

Sir2 mediates longevity in the fly through a pathway related to calorie restriction

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Calorie restriction can extend life span in a variety of species including mammals, flies, nematodes, and yeast. Despite the importance of this nearly universal effect, little is understood about the molecular mechanisms that mediate the life-span-extending effect of calorie restriction in metazoans. Sir2 is known to be involved in life span determination and calorie restriction in yeast mother cells. In nematodes increased Sir2 can extend life span, but a direct link to calorie restriction has not been demonstrated. We now report that Sir2 is directly involved in the calorie-restriction life-span-extending pathway in *Drosophila*. We demonstrate that an increase in *Drosophila* Sir2 (*dSir2*) extends life span, whereas a decrease in *dSir2* blocks the life-span-extending effect of calorie reduction or *rpd3* mutations. These data lead us to propose a genetic pathway by which calorie restriction extends life span and provides a framework for genetic and pharmacological studies of life span extension in metazoans.

aging | life span | Rpd3 | histone deacetylase | *Drosophila melanogaster*

It has been known for ≈ 70 years that calorie restriction can dramatically extend the life span of rodents (1). In primates calorie restriction causes a number of physiological changes with positive health benefits (2, 3). Although calorie restriction has been the subject of intense investigation, little is understood about the molecular and cellular mechanisms by which a reduction in calorie intake effects life span extension. Recently it has been shown that the calorie-restriction life-span-extending effect is conserved across distant species from yeast to mammals (2, 4–12). Powerful molecular genetic techniques and the relatively short life span of model organisms such as yeast, nematodes, and flies provide the opportunity to uncover the molecular and cellular mechanisms underlying this universal effect on life span. Indeed, the use of these model organisms has implicated insulin-signaling, nutrient-sensing, and chromosome-remodeling proteins in either triggering calorie restriction or contributing to its life-span-extending effect (13–19).

The Rpd3/Sir2 histone deacetylases have been implicated in both life span determination and calorie restriction in yeast (15). Rpd3 and Sir2 can effect the activity of a variety of genes and physiological systems by deacetylating histones and other proteins such as p53 (12, 20). A decrease in Rpd3 or an increase in Sir2 extends mother cell life span in yeast (11, 21), and the effect of Sir2 on yeast life span is linked to calorie restriction (22). A similar mechanism may operate in metazoans, because an increase in Sir2 extends life span in nematodes (23), and a decrease in Rpd3 extends life span in flies (16). The increase in life span associated with decreased Rpd3 in flies is thought to occur through a mechanism related to calorie restriction (16). The finding of an increase in *Drosophila* Sir2 (*dSir2*) transcription in both long-lived *rpd3* mutant flies and long-lived calorie-restricted normal flies implicates *dSir2* as a potential member of the calorie-restriction life-span-extending pathway (16). Further evidence of a role for Sir2 in the determination of life span is the finding that the Sir2 agonist resveratrol extends life span in yeast, nematodes, and flies in a Sir2- and calorie-restriction-dependent manner (24, 25). These data suggest that Sir2 may be one of the

primary elements of the calorie-restriction-induced life span extension in flies and other metazoans.

Materials and Methods

Fly Strains. The *dSir2*^{4.5}- and *dSir2*^{5.26}-null mutant lines were obtained from S. Smolik (Oregon Health & Science University, Portland); the *dSir2*¹⁷-null mutant line was obtained from S. Astrom (Stockholm University, Stockholm); the ELAV-GeneSwitch line was obtained from H. Keshishian (Yale University, New Haven, CT); the Canton-S, armadillo-GAL4 driver, tubulin-GAL4 driver, and ELAV-GAL4 driver and the *dSir2*^{EP2300}, *dSir2*^{EP2384}, and *dSir2*^{KG00871} mutant lines were obtained from the Bloomington *Drosophila* Stock Center at Indiana University; the *dSir2*^{EYO3602} line was obtained from H. Bellen (Baylor College of Medicine, Houston); and the D42 driver was obtained from G. Boulianne (Hospital for Sick Children Research Institute, Toronto).

