Cell Metabolism, Volume *16*

Supplemental Information

Intramyocellular Fatty-Acid Metabolism Plays a Critical Role in Mediating Responses to Dietary Restriction in *Drosophila melanogaster*

Subhash D. Katewa, Fabio Demontis, Marysia Kolipinski, Allan Hubbard, Matthew S. Gill, Norbert Perrimon, Simon Melov, and Pankaj Kapahi

Figure S1

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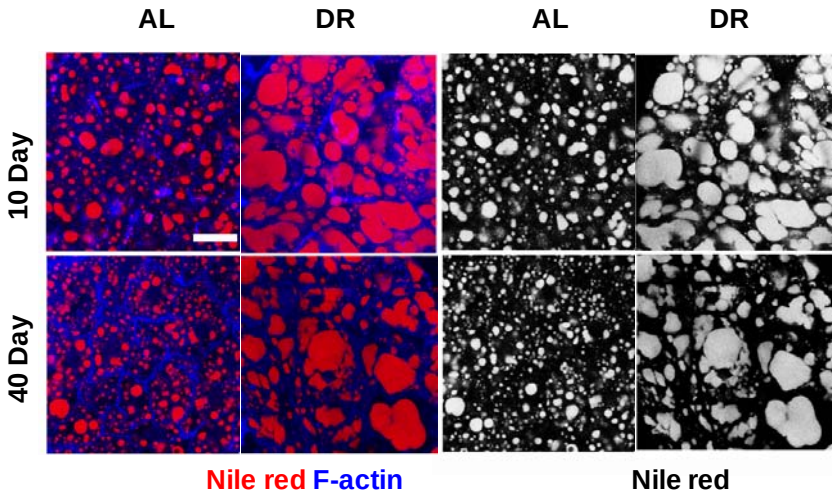
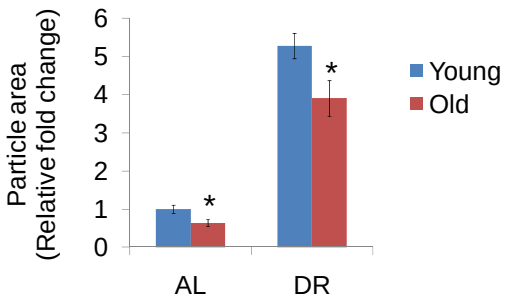


(ii)



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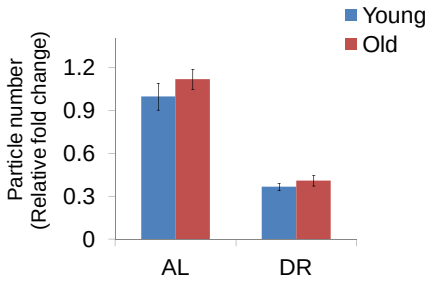

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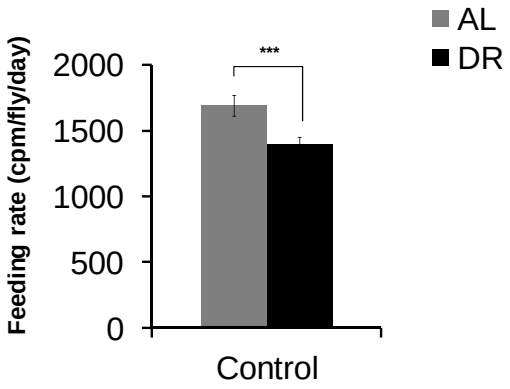


Figure S2

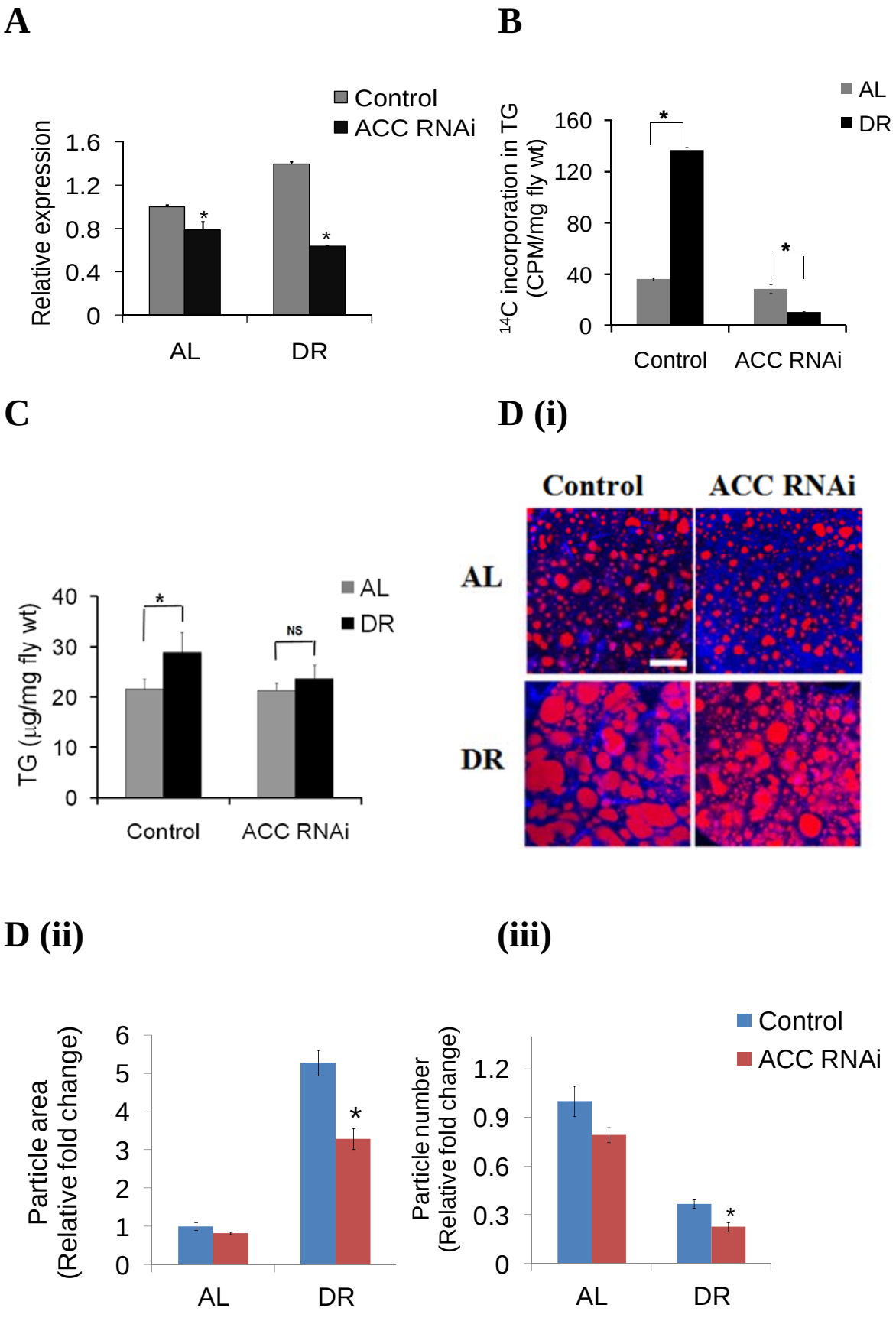
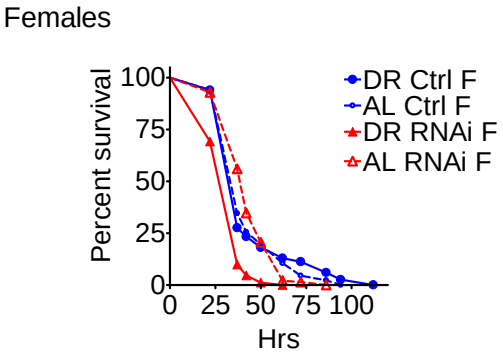
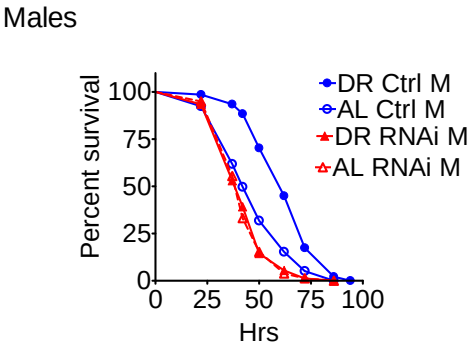

A
Control
ACC RNAi
Relative expression
1.6
1.2
0.8
0.4
0
AL
DR
B
AL
DR
^{14}C incorporation in TG (CPM/mg fly wt)
160
120
80
40
0
Control
ACC RNAi
C
AL
DR
TG (µg/mg fly wt)
40
30
20
10
0
NS
Control
ACC RNAi
D (i)
Control
ACC RNAi
AL
DR
D (ii)
Particle area (Relative fold change)
6
5
4
3
2
1
0
AL
DR
(iii)
Control
ACC RNAi
Particle number (Relative fold change)
1.2
0.9
0.6
0.3
0
AL
DR

Figure S2 (continued)

E (i)





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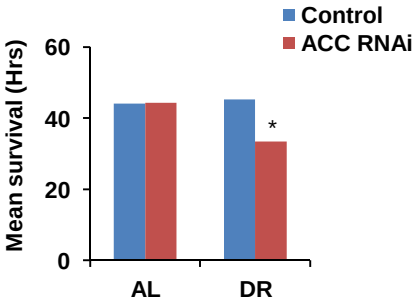

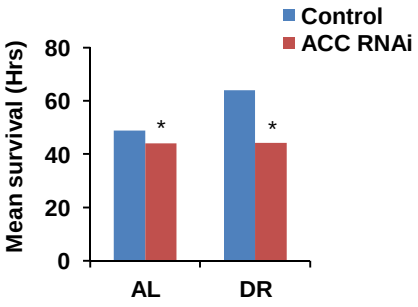

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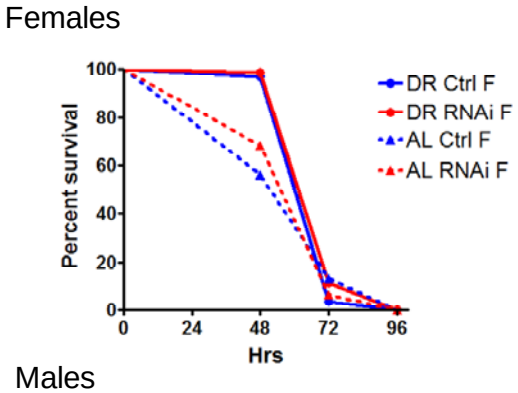

