P* values above 0.05 are displayed in black, $0.05 > P > 0.01$ is displayed in red, $0.01 > P > 0.001$ is displayed in blue, and $P < 0.001$ is displayed in green. KO, knockout.