Genetic Crosses. The GAL4 and UAS binary system was used to drive overexpression of *dSir2* (26, 27). To generate flies that ubiquitously overexpressed *dSir2*, >90 tubulin-GAL4/*TM3* male flies were crossed to >100 virgin *dSir2*^{EP2300}/*CyO*, *dSir2*^{EP2384}/*CyO*, or *dSir2*^{EYO3602}/*CyO* female flies. To generate flies that ubiquitously overexpressed *dSir2* at a lower level, >90 armadillo-GAL4 male flies were crossed to >100 virgin *dSir2*^{EP2300/+} females. *dSir2*^{EP2300/+}; tubulin-GAL4^{+/+}, *Sir2*^{EP2384/+}; tubulin-GAL4^{+/+}, *dSir2*^{EYO3602/+}; tubulin-GAL4^{+/+}, armadillo-GAL4/*dSir2*^{EP2300} progeny were used for the experimental life spans or semiquantitative RT-PCR. Control flies were obtained by mating >50 virgin F₁ female flies and >50 F₁ male flies from each condition to obtain white-eyed flies lacking balancer chromosomes, the tubulin-GAL4 driver chromosome, or chromosomes containing *dSir2*^{EP2300}, *dSir2*^{EP2384}, or *dSir2*^{EYO3602}. A similar set of crosses was performed to obtain experimental and control flies for the neuronal overexpression of *dSir2* in the ELAV-GAL4 studies, except that the ELAV-GAL4 driver stock was homozygous (ELAV-GAL4 is on the X chromosome), and >50 virgin F₁ females flies were backcrossed to the ELAV-GAL4 stock so that control flies had an ELAV-GAL4 chromosome but no UAS-*dSir2*.

The conditional ELAV-GeneSwitch driver was combined with the *dSir2*^{EP2300} line to drive overexpression of *dSir2*. A single cohort of F₁ *dSir2*^{EP2300/+}; ELAV-GeneSwitch^{+/+} male and female adult flies was collected and placed on a diet of either food with RU-486 (mifepristone, Sigma) at a concentration of 200 μ M or food with only diluent from the first day after eclosion.

Flies heteroallelic for *dSir2*-null mutations, *dSir2*^{4.5}/*dSir2*^{5.26} flies, were generated by crossing *dSir2*^{4.5}/*CyO* to *dSir2*^{5.26}/*CyO*. Flies heterozygous for the null allele of *rpd3* (*rpd3*^{def24}) and the null allele *dSir2*¹⁷ (28) or the hypomorphic allele *dSir2*^{EP2300} were crossed to Canton-S flies to generate a matched genetic background. *dSir2*¹⁷/*dSir2*⁺; *rpd3*^{def24}/*rpd3*⁺ flies were generated by

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crossing *dSir2/CyO* and *rpd3/TM6Sb* flies. *dSir2^{EP2300}/dSir2⁺; rpd3^{def24}/rpd3⁺* flies possessing a copy of the *dSir2*-hypomorphic allele *dSir2^{EP2300}* and a copy of the *rpd3*-null allele *rpd3^{def24}* were generated in the same way. Control flies, generated in a similar manner, were obtained by mating >50 F₁ *CyO; Sb* (without *dSir2^{EP2300}* or *rpd3^{def24}*) male flies and >50 F₁ virgin *CyO; Sb* (without *dSir2^{EP2300}* or *rpd3^{def24}*) female flies to obtain flies with no balancer chromosome.

Life Span Determinations. Newly eclosed adults were collected, and ≈20 males and 20 females were placed into each vial with normal cornmeal sucrose food (per refs. 16 and 17) or high-calorie (15% yeast, 15% sucrose, and 2% agar) or low-calorie (5% yeast, 5% sucrose, and 2% agar) food (per ref. 7). Studies with the RU-486-ELAV-GeneSwitch driver used food in which RU-486, dissolved in 100% EtOH, was added during preparation when the food had cooled to 50°C, at a final concentration of 200 μM. Control food for the RU-486 experiments was made by adding the same amount of EtOH without RU-486 when the food had cooled to 50°C. Flies were maintained in a humidified temperature-controlled environmental chamber at 25°C. Every 2 days, flies were passed into new vials, and the number of dead flies was counted as described in ref. 16. Median and mean life spans and statistical log rank analyses were performed with STATVIEW (SAS Institute, Cary, NC). Maximum life spans were calculated as the mean of 10% survival, except for the studies with RU-486, in which 1% maximum life span was used.