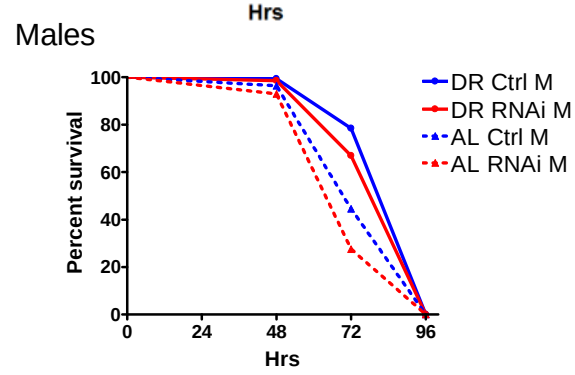

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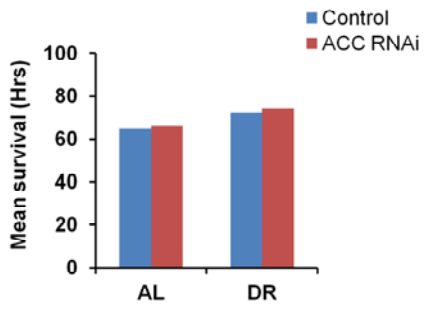

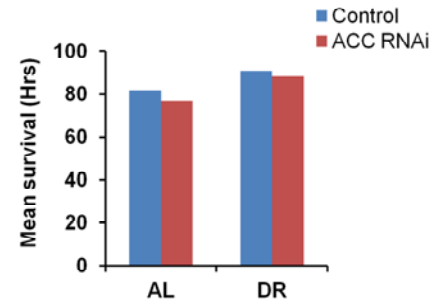

Figure S2 (continued)

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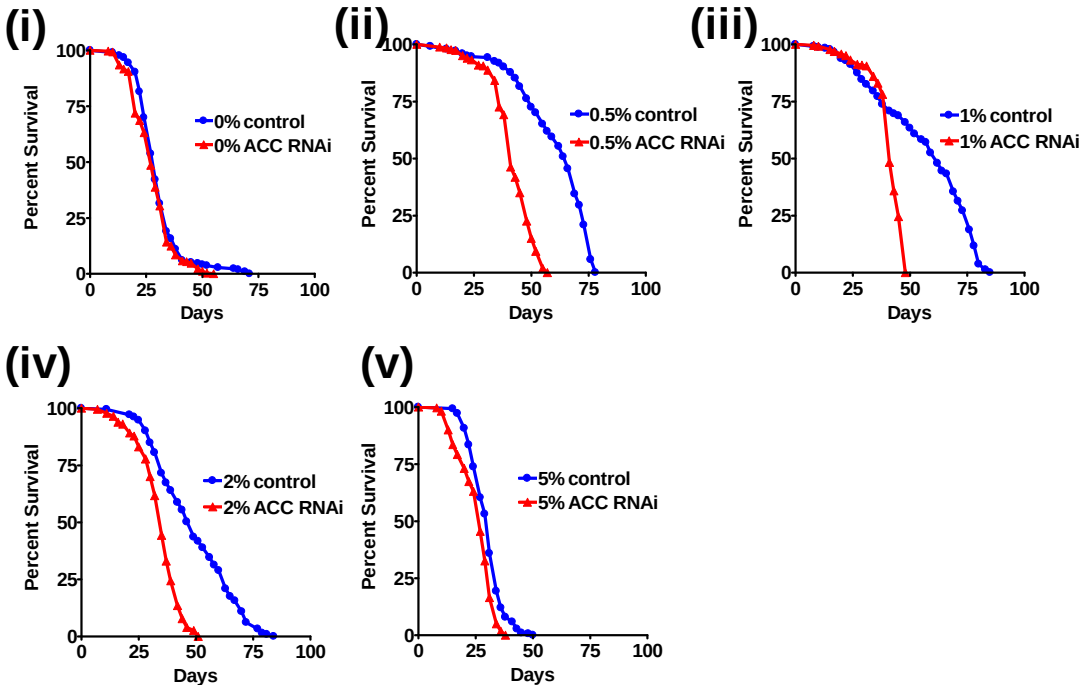

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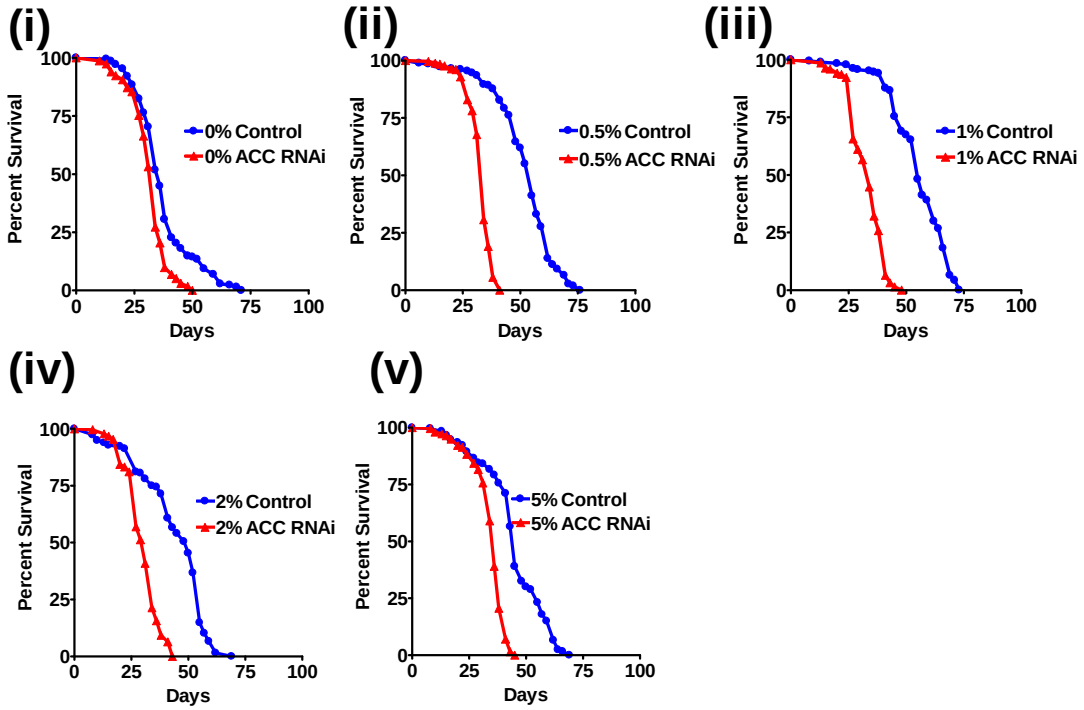

Figure S2 (continued)

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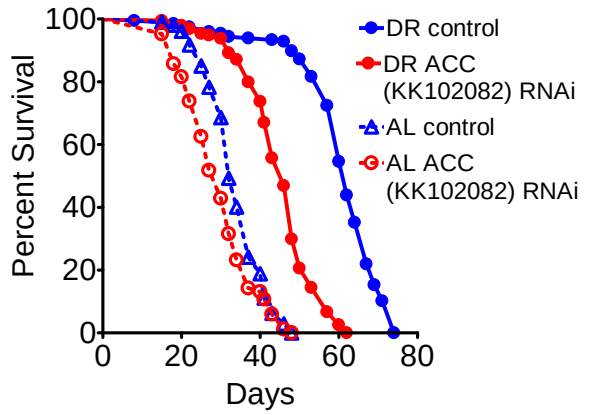
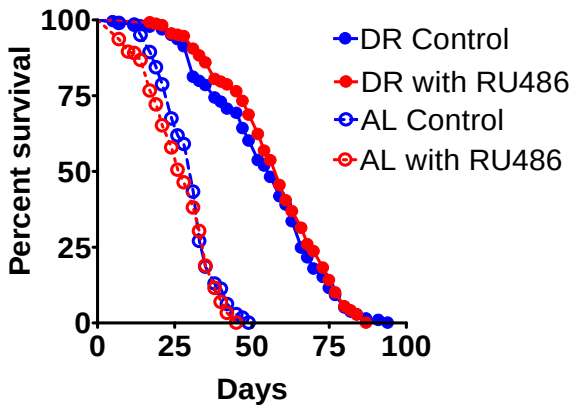

Percent Survival
100
80
60
40
20
0
0
20
40
60
80
Days
DR control
DR ACC (KK102082) RNAi
AL control
AL ACC (KK102082) RNAi

J


Percent survival
100
75
50
25
0
0
25
50
75
100
Days
DR Control
DR with RU486
AL Control
AL with RU486

K

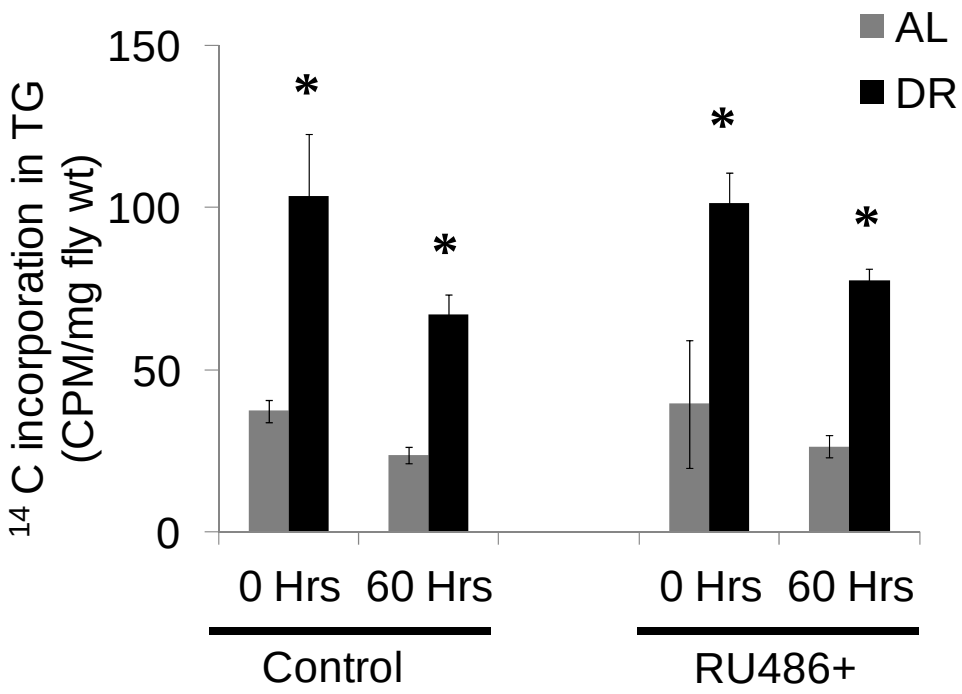
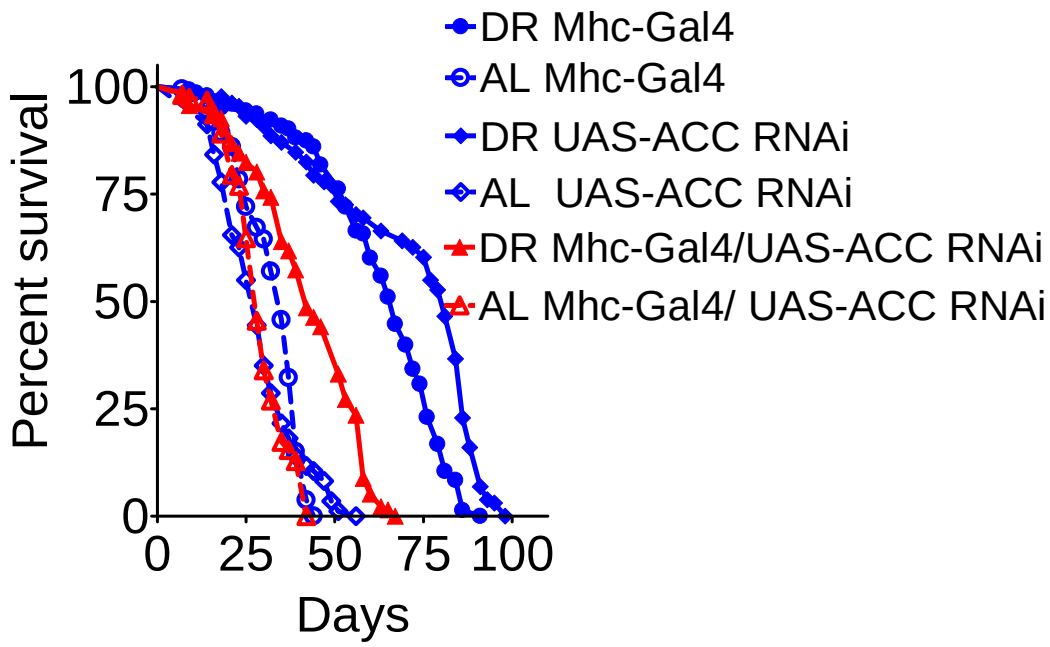

AL
DR
^{14}C incorporation in TG (CPM/mg fly wt)
150
100
50
0
*
*
*
*
0 Hrs
60 Hrs
0 Hrs
60 Hrs
Control
RU486+

Figure S2 (continued)

L



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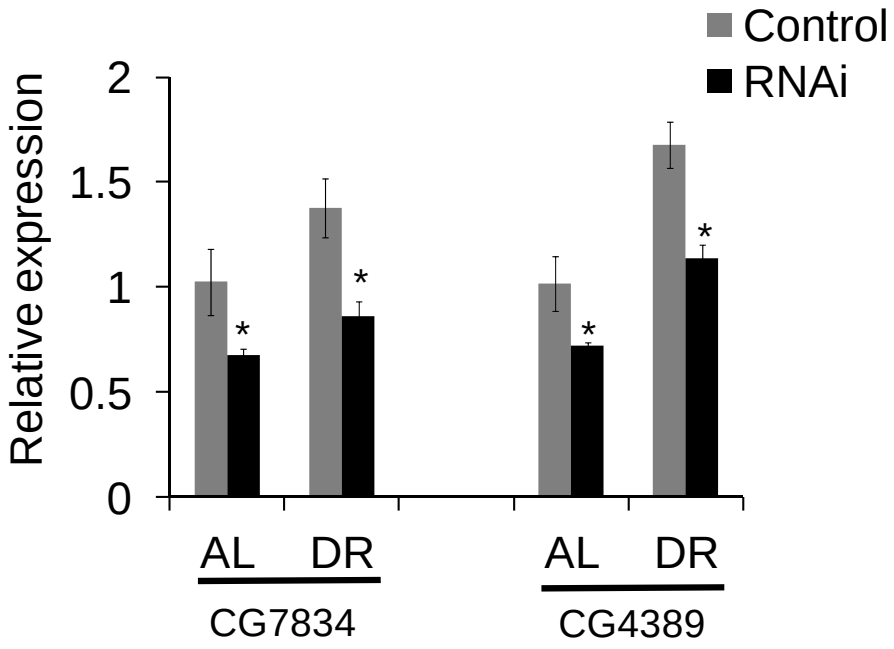


Figure S3

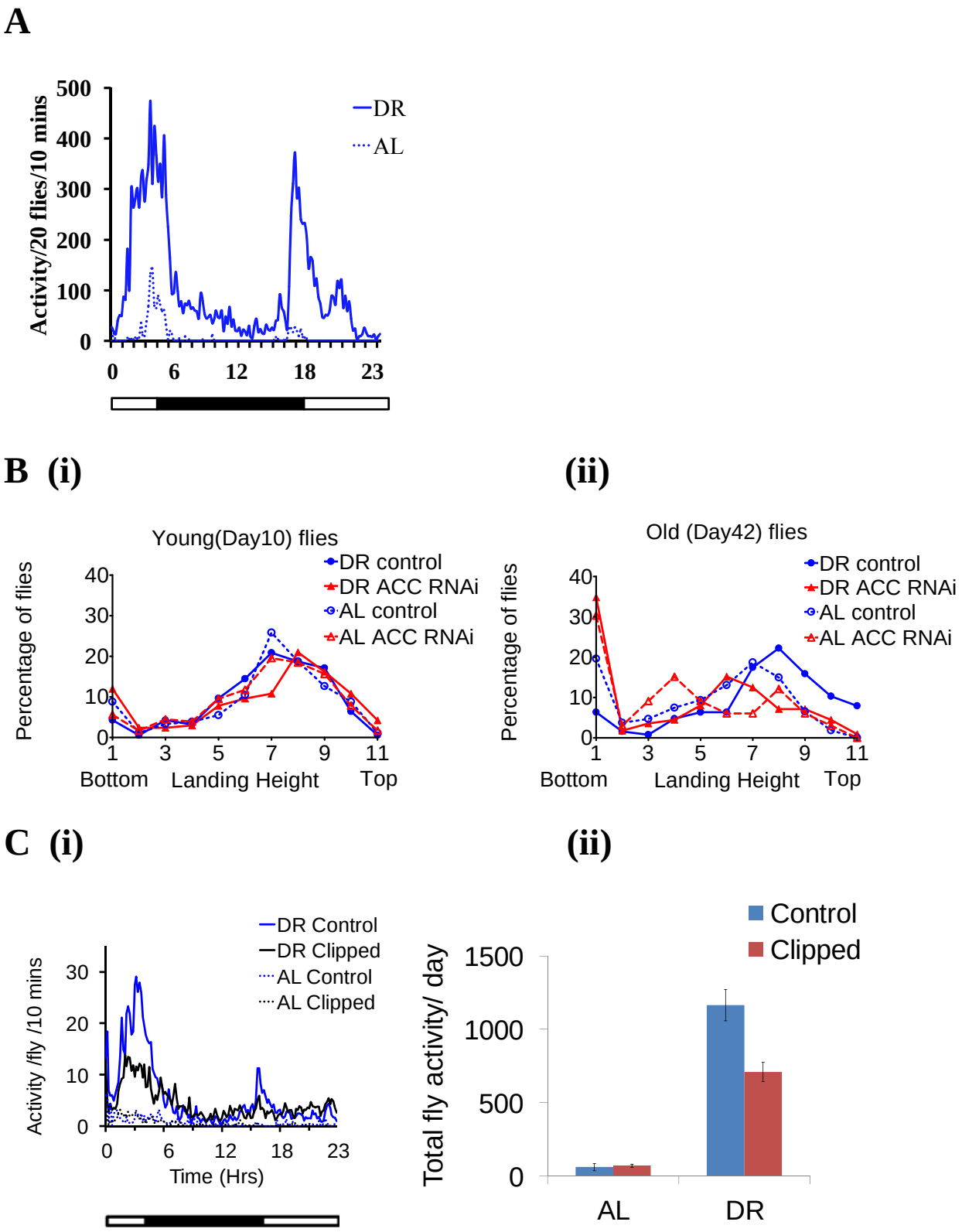

A
Activity/20 flies/10 mins
500
400
300
200
100
0
0
6
12
18
23
DR
AL
B (i)
(ii)
Young(Day10) flies
Old (Day42) flies
Percentage of flies
DR control
DR ACC RNAi
AL control
AL ACC RNAi
Bottom
Landing Height
Top
C (i)
(ii)
Activity /fly /10 mins
Time (Hrs)
DR Control
DR Clipped
AL Control
AL Clipped
Total fly activity/ day
Control
Clipped
AL
DR

Figure S3 (continued)