Results

Increasing Sir2 Expression Extends Life Span in *Drosophila*. To test whether *dSir2* is involved in longevity determination in the fly, we examined the life span of flies in which the level of *dSir2* had been increased by using molecular genetic techniques. Flies were constructed that ubiquitously overexpressed *dSir2* by combining, in individual flies, the *Drosophila* tubulin promoter fused to the gene for the yeast GAL4 activator protein (tubulin-GAL4 driver) with a native *dSir2* gene that has a *P* element with GAL4-binding sites (EP-UAS) inserted just upstream (26, 27). Flies carrying the tubulin-GAL4 driver and each of the different EP-UAS-*dSir2* genes, *dSir2^{EP2300}*, *dSir2^{EP2384}*, or *dSir2^{EYO3602}*, had a >4-fold increase in *dSir2* mRNA expression over the endogenous level (Fig. 5, which is published as supporting information on the PNAS web site). Consistent with our hypothesis that an increase in *dSir2* in flies will increase life span, up to a 57% increase in average life span was seen in the tubulin-GAL4/*dSir2^{EP2300}*, tubulin-GAL4/*dSir2^{EP2384}*, and tubulin-GAL4/*dSir2^{EYO3602}* flies, with an increase across all lines of 29% for females and 18% for males (Fig. 1 *a–d* and Table 1; see also Figs. 6 and 7, which are published as supporting information on the PNAS web site).

To determine whether a threshold level of *dSir2* expression is required for life span extension in the fly, we examined flies in which the armadillo-GAL4 driver was combined with the *dSir2^{EP2300}* chromosome. The armadillo-GAL4 driver is a weaker driver than the tubulin-GAL4 driver: compared with control flies, armadillo-GAL4/*dSir2^{EP2300}* flies showed only a 10–20% increase in *dSir2* mRNA levels and no life span extension, suggesting that a significant increase in *dSir2* mRNA is required to cause an extension in life span (data not shown).

Increasing Sir2 Expression in Neurons Extends Life Span. Knowing that ubiquitous overexpression of *dSir2* increases life span, we wanted to determine which tissues normally express *dSir2* in adults and whether an effect in a single tissue could mediate the life span extension caused by *dSir2* overexpression. Using anti-*dSir2* antibodies (28, 29), we found that, similar to embryos and larvae (29), in adults *dSir2* protein is found at high levels in the nuclei of neurons and in the nuclei and cytoplasm of fat body