D

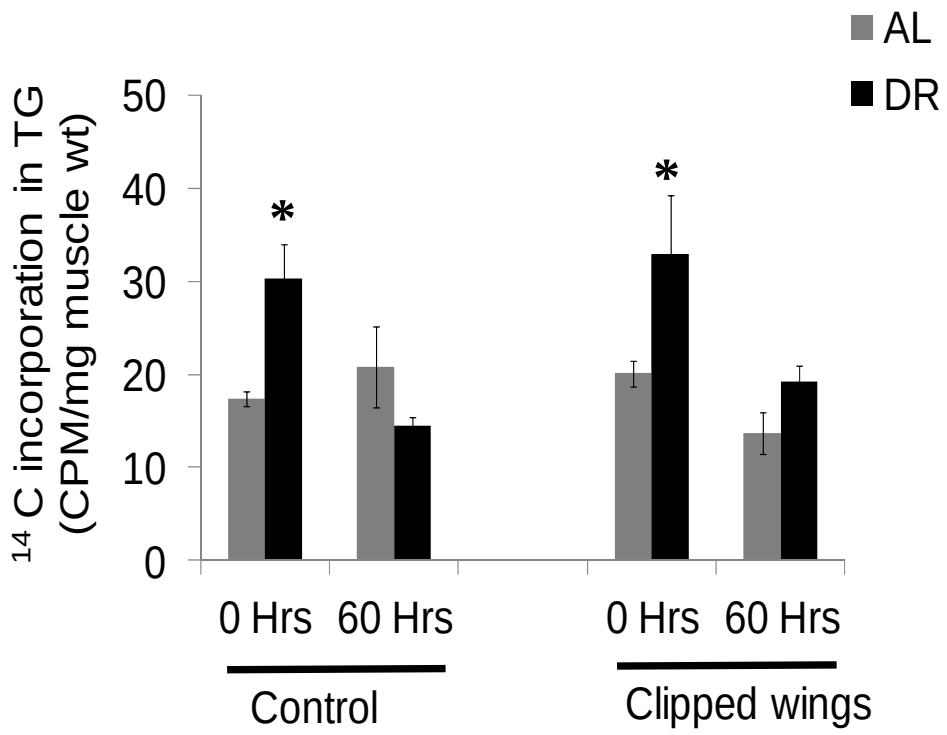
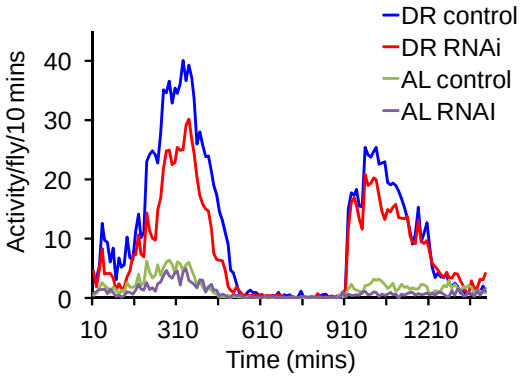
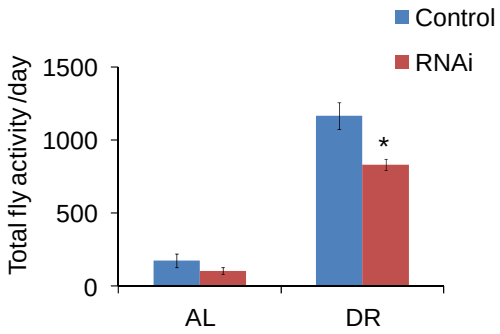


Figure S3 (continued)

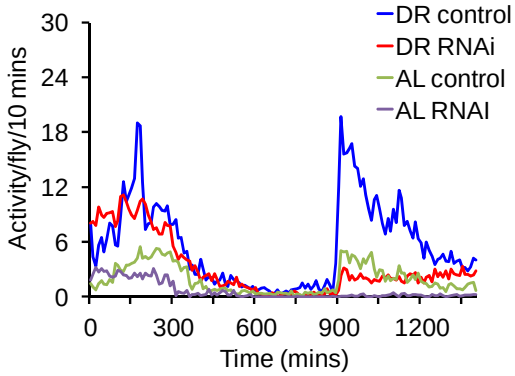
E **(i)** **(ii)**

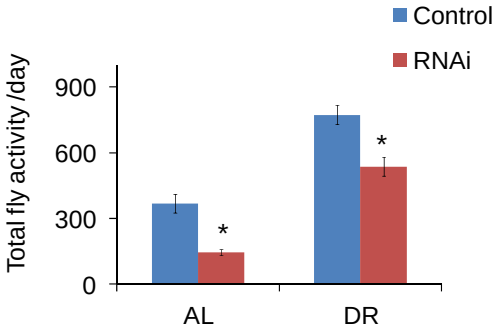
CG11198



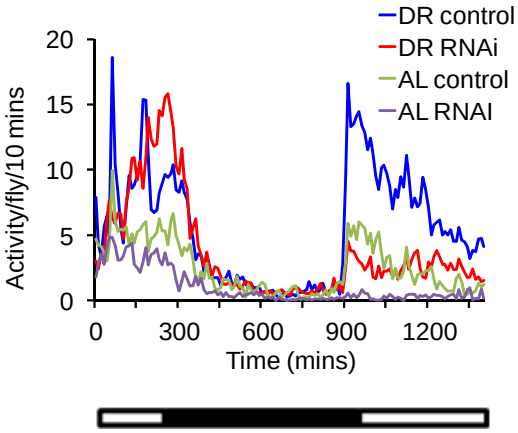


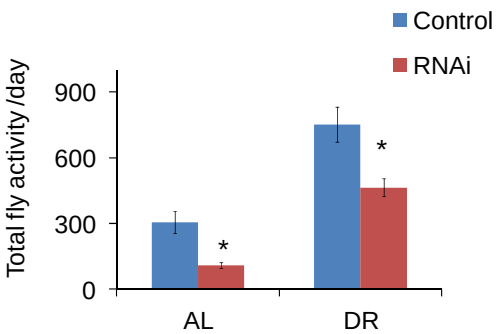
CG7834





CG4389





Supplementary figures legends

Figure S1. Comparison of fat remobilization in young and old flies, Related to Figure 1. (A) Fat body staining for the status of remobilization of lipid stores in control flies (*+/+; Act5c-GS-GAL4/+; UAS-CG11198 RNAi/+*, without RU486) upon DR and AL feeding. The peripheral fat bodies of the abdomen were fixed and stained with Nile Red and Alexa633-conjugated Phalloidin. The scale bar is 20 μm. Red are triglycerides (Nile red) and blue is F-actin (Phalloidin) (panel i). The Bar graphs represent the quantification (from 10-15 individual animals per treatment) of the particle area (panel ii) and particle number for the figures and statistical analysis by students' t test. * indicates $p < 0.05$ (panel iii). **(B)** Feeding rates in control flies is not higher under DR. Control (*+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+*, without RU486) female flies were fed with AL and DR diets spiked with ^{32}P labeled CTP for 24 hours and the incorporation of ^{32}P in whole flies was measured. The error bars indicate S.E.M of 4-5 independent preparations (* indicates $p < 0.05$).

Figure S2. Effect of *dACC* inhibition on fat metabolism, stress resistance and lifespan, Related to Figure 2. (A) Relative levels of *dACC* mRNA upon *dACC* inhibition in whole flies. 10-day old female control flies (*+/+; Act5c-GS-GAL4/+; UAS-CG11198 RNAi/+*, without RU486) and RNAi flies (*+/+; Act5c-GS-GAL4/+; UAS-CG11198 RNAi/+*, with RU486) were used to measure the relative levels of *dACC* mRNA upon DR and AL feeding. The error bars indicate S.E.M of 3 independent preparations (* indicates $p<0.05$). **(B)** Measurement of *de novo* triglyceride synthesis in *dACC* RNAi flies using ^{14}C labeled glucose under DR and AL conditions. The error bars indicate S.E.M of 5-6 independent preparations. * indicates $p < 0.05$. **(C)** Measurement of triglyceride content in *dACC* RNAi flies upon DR and AL feeding for 10 days. The error bars indicate S.E.M of 4-5 independent preparations (* indicates $p<0.05$). **(D)** Fat body staining for the status of remobilization of lipid stores in *dACC* RNAi flies upon DR and AL feeding. Scale bar is 20 μm. Red are triglycerides (Nile red) and blue is F-actin (Phalloidin) (panel i). The Bar graphs represent the quantification (from 10-15 individual animals per treatment) of the particle area (panel ii) and particle number for the figures and statistical analysis by students't test (error bars indicate S.E.M, * indicates $p<0.05$) (panel iii). **(E)** Effect of paraquat stress in control and *dACC* RNAi flies. 10 day old flies were transferred and maintained in vials with filter paper soaked in 5% sucrose with 20 mM paraquat. Kaplan Meier survival analysis for survival under paraquat fed conditions was measured in control flies (*+/+; Act5c-*