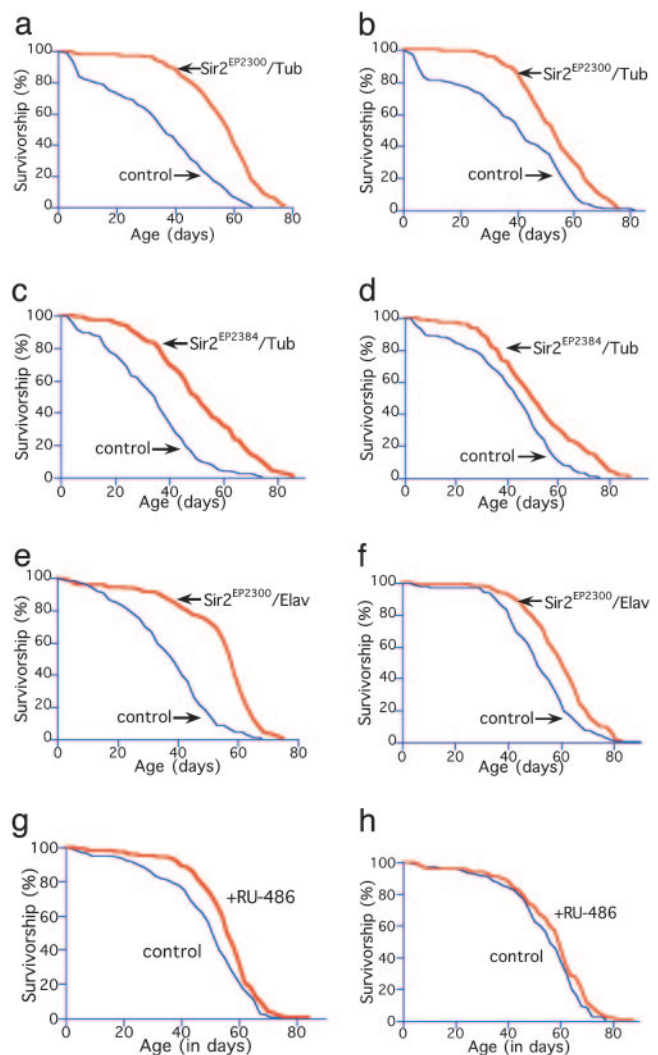


Fig. 1. Increased expression of *dSir2* extends life span in the fly. Life span extension is seen in female (*a*, *c*, *e*, and *g*) and male (*b*, *d*, *f*, and *h*) flies in which *dSir2* expression is increased (red line) in a ubiquitous manner (*a–d*) or in the nervous system (*e–h*) compared with genetically matched controls (blue line). Survivorship curves for genetically matched controls and tubulin-GAL4 driver/*dSir2^{EP2300}* flies (*a* and *b*), tubulin-GAL4 driver/*dSir2^{EP2384}* flies (*c* and *d*), ELAV-GAL4 driver/*dSir2^{EP2300}* flies (*e* and *f*), and RU-486-ELAV-GeneSwitch driver/*dSir2^{EP2300}* flies (*g* and *h*). Genetically matched controls are flies derived from a second-generation cross of the offspring from the cross that generated the experimental flies in *a–f*. Controls in *a–f* are flies containing a mix of the chromosomes found in the experimental flies but which do not contain the GAL4 chromosome, the UAS-containing chromosome, or any balancer chromosome. Controls in *g* and *h* are flies from the same cohort fed only diluent and not RU-486. Each life span included at least 149 male and 159 female flies (16).

cells (Fig. 8, which is published as supporting information on the PNAS web site). The finding of a prominent expression of *dSir2* in the nervous system of normal animals led us to examine whether an increase in *dSir2* in neurons may be one of the primary mediators of the Sir2-related life span extension. Neuronal *dSir2* overexpression in flies carrying the pan-neuronal promoter ELAV-GAL4 driver and *dSir2^{EP2300}* extended the average life span by 52% in females and 20% in males (Fig. 1 *e* and *f*, respectively, and Table 1).

ELAV-GAL4 drives expression in embryos and larvae as well as adults. A different system for overexpressing *dSir2* only in adult

Table 1. Life span is extended when Sir2 expression is increased

Gender	Genotype	n	Median life span (% change)	χ^2	Maximal life span (% change)
F	Tub/ <i>dSir2</i> ^{EP2300}	196	58 (57)	139.754	73.62 (19)
F	Control	171	37		62.1
M	Tub/ <i>dSir2</i> ^{EP2300}	194	54 (32)	37,024	73.57 (14)
M	Control	179	41		65.43
F	Tub/ <i>dSir2</i> ^{EP2384}	185	52 (44)	84.391	78.96 (25)
F	Control	159	36		62.75
M	Tub/ <i>dSir2</i> ^{EP2384}	180	50 (14)	24.597	81.7 (21)
M	Control	149	44		67.53
F	ELAV/ <i>dSir2</i> ^{EP2300}	208	59 (52)	135.190	70 (19)
F	Control	191	39		59.08
M	ELAV/ <i>dSir2</i> ^{EP2300}	179	61 (20)	28.347	80.56 (8)
M	Control	202	51		75.29
F	Tub/ <i>dSir2</i> ^{EY03602}	193	41 (–15)	37.803	55.25 (–11)
F	Control	183	47		62.24
M	Tub/ <i>dSir2</i> ^{EY03602}	184	47 (9)	24.461	74.26 (25)
M	Control	161	43		58.79
F	ELAV-GS/ <i>dSir2</i> ^{EP2300}	232	56 (12)	19.10	84 (9)
F	Control	202	51		77
M	ELAV-GS/ <i>dSir2</i> ^{EP2300}	234	60 (–5)	0.319*	86 (–1)
M	Control	187	63		87
F	ELAV-GS/ <i>dSir2</i> ^{EP2300}	186	60 (5)	6.260**	88 (16)
F	Control	200	56		76
M	ELAV-GS/ <i>dSir2</i> ^{EP2300}	187	62 (–3)	2.659***	84 (11)
M	Control	185	64		76

The median and maximal life spans of male (M) and female (F) flies with increased expression of *dSir2* ubiquitously (tubulin-GAL4/*dSir2*^{EP2300}, tubulin-GAL4/*dSir2*^{EP2384}, and tubulin-GAL4/*dSir2*^{EY03602}), expression restricted to neurons throughout life (ELAV-GAL4/*dSir2*^{EP2300}), or expression restricted to adult neurons (ELAV-GeneSwitch/*dSir2*^{EP2300}) compared with their genetically matched controls. n, Number of flies in each life span; Tub, tubulin-GAL4 driver; ELAV, ELAV-GAL4 driver; ELAV-GS, ELAV-GeneSwitch driver. (For example, Tub/*dSir2*^{EP2300} is a fly that possesses one copy of the tubulin-GAL4 driver and one copy of the *dSir2*^{EP2300} chromosome.) Controls are described in *Materials and Methods*. Control flies for ELAV-GeneSwitch driver flies were from the same cohort and fed only diluent, without RU-486. Statistical log rank analyses were performed with STATVIEW. Maximum life span is the mean of 10% survival, except for ELAV-GeneSwitch flies, which have a 1% maximum life span. $P < 0.0001$ for each experiment except where noted. *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.10$.

neurons might lead to life span extension. The RU-486 Gene-Switch system allows for the comparison of genetically identical animals from the same cohort, one group receiving RU-486, which induces expression of the EP-UAS gene, and the other group receiving only diluent (30, 31). In two independent trials the maximum life span of adult *dSir2*^{EP2300}/ELAV-GeneSwitch flies receiving RU-486 was increased by 9% and 16%, respectively, in females and was decreased by 1% and increased by 10%, respectively, in males (Fig. 1g and h, respectively, and Table 1). In females, respective 5% and 12% increases in median life span were also seen. The smaller increase in life span in flies with the ELAV-GeneSwitch driver, relative to those with the ELAV driver, is consistent with a lower level of *dSir2* induction with the ELAV-GeneSwitch driver at the dose of RU-486 used (200 μ M). In addition, preliminary studies suggest that RU-486 itself may have some mild deleterious effects on life span in flies, especially on males (unpublished results). The greater life span extension obtained by using the standard ELAV driver could be due partly to increased *dSir2* activity before eclosion in adult ELAV-GAL4/*dSir2*^{EP2300} flies. Regardless, the results demonstrate that pan-neuronal overexpression of *dSir2* during only the adult stage is sufficient to produce a modest extension of maximal life span.

To further explore the possibility that a subset of neurons may be important in *dSir2*-mediated life span extension, we examined the life span of flies containing the D42-GAL4 motoneuron-specific driver (32) and *dSir2*^{EP2300}. No life span extension was seen in the D42/*dSir2*^{EP2300} flies (data not shown).

Decreases in physical activity, reproductive status (especially in females), or calorie intake are known to increase life span in

the fly (reviewed in ref. 33). Although we did not perform quantitative studies, visual inspection suggested no obvious decrease in physical activity or fertility in the long-lived *dSir2*-overexpressing flies compared with their matched controls. Furthermore, the fact that life span was found to be significantly increased in both males and females suggests that a potential decrease in female reproduction is unlikely to be the primary cause of the observed life span extension.

Considered together, the results of experiments driving *dSir2* demonstrate that overexpression of *dSir2* correlates well with increased life span in flies. In four different driver-GAL4/UAS-*dSir2* lines in which *dSir2* was substantially overexpressed either ubiquitously or in neurons, the life span of flies was extended significantly. However, when the driver caused only a small ubiquitous increase in *dSir2* or an increase only in motor neurons, life span was not extended. Furthermore, an intermediate increase in *dSir2* in the adult nervous system caused by the ELAV-GeneSwitch driver caused an intermediate increase in life span.