GS-Gal4/+; UAS-CG11198 /+, without RU486, blue) and *dACC* RNAi flies (*+/+; Act5c-GS-Gal4/+; UAS-CG11198/+*, with RU486, red) under DR (solid line) and AL (dashed line) conditions (panel i). The bar graph represents the mean survival time (hrs) (panel ii). **(F)** Effect of hyperoxia stress in control and *dACC* RNAi flies. 10 day old flies were maintained in a small chamber under a constant stream of 100% oxygen. Kaplan Meier survival analysis for survival under 100% oxygen was measured in control flies (*+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+*, without RU486, blue) and *dACC* RNAi flies (*+/+; Act5c-GS-Gal4/+; UAS-CG11198/+*, with RU486, red) under DR (solid line) and AL (dashed line) conditions (panel i). The bar graph represents the mean survival time (hrs). * indicates significance of $p<0.05$ by students't test (panel ii). **(G)** Kaplan Meier survival analysis of female flies upon *dACC* RNAi in whole body under different yeast (YE) concentrations (0% YE (panel i); 0.5% YE (panel ii); 1% YE (panel iii); 2% YE (panel iv) and 5% YE(panel v)); control flies (*+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+*, without RU486, blue) and RNAi flies (*+/+; Act5c-GS-Gal4/+; UAS-CG11198/+*, with RU486, red). The following median lifespan in days (d) were observed with at least 150 flies used in each trial: Control flies: 0% YE, 29d; 0.5% YE, 66d; 1% YE, 62d; 2%YE, 49d; 5% YE, 31d. *dACC* RNAi flies: 0% YE, 27d; 0.5% YE, 41d; 1% YE, 41d; 2% YE, 35d; 5% YE, 27d. **(H)** Kaplan Meier survival analysis of male flies upon *dACC* RNAi in whole body under different yeast concentrations (0% YE (panel i); 0.5% YE (panel ii); 1% YE (panel iii); 2% YE (panel iv) and 5% YE(panel v)); control flies (*+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+*, without RU486, blue) and RNAi flies (*+/+; Act5c-GS-Gal4/+; UAS-CG11198/+*, with RU486, red). The following median lifespan in days were observed with at least 150 flies used in each trial: Median lifespan in days (d); Control flies: 0% Y, 36d; 0.5% Y, 55d; 1% Y, 55d; 2%Y, 50d; 5% Y, 45d. *dACC* RNAi flies: 0% Y, 34d; 0.5% Y, 34d; 1% Y, 34d; 2% Y, 31d; 5% Y, 36d. (Complete statistical analyses are provided in Table S1). **(I)** The effects of *dACC* RNAi (using a different construct (w[1118]; P{KK102082}v108631) on nutrient dependent changes on lifespan in female flies. Kaplan Meier survival analysis for control flies (*+/+; Act5c-GS-Gal4/ UAS-CG11198(KK102082); + /+*, without RU486, blue) and RNAi flies (*+/+; Act5c-GS-Gal4/ UAS-CG11198 (KK102082); +/+*, with RU486, red) under DR and AL conditions. The following median lifespan in days (d) were observed: Control flies; AL, 32d (n=207); DR, 62d (n=196), *dACC* RNAi flies: AL, 30d (n=168); DR, 46d (n= 194). Percentage extension upon DR treatment was 94% in the control flies and 53% in *dACC* RNAi flies. (**J)** Effect of the drug Ru486 on lifespan in the female flies. Kaplan Meier survival analysis of female flies (*+/+; Act5C-GS-*

GAL4/+;+ /+) upon feeding of 200uM RU486 under different nutrient conditions, control flies (without RU486, blue) and RU486 fed flies (with RU486, red). The following median lifespan in days (d) were observed: Control flies: AL, 31d (n=178); DR, 59d (n= 214); RU486 fed flies: AL, 28d (n=218); DR, 56d (n=214). Log rank analysis suggested no significant difference between the control and RU486 fed flies on respective diets. The percentage extension observed upon DR treatment was 90% in control flies and in 100% in the RU486 fed group. **(K)** Effect of the drug Ru486 on triglyceride turnover in control flies. Triglyceride turnover rates were measured in control female flies (*+/+; Act5C-GS-GAL4/+; + /+)* upon feeding of 200uM RU486 under different nutrient conditions. Flies were fed with AL and DR diets spiked with ^{14}C labeled glucose for 24 hours (0 hrs fraction) and then were transferred to non-labeled food for 60 hours (60 hrs fraction) and incorporation of ^{14}C glucose in triglyceride fraction was measured. The error bars indicate S.E.M of 4-5 independent preparations (* indicates $p < 0.05$). **(L)** Effect of *dACC* RNAi in muscle on DR-dependent lifespan extension using a non-inducible driver. Kaplan Meier survival analysis of female flies upon muscle specific RNAi of *dACC* with Mhc-Gal4 (a non-inducible Gal4), control flies are Mhc-Gal4 (*+/+; +/+; Mhc-Gal4/+*) and UAS-ACC RNAi (*+/+; +/+; UAS-CG11198/+*), RNAi induced flies were (*+/+; +/+; Mhc-Gal4/ UAS-CG11198*). Percentage extension upon DR treatment was 80% in control flies (Mhc-Gal4) and 50% upon RNAi induction. **(M)** Relative levels of *CG7834 and CG4389* mRNA upon muscle specific inhibition. RNAi induction reduced the levels of mRNA of both CG7834 ((control flies (*+/+; Mhc-GS-Gal4/+; UAS-CG4389/+*, without RU486) and RNAi flies (*+/+; Mhc-GS-Gal4/+; UAS-CG4389/+*, with RU486)) and CG4389 ((control flies (*+/+; Mhc-GS-Gal4/+; UAS-CG7834/+*, without RU486) and RNAi flies (*+/+; Mhc-GS-Gal4/+; UAS-CG7834/+*, with RU486)) knockdown in muscles. Percentage inhibition in CG7834 RNAi flies was 37% (DR) and 34% (AL) and in CG4389 flies was 32% (DR) and 29% (AL). The error bars indicate S.E.M of 3 independent preparations (* indicates $p<0.05$).

Figure S3. Spontaneous movement is different in adult *D. melanogaster* females under different nutrient conditions, Related to Figure 3. (A) Measurement of total spontaneous activity in very young (day 6) old female flies on DR (solid line) and AL (dashed line) food conditions. The black and white bars represent the dark and light cycle to which flies were entrained. **(B)** Flying ability of young (day 10, panel i) and old (day 42, panel ii)female flies on DR (solid line) and AL (dashed line) conditions was measured and were plotted as percentage of

flies at respective landing heights; control flies (*+/+; Act5c-GS-GAL4/+; UAS-CG11198 /+*, without RU486, blue) and RNAi flies (*+/+; Act5c-GS-GAL4/+; UAS-CG11198/+*, with RU486, red). The mean landing height was calculated and is plotted in Figure 4B. **(C)** Measurement of spontaneous activity in clipped-wing female flies under different nutrition conditions. 3 day post-eclosion female flies were sorted under light CO_2 and wings were partially clipped (about one third from the top). The flies were then transferred to AL and DR food (without RU486) and maintained for 10 days and then the activity was monitored for 24 hrs on DR/AL food. The X axis represents time (in Hrs) after the flies were moved to the activity monitors at 4:00pm. The black and white bars at the bottom represent the dark and light cycle to which flies were entrained (panel i). The data in the graph is also plotted as bar graphs representing the total activity/fly/day. Error bar indicates S.E.M, with n=4 for each group, * indicates $p < 0.05$ (panel ii). **(D)** Muscle specific inhibition of fat metabolism related genes decreases spontaneous activity in flies. 3 day post-eclosion female flies were sorted under light CO_2 and were transferred to AL and DR food (with and without RU486) and maintained for 10 days and then the activity was monitored for 24 hrs on DR/AL food (panel i). The data in the graph is also plotted as bar graphs representing the total activity/fly/day. Error bar indicates S.E.M, with n=4 for each group, * indicates $p < 0.05$ (panel ii). The three groups are CG11198 (control flies (*+/+; Mhc-GS-Gal4/+; UAS-CG11198/+*, without RU486) and RNAi flies (*+/+; Mhc-GS-Gal4/+; UAS-CG11198/+*, with RU486)), CG7834 (control flies (*+/+; Mhc-GS-Gal4/+; UAS-CG7834/+*, without RU486) and RNAi flies (*+/+; Mhc-GS-Gal4/+; UAS-CG7834/+*, with RU486), CG4389 (control flies (*+/+; Mhc-GS-Gal4/+; UAS-CG4389/+*, without RU486) and RNAi flies (*+/+; Mhc-GS-Gal4/+; UAS-CG4389/+*, with RU486)), and CG4389. The X axis represents time (in mins) after the flies were moved to the activity monitors at 4:00pm. The black and white bars at the bottom represent the dark and light cycle to which flies were entrained.

Supplemental Tables

Table S1. Statistical analyses of survival curves and summary of the independent repeats of the lifespan analyses of the survival curves, Related to Figure 2-4.

Table S2. Genes showing significant changes in expression upon DR and are reversed upon *dACC* RNAi, Related to Figure 2D. Both control and *dACC* knockdown female flies were fed AL and DR food for 10 days before assessing transcript changes via a genome-wide transcriptional analysis. Total RNA was extracted from approximately 35 flies collected per replicate per group. Six independent samples were collected per group and expression array analysis was carried out on six individual replicates per group. Details of RNA extraction, amplification, labeling and hybridization are given in supplementary text. Differentially expressed genes were determined and clustered. To identify gene functions that mediate lifespan extension upon DR in a *dACC* dependent manner, expression changes that correlated with lifespan for the four groups were identified. Differentially expressed genes were determined by a significant association of expression versus mean longevity (based on the strain) and statistical inference was based upon the limma empirical Bayes procedure (see supplemental experimental procedures for details).

Table S3. GO analysis of genes that change upon DR but are reversed upon *dACC* inhibition, Related to Figure 2D. Control and *dACC* RNAi flies were fed on AL and DR food for 10 days before assessing transcript changes via a genome-wide transcriptional analysis. *dACC* knockdown was achieved by using the drug inducible *Act5C-GS- Gal4* driver and six independent biological replicate samples were prepared per group. To identify genes that mediate lifespan extension upon DR in a *dACC*-dependent manner, expression changes that correlated with lifespan of the four groups were identified (Figure 2D). GO analysis identified a number of genes whose products are involved in structure and function of muscle (bold).

GO.ID	Term	Significant	Rank in classic*	classic**	elimination***
GO:0006811	ion transport	28	18	2.20E-06	0.00548
GO:0007186	G-protein coupled receptor protein signaling	21	29	7.80E-06	0.00022
GO:0007517	**muscle development**	**18**	**31**	**1.20E-05**	**0.00563**
GO:0030030	cell projection organization	29	32	1.30E-05	0.0329
GO:0006928	cell motion	24	44	2.00E-05	0.00341
GO:0014866	**skeletal myofibril assembly**	**4**	**45**	**2.10E-05**	**0.00121**
GO:0007409	axonogenesis	19	49	2.80E-05	0.02829
GO:0007165	signal transduction	57	54	5.50E-05	0.02285
GO:0007268	synaptic transmission	17	59	0.0001	0.0278
GO:0006936	**muscle contraction**	**5**	**63**	**0.00016**	**0.00016**
GO:0006030	chitin metabolic process	11	66	0.00028	0.00313
GO:0007604	phototransduction, UV	3	70	0.0004	0.0004
GO:0008344	**adult locomotory behavior**	**7**	**72**	**0.00041**	**0.00766**
GO:0006816	calcium ion transport	6	78	0.00051	0.00051

***** Rank of the category in the hierarchy of GO term classification (using *topGO* analysis).
****** Adjusted *p* value based on the false discovery rate using Benjamini and Hochberg method.
******* Statistical significance based on the *elim* procedure (where the hierarchy of GO terms is used in determination of statistical significance).

Table S4. GO analysis of genes that change upon DR treatment in control flies, Related to Figure 2D. Control female flies were fed AL and DR food for 10 days before assessing transcript changes via a genome-wide transcriptional analysis. Total RNA was extracted from approximately 35 flies collected per replicate per group. Six independent samples were collected per group and expression array analysis was carried out on six individual replicates per group. Details of RNA extraction, amplification, labeling and hybridization are given in supplementary text. Differentially expressed genes were determined and clustered. We then used topGO (Alexa et al., 2006) and the definition of statistical significance based on the so-called elim procedure (where the hierarchy of GO terms is used in determination of statistical significance).

Supplemental experimental procedures

Fly husbandry and lifespan analysis

Flies were developed on standard lab food (Caltech food recipe), and for lifespan analysis the adults were transferred within 2-3 days of eclosion to yeast extract (YE) diet (variable concentrations of YE) as described previously (Zid et al., 2009). The AL diet was 5% yeast extract while the DR diet had 0.5% yeast extract. Males from *dACC* RNAi (*UAS-CG11198*) lines (from VDRC, Vienna) were crossed to virgin females carrying the RU486 inducible *Act5C-GS-Gal4* driver. VDRC offers two different RNAi constructs for *dACC* (v8051 (w[1118]; P{GD3482}v8105) and v108631(w[1118]; P{KK102082}v108631)). We observed similar effect on lifespan with both the two constructs (Figures 2D and S2I) and so used v8051 for all other studies. Adults from the progeny were then transferred to food with varying concentrations of YE in the absence and presence of 200μM RU486 and were maintained at 25°C for lifespan and other biochemical measurements. Muscle specific inhibition was achieved by crossing the RNAi lines UAS-CG11198 (v8051 (w[1118]; P{GD3482}v8105); UAS-CG4389(w[1118]; P{GD11299}v21845), UAS-CG7834(w[1118]; P{GD14970}v36661/TM3); obtained from VDRC, Vienna) with a RU486 inducible *Mhc-GS-Gal4*. For pan-neuronal and fat body specific inhibition, we used *Elav-GS-Gal4* and *$S_1$106-GS-Gal4* respectively. *UAS-dAKH* (y[1]w[*]; p{w[+mc]=UAS-AKH.L}2) was obtained from Bloomington stock center and was used for over-expression of AKH.

Genotype of the fly strains used:

Figure panel	***Genotype***
Figure 2A	*(+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+)* with and without RU486
Figure 2B	*(+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+)* with and without RU486
Figure 2C	*(+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+)* with and without RU486
Figure 2D	*(+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+)* with and without RU486
Figure 2E (i)	*(+/+; +/+; $S_1$106-Gal4/ UAS-CG11198)* with and without RU486
Figure 2E (ii)	*(+/+; +/+; Elav-GS-Gal4 / UAS-CG11198)* with and without RU486
Figure 2E (ii)	*(+/+; Mhc-GS-Gal4/+; UAS-CG11198/+)* with and without RU486
Figure 2F	*(+/+; Mhc-GS-Gal4/+; UAS-CG11198/+)* with and without RU486
Figure 2G(i)	*(+/+; Mhc-GS-Gal4/+; UAS-CG4389/+)* with and without RU486
Figure 2G(ii)	*(+/+; Mhc-GS-Gal4/+; UAS-CG7834/+)* with and without RU486

Figure 3A	(*+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+)* with and without RU486
Figure 3B	(*+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+)* with and without RU486
Figure 3B	Wings ablated flies *(1096-Gal4/+; UAS-rpr/+; +/+)* Control flies (*1096-Gal4/+; +/+; +/+*)
Figure 3D	(*+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+)* without RU486
Figure 3E	(*+/+; Act5c-GS-Gal4/+; UAS-CG11198 /+)* with RU486
Figure 4A	(+/+; *Act5c-GS-GAL4*/*UAS-AKH*; + /+) with and without RU486
Figure 4B	(+/+; *Act5c-GS-GAL4*/*UAS-AKH*; + /+) with and without RU486
Figure 4C	(+/+; *Act5c-GS-GAL4*/*UAS-AKH*; + /+) with and without RU486

Lipid analysis

Measurement of triglyceride and free fatty acid content - Triglyceride and free fatty acid were measured using commercially available kits (Stanbio labs, Boerne, TX). Flies that were fed AL or DR diet for 10 days were anaesthetized using CO_2 (less than 2 minutes), separated in batches of 4-5, weighed and then snap frozen in liquid nitrogen. Frozen flies were then homogenized in PBS for measurement of triglyceride and free fatty acid as per the instructions of the manufacturer.

Fat body staining - Peripheral fat bodies of the abdomen were fixed in PBS + 4% paraformaldehyde for 30 minutes, washed, and incubated overnight in a freshly prepared 0.5 mM solution of Nile Red (Sigma), together with Alexa633-conjugated Phalloidin (1:200, Molecular Probes). Following extensive washes, fat bodies were mounted in Vectashield (Vector Labs) and imaged with a laser scanning confocal microscope (Leica SP2). Image analysis was done with ImageJ and Photoshop.

Gene-array expression analysis

Total RNA extraction: Total RNA was extracted from approximately 35 flies collected in 1.5ml tubes for each experimental sample using Qiagen's Rneasy Lipid Tissue Mini Kit (74804). The samples were initially transferred to a Qiagen 2ml sample tube (990381) and then digested using

Qiagen's Qiazol lysis buffer. Lysis was aided with the use of Qiagen's TissueLyser, which pulverized the samples in lysis buffer for nine minutes at 20Hz. The samples were then processed on Qiagen's QIAcube robot, with an on-column Dnase digestion, using a modified version of Qiagen's QIAcube standard protocol, "RNeasy Lipid Tissue Mini-Animal Tissues-Aqueous Phase." The total RNA samples were processed according to the manufacturers' protocol, and their quality and concentration were assessed using a Nanodrop and Agilent's bioanalyzer (RNA 6000 Nano Kit (5067-15811)). Total RNA extraction was randomized across experimental groups.

*dscDNA amplification***:** 150ng of total RNA was amplified for each experimental sample using Sigma's TransPlex Complete Whole Transcriptome Amplification Kit (WTA2). Only 1/5th of the amplified sample library was used for the secondary amplification reaction. Amplified *dscDNA* samples were then purified using Qiagen's QIAquick PCR Purification Kit (28104) and processed on the QIAcube according to the manufacturers' standard protocol, "QIAquick PCR Purification-Standard." Sample quality and concentration were assessed using a Nanodrop and Agilent's Bioanalyzer (DNA 7500 Kit (5067-1506)). Amplification was randomized across experimental groups.

Gene expression labeling: 1µg of *ds*-cDNA was labeled with Cy3 for each experimental sample, using the NimbleGen (Roche) One-Color DNA Labeling Kit (05223555001) according to the manufacturers' protocol. Cy3 labeling was randomized across experimental groups.

Hybridization to NimbleGen 12-Plex gene expression arrays: Sample Hybridization was done according to the manufacturers' protocol for the 12-plex Gene Expression array platform. NimbleGen's Sample Tracking Controls (05223512001), Hybridization Kit reagents (05583683001) and Wash Buffers (05584507001) were used. Each 12-plex array was sealed with a H12 mixer, provided with the Gene Expression array. 4µg of each Cy3 labeled sample was loaded onto an array on the 12-plex chip, using a Gilson M100 pipette. Hybridization was done overnight on a NimbleGen Hybridization System 4 unit, at 42°C for 16hrs. After the 12-plex chip was washed and dried, the chip was scanned on a GenePix 4200A scanner at 300PMT, 100POW. Arrays were then quantitated using NimbleGen's NimbleScan2 software.

Gene-array expression analysis - Analyses were conducted in R (http://www.r-project.org) and statistical procedures part of *Bioconductor* (Gentleman et al., 2004). The expression data was loess-normalized using the *normalize loess* function as part of the *Affy* package. Differentially expressed genes were determined by a significant association of expression versus mean longevity (based on the strain) and statistical inference was based upon the *limma* empirical Bayes procedure. Significantly differentially expressed genes were defined as having an adjusted q-value (based on false discovery rate, or FDR) of < 0.05 based on Benjamini and Hochberg (Benjamini and Hochberg, 1995). To explore clusters of genes with similar expression patterns, we used both descriptive displays based on heatmaps as well hierarchical clustering based on the HOPACH algorithm (Van der Laan and Pollard, 2003). Once clusters of interest were identified that defined patterns of interest. We chose to examine only the significantly differentially expressed genes that were defined to be close (based on the cosine angle distance) to the medoids (representative genes) of the target clusters; close was defined to be smaller than the < than the 0.02 quantile of all pairwise distances among the differentially expressed genes. We then used *topGO* (Alexa et al., 2006) and the definition of statistical significance based on the so-called *elim* procedure (where the hierarchy of GO terms is used in determination of statistical significance).

Spontaneous activity measurements, muscle strength assays and stress resistance assays

Spontaneous activity measurements - For measurement of spontaneous activity we used *Drosophila* activity monitors (Trikinetics Inc., Waltham, MA). The instrument measures the movement of flies in the vertical direction and at three equidistant points over the length of a vial (approximately 2 cms, 5 cms and 8 cms above food surface). For a 24 hr measurement, the flies were first transferred to fresh food in the morning at 9:00 am and then moved to the counters at 4:00 pm for measurements for the next 24 hours. The data was collected, pooled and recorded every 10 minutes.

Muscle strength assay - Flight assays were based on previously described methods (Benzer, 1973; Elkins et al., 1986; Palladino et al., 2002). Briefly, both young (day 10) and old (day 42) female flies were dropped into the top of a 2L glass graduated cylinder through a glass funnel (n = 50-60 per replicate per group). The inside surface of the cylinder was coated with paraffin oil (Sigma, St. Louis, MO), causing flies to become stuck where they strike the wall. The strongest

fliers initiate flight immediately and stick near the top of the cylinder whereas weaker fliers fall further down and become stuck near the bottom of the cylinder. The vertical distribution of each group of flies over the length of the cylinder was determined to measure flying ability. Performance coefficients were calculated by assigning numerical scores for the distance fallen by each fly before becoming stuck according to the following scale: 1, bottom; 2, <4 cm; 3, 4-8 cm; 4, 8-12 cm; 5, 12–16 cm; 6, 16-20 cm; 7, 20-24 cm; 8, 24-28 cm; 9, 28–32 cm; 10, 32-36 cm; 11, 36-40 cm; 12 >40 cm. These scores were then averaged for each group of flies for statistical analysis using Student's t-test. Data are presented as mean ± SEM.

Cold shock resistance assay - Cold coma assay was carried out following the protocol described previously (Ballard et al., 2007). Briefly, female flies (day 10 on AL or DR diet) were kept at 0°C for 16 hours and then moved back to 25°C, and their ability to recover in the next two hours was monitored.

Starvation assay - For starvation assays female flies (day 10 on AL or DR diet) were transferred to vials containing 1% agar. The flies were transferred to fresh vials every 24 hours and deaths were recorded every 6-12 hours.

Supplementary statistical analysis

Differential effect of diet on longevity with clipped (vs. unclipped) wings among control and ACC RNAi flies:

Cox regression analysis was performed with robust standard errors (Huber/White/sandwich estimator) of the form (for regression on the hazard of death):

$$\lambda^{strain}(t \mid DR, CLP) = \lambda_0^{strain}(t) * \exp(\beta_1^{strain} DR + \beta_2^{strain} CLP + \beta_3^{strain} DR * CLP)$$

In this case, we are interested in whether clipping the wings has an effect of DR on longevity, so that is the coefficient β_3^{strain} for either strain = control or mutant. This coefficient is the ratio of hazard ratios (relative rates of disease) related to DR of those with clipped wings over those without. We report the test of interaction, or H_0: $\beta_3^{strain}=0$, which if not significant, indicates that there is no evidence that clipping wings modifies the effect of dietary restriction. As indicated, we repeat this for control and mutant flies. All analyses were made using STATA vers. 11 (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP.).

The results for controls are:

No. of subjects	=	693	Number of obs	=	693
No. of failures	=	693			
Time at risk	=	34891			
			Wald chi2(3)	=	448.94

Log pseudolikelihood = -3627.8457 Prob > chi2 = 0.0000

_t	Coef.	Robust Std. Err.	z	P>|z|	[95% Conf.	Interval]
dr	-2.895527	.1504598	-19.24	0.000	-3.190423	-2.600631
clipped	-.4090053	.1066883	-3.83	0.000	-.6181105	-.1999001
drclip	**1.255214**	**.1636651**	**7.67**	**0.000**	**.9344362**	**1.575991**

HR of DR among those without Clipped Wings

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	.0552699	.0083159	-19.24	0.000	.0411545	.0742267

HR of DR among those *with* Clipped Wings

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
(1)	.1939193	.0233235	-13.64	0.000	.1531947	.2454701

Thus, a significant change, such that though DR still reduced the rate of death among those with clipped wings, this reduction is 4 fold less among those without clipped wings (Highly significant).

The results for ACC RNAi flies are:

Cox regression -- Breslow method for ties

No. of subjects = 674 Number of obs = 674
No. of failures = 674
Time at risk = 24545
Wald chi2(3) = 192.08
Log pseudolikelihood = -3650.9433 Prob > chi2 = 0.0000

_t	Coef.	Robust Std. Err.	z	P>|z|	[95% Conf.	Interval]
dr	-1.258737	.1224566	-10.28	0.000	-1.498748	-1.018727
clipped	-.1370436	.1216592	-1.13	0.260	-.3754911	.101404
drclip	**-.1172463**	**.1533441**	**-0.76**	**0.445**	**-.4177952**	**.1833025**

HR of DR among those without Clipped Wings

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]	
(1)	.2840124	.0347792	-10.28	0.000	.2234097	.3610544

HR of DR among those *with* Clipped Wings

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]	
(1)	.252591	.0308582	-11.26	0.000	.198806	.320927

We noted that the results are very different compared to the controls. Though as above, DR is associated with a reduction in the rate of death (75% reduction, so not as much), clipping wings appears to have no impact on the effect of DR at all (note that the HR's are nearly equal within the two groups). If the interaction is removed, clipped wings only has a very marginal association with reduced rate of death (an 18% reduction, p-value = 0.01). Thus, the Cox-regression analysis suggests that the impact of clipped wings on the effect of DR on longevity is substantially less (non-existent) in *dACC* RNAi flies, but statistically significant in control flies.

Table S1. Statistical analyses of survival curves and summary of the independent repeats of the lifespan analyses of the survival curves

Group 1/Group 2 [a]	Chi square [b]	*p* value [b]	# of flies n1	n2	Median survival (MS) Group 1	Group 2	% change in MS
Statistical analysis for Fig. 2A.							
AL control / AL ACC RNAi	0.242	0.623	159	126	48*	48*	0
DR control /DR ACC RNAi	139.0	P<0.0001	158	193	86*	65*	-24.4
AL control /DR control	291.4	P<0.0001	159	158	48*	86*	+79.1
AL ACC RNAi /DR ACC RNAi	115.7	P<0.0001	126	193	48*	65*	+35.4
Control flies (+/+*; Act5c-GS-GAL4/+; UAS-CG11198 /+*, without RU486) and RNAi flies (+/+*; Act5c-GS-GAL4/+; UAS-CG11198/+*, with RU486)							
Statistical analysis for Fig. 2B.							
AL control /AL ACC RNAi	1.768	0.1837	115	143	73.2**	102.1**	+39.4
DR control /DR ACC RNAi	90.05	P<0.0001	116	142	57.4**	118.2**	+105.9
AL control /DR control	47.51	P<0.0001	115	116	73.2**	57.3**	-21.7
Al ACC RNAi / DR ACC RNAi	0.154	0.6947	143	142	102.1**	118.2**	+15.7
Control flies (+/+*; Act5c-GS-GAL4/+; UAS-CG11198 /+*, without RU486) and RNAi flies (+/+*; Act5c-GS-GAL4/+; UAS-CG11198/+*, with RU486)							
Statistical analysis for Figure 2E, panel i.							
AL control /AL ACC RNAi	19.77	P<0.0001	225	224	24	31	+29.2
DR control /DR ACC RNAi	6.574	0.0103	263	229	40	45	+12.5
AL control /DR control	173.5	P<0.0001	225	263	24	40	+66.7

AL ACC RNAi /DR ACC RNAi	75.79	P<0.0001	224	229	31	45	+45.2
Control flies (*+/+; +/+; $S_1$106-GAL4/ UAS-CG11198*, without RU486) and RNAi flies (*+/+;+/+; $S_1$106-GAL4/ UAS-CG11198*, with RU486).							
Statistical analysis for Figure 2E, panel ii.							
AL control /AL ACC RNAi	8.084	0.0045	205	191	42	46	+9.5
DR control /DR ACC RNAi	13.55	0.0002	206	161	57	63	+10.5
AL control /DR control	73.81	P<0.0001	205	206	42	57	+35.7
AL ACC RNAi / DR ACC RNAi	92.78	P<0.0001	191	161	46	63	+36.9
Control flies (*+/+; +/+; Elav-GS-GAL4 / UAS-CG11198*, without RU486) and RNAi flies (*+/+;+/+; Elav-GS-GAL4 / UAS-CG11198*, with RU486)							
Statistical analysis for Figure 2E, panel iii.							
AL control /AL ACC RNAi	71.34	P<0.0001	145	147	36	22	-38.9
DR control /DR ACC RNAi	144.8	P<0.0001	164	170	53	22	-58.5
AL control /DR control	60.34	P<0.0001	145	164	36	53	+47.2
AL ACC RNAi / DR ACC RNAi	4.3	0.0385	147	170	22	22	00.0
Control flies (*+/+; Mhc-GS-GAL4/+; UAS-CG11198/+*, without RU486) and RNAi flies (*+/+; Mhc-GS-GAL4/+; UAS-CG11198/+*, with RU486)							
Statistical analysis for Figure 2G, panel i.							
AL control /AL CG4389 RNAi	34.66	P<0.0001	174	79	30	27	-10.0
DR control /DR CG4389 RNAi	159.6	P<0.0001	109	88	67	33	-51.5
AL control /DR control	159.2	P<0.0001	174	109	30	67	+123.3
AL CG4389 RNAi/ DR	8.755	0.0031	79	88	27	33	+20.3

CG4389 RNAi							
Control flies (*+/+; Mhc-GS-GAL4/ UAS-CG4389; +/+*, without RU486) and RNAi flies (*+/+; Mhc-GS-GAL4/ UAS-CG4389; +/+*, with RU486)							
Statistical analysis for Figure 2G, panel i.							
AL control /AL CG7834 RNAi	176.3	P<0.0001	193	179	33	21	-36.4
DR control /DR CG7834 RNAi	344.6	P<0.0001	179	171	51	24	-52.9
AL control /DR control	223.1	P<0.0001	193	179	33	51	+54.5
AL CG7834 RNAi /DR CG7834 RNAi	4.934	0.0263	179	171	21	24	+14.3
Control flies (*+/+; Mhc-GS-GAL4/+; UAS-CG7834/+*, without RU486) and RNAi flies (*+/+; Mhc-GS-GAL4/+; UAS-CG7834/+*, with RU486)							
Statistical analysis for Figure. S2I.							
AL control UAS /AL ACC RNAi	0.496	NS	171	156	28	28	0
AL control GAL4 /AL ACC RNAi	16.26	P<0.0001	186	156	35	28	-20.0
DR control UAS /DR ACC RNAi	127.8	P<0.0001	131	136	81	42	-48.2
DR control GAL4 /DR ACC RNAi	123.0	P<0.0001	143	136	67	42	-37.3
AL control GAL4 /DR control GAL4	249.2	P<0.0001	186	143	35	67	+80.0
AL control UAS /DR control UAS	218.5	P<0.0001	171	131	28	82	+192.0
AL ACC RNAi / DR ACC RNAi	99.85	P<0.0001	156	136	42	28	+50.0
Control UAS flies (*+/+; +/+; +/ UAS-CG11198*), Control GAL4 flies (*+/+; +/+; +/ Mhc-GAL4*) and RNAi flies (*+/+; +/+; Mhc-GAL4/ UAS-CG11198*).							
Statistical analysis for Figure 3C.							

AL control /AL wings ablated	321.6	P<0.0001	182	216	46	14	-69.6
DR control /DR wings ablated	360.4	P<0.0001	193	64	74	16	-78.4
AL control /DR control	352.1	P<0.0001	182	193	46	74	+60.9
AL wings ablated/ DR wings ablated	5.405	0.0201	216	64	14	16	+14.3
Control flies (*1096-Gal4/+; +/+; +/+*)) and wings ablated flies(*1096-Gal4/+; UAS-rpr/+; +/+*))							
Statistical analysis for Figure 3D.							
AL control /AL clipped	8.251	0.0041	54	68	39	49	+25.6
DR control /DR clipped	37.89	P<0.0001	53	62	77	65	-15.6
AL control /DR control	99.85	P<0.0001	54	53	39	77	+97.4
AL clipped/ DR clipped	60.93	P<0.0001	68	62	49	65	+32.7
Both control and clipped flies (*+/+; Act5c-GS-GAL4/+; UAS-CG11198 /+*, without RU486)							
Statistical analysis for Figure 3E.							
AL ACC RNAi /AL ACC RNAi clipped	2.724	0.0989	55	69	33	30	-9.1
DR ACC RNAi /DR ACC RNAi clipped	1.221	0.2692	56	67	44	44	0
AL ACC RNAi /DR ACC RNAi	46.64	P<0.0001	55	56	33	44	+33.3
Al ACC RNAi clipped /DR ACC RNAi clipped	64.74	P<0.0001	69	67	30	44	+46.7
Both ACC RNAi and ACC RNAi clipped flies (*+/+; Act5c-GS-GAL4/+; UAS-CG11198/+*, with RU486)							

Statistical analysis for Figure 4C.							
AL control / AL AKH overexpression	51.53	P<0.0001	159	153	43	57	+32.6
DR control /DR AKH overexpression	6.96	P<0.0083	151	143	76	76	0.0
AL control /DR control	264.1	P<0.0001	159	151	43	76	+76.7
AL AKH overexpression /DR AKH overexpression	172.2	P<0.0001	153	143	57	76	+33.3
Control flies (+/+*; Act5c-GS-GAL4/ UAS-AKH;* + /+, without RU486) and RNAi flies (+/+*; Act5c-GS-GAL4/ UAS-AKH;* +/+, with RU486)							
Statistical analysis for Figure S2G.							
0% (control / ACC RNAi)	3.924	0.0476	216	274	29	27	-6.9
0.5% (control / ACC RNAi)	270.5	P<0.0001	244	266	66	41	-37.9
1% (control / ACC RNAi)	160.7	P<0.0001	240	265	62	41	-33.9
2% (control / ACC RNAi)	139.4	P<0.0001	211	230	49	35	-28.6
5% (control / ACC RNAi)	47.09	P<0.0001	290	279	31	27	-12.9
AL control /DR control	451.9	P<0.0001	290	244	31	66	+112.9
AL ACC RNAi /DR ACC RNAi	413.2	P<0.0001	279	266	27	41	+51.8
Control flies (+/+*; Act5c-GS-GAL4/+; UAS-CG11198 /+*, without RU486) and RNAi flies (+/+*; Act5c-GS-GAL4/+; UAS-CG11198/+*, with RU486)							
Statistical analysis for Figure S2H.							
0% (control / ACC RNAi)	45.83	P<0.0001	216	235	36	34	-5.6
0.5% (control / ACC RNAi)	406.1	P<0.0001	260	251	55	34	-38.2

1% (control / ACC RNAi)	336.5	P<0.0001	187	221	55	34	-38.2
2% (control / ACC RNAi)	211.8	P<0.0001	196	281	50	31	-38.0
5% (control / ACC RNAi)	217.9	P<0.0001	246	256	45	36	-20.0
AL control /DR control	42.2	P<0.0001	246	260	45	55	+22.2
AL ACC RNAi /DR ACC RNAi	36.40	P<0.0001	256	251	36	34	-5.6
Control flies (+/+*; Act5c-GS-GAL4/+; UAS-CG11198 /+*, without RU486) and RNAi flies (+/+*; Act5c-GS-GAL4/+; UAS-CG11198/+*, with RU486)							

[a] Group 1 and group 2 represents the group involved for the comparison.

[b] Survival curves were plotted and statistical analyses (log-rank tests) were performed using the Prism 4 software (Graphpad Software, Inc., San Diego, CA, USA)

* median starvation survival is in hrs

** median recovery time is in minutes

Summary of the independent repeats of the lifespan analyses of the survival curves.

		Median lifespan (in days)			
Group (Cross genotype)	Repeat #	Control DR (n) (Without RU486)	RNAi DR (n) (With RU486)	Control AL (n) (Without RU486)	RNAi AL (n) (With RU486)
(*+/+; Act5c-GS-GAL4/+; UAS-CG11198 /+*) **(Figure 2C)**	1	66(244)	41(266)	31(290)	27(279)
	2	64(92)	47(104)	38(85)	30(64)
(*+/+; +/+; $S_1$106-GAL4/ UAS-CG11198)* **(Figure 2E, panel i)**	1	40(263)	45(229)	24(225)	31(224)
	2	46(169)	46(173)	28(153)	32(167)
(*+/+; +/+; Elav-GS-GAL4 / UAS-CG11198)* **(Figure 2E, panel ii)**	1	57(206)	63(161)	42(205)	46(191)
	2	68(91)	61(92)	36(92)	38(95)
(*+/+; Mhc-GS-GAL4/+; UAS-CG11198/+)* **(Figure 2E, panel iii)**	1	53(164)	22(170)	36(145)	22(147)
	2	41(207)	25(209)	25(208)	20(205)
	3	62(129)	34(116)	27(108)	20(117)
(*+/+; Mhc-GS-GAL4/ UAS-CG4389; +/+)* **(Figure 2G, panel i)**	1	67(109)	33(88)	30(174)	27(79)
	2	57(141)	34(135)	34(139)	20(141)
	3	73((179)	26(178)	31(174)	21(160)
(*+/+; Mhc-GS-GAL4/+; UAS-CG7834/+)* **(Figure 2G, panel ii)**	1	51(179)	24(171)	33(193)	21(179)
	2	63(43)	38.5(36)	37(29)	28(41)
For clipped wing flies experiment: **(Figure 3D and 4E)**					

(+/+; *Act5c-GS-GAL4/+; UAS-CG11198 /+) control*	1	77(53)	44(56)	39(54)	33(55)
(+/+; *Act5c-GS-GAL4/+; UAS-CG11198 /+) clipped*		65(62)	44(67)	49(68)	30(69)
(+/+; *Act5c-GS-GAL4/+; UAS-CG11198 /+) control*	2	60 (119)	43(112)	29(104)	29(91)
(+/+; *Act5c-GS-GAL4/+; UAS-CG11198 /+) clipped*		58(120)	50 (115)	36(113)	29(109)
(+/+; *Act5c-GS-GAL4/ UAS-AKH; + /+)* **(Figure 4C)**	1	76(151)	76(143)	43(159)	57(153)
	2	67(178)	67(162)	43(158)	60(167)

Table S3. GO analysis of genes that change upon DR but are reversed upon *dACC* inhibition, Related to Figure 2D. Control and *dACC* RNAi flies were fed on AL and DR food for 10 days before assessing transcript changes via a genome-wide transcriptional analysis. *dACC* knockdown was achieved by using the drug inducible *Act5C-GS- Gal4* driver and six independent biological replicate samples were prepared per group. To identify genes that mediate lifespan extension upon DR in a *dACC*-dependent manner, expression changes that correlated with lifespan of the four groups were identified (Figure 2D). GO analysis identified a number of genes whose products are involved in structure and function of muscle (bold).

GO.ID	Term	Significant	Rank in classic*	classic**	elimination***
GO:0006811	ion transport	28	18	2.20E-06	0.00548
GO:0007186	G-protein coupled receptor protein signaling	21	29	7.80E-06	0.00022
GO:0007517	**muscle development**	**18**	**31**	**1.20E-05**	**0.00563**
GO:0030030	cell projection organization	29	32	1.30E-05	0.0329
GO:0006928	cell motion	24	44	2.00E-05	0.00341
GO:0014866	**skeletal myofibril assembly**	**4**	**45**	**2.10E-05**	**0.00121**
GO:0007409	axonogenesis	19	49	2.80E-05	0.02829
GO:0007165	signal transduction	57	54	5.50E-05	0.02285
GO:0007268	synaptic transmission	17	59	0.0001	0.0278
GO:0006936	**muscle contraction**	**5**	**63**	**0.00016**	**0.00016**
GO:0006030	chitin metabolic process	11	66	0.00028	0.00313
GO:0007604	phototransduction, UV	3	70	0.0004	0.0004
GO:0008344	**adult locomotory behavior**	**7**	**72**	**0.00041**	**0.00766**
GO:0006816	calcium ion transport	6	78	0.00051	0.00051

***** Rank of the category in the hierarchy of GO term classification (using *topGO* analysis).
****** Adjusted *p* value based on the false discovery rate using Benjamini and Hochberg method.
******* Statistical significance based on the *elim* procedure (where the hierarchy of GO terms is used in determination of statistical significance).

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