***dSir2* Is Necessary for the Life-Span-Extending Effect of Calorie Restriction.** Flies given low-calorie food, in addition to showing an increase in life span, showed an increase in *dSir2* mRNA expression (16). To determine whether *dSir2* is directly in the calorie-restriction life-span-extending pathway in flies, we compared the life span of flies that had reduced or no *dSir2* expression on a diet of low-calorie food with that of genetically identical flies from the same cohort on a diet of normal or high-calorie food. Flies with either no *dSir2* gene function [e.g.,

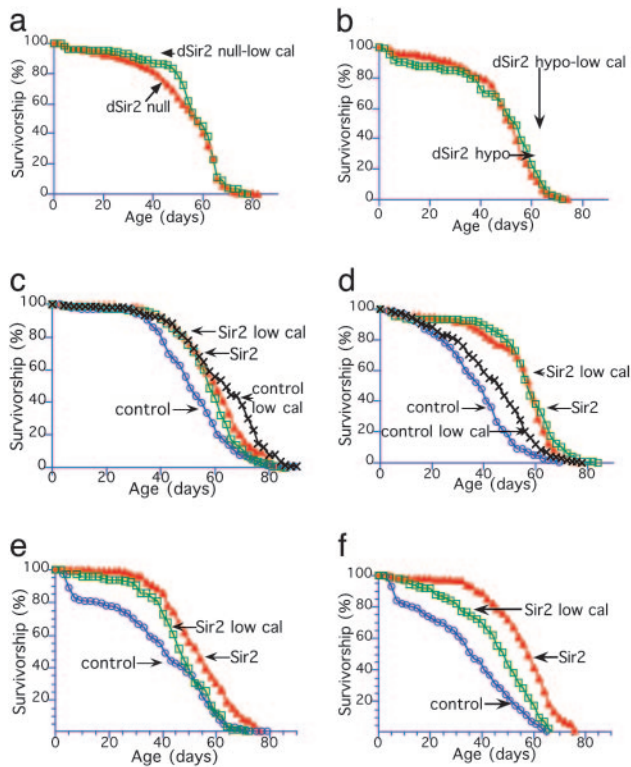


Fig. 2. Calorie-reduction life span extension is mediated by dSir2. (a and b) The expected life span extension with calorie reduction was blocked by mutations in dSir2. Survivorship curves of dSir2-null [*dSir2^{4.5}/dSir2^{5.26}*] (a) and dSir2-hypomorphic (*dSir2^{KG00871}/dSir2^{KG00871}*) (b) female flies on normal (red filled triangles) and low-calorie (green open boxes) food. Male flies mutant for dSir2 also had no life span extension in response to caloric reduction. (c–f) The life span of long-lived dSir2-overexpressing flies was not further extended by calorie reduction. Survivorship curves of long-lived ELAV-GAL4/*dSir2^{EP2300}* (c and d) and tubulin-GAL4/*dSir2^{EP2300}* (e and f) male (c and e) and female (d and f) flies on normal (red filled triangles) and low-calorie (green open boxes) food and those of genetically matched controls (c and d) on normal (blue open circles) and low-calorie (black X's) food. Low-calorie food was as described in ref. 16 and *Materials and Methods*. Each life span included at least 179 male and 171 female flies (16).

dSir2^{4.5}/dSir2^{5.26} (29)] or with severely decreased *dSir2* gene function (e.g., *dSir2^{KG00871}/dSir2^{KG00871}*) showed no life span extension on a diet of low-calorie food (Fig. 2 a and b) relative to genetically identical flies on a diet of normal or high-calorie food. The inability of *dSir2* mutant flies to increase their life span in response to a low-calorie diet demonstrates that a sufficient level of *dSir2* must be available for the activation of life span extension by calorie reduction and, furthermore, that dSir2 is an important element in the calorie-reduction life-span-extending pathway.

Calorie Restriction Does Not Further Extend the Life Span of Long-Lived dSir2-Overexpressing Flies. If *dSir2* mediates the effect of calorie-restriction-induced life span extension, we would also expect that calorie restriction would not further increase life span in flies in which dSir2 activity is already elevated. We examined the life span of two of the long-lived *dSir2*-overexpressing lines, tubulin-GAL4/*dSir2^{EP2300}* and ELAV-GAL4/*dSir2^{EP2300}*, under normal and low-calorie food conditions. Under our husbandry conditions, a decrease in caloric content in the food typically increased the life span of normal flies by 35–40% or more (16). However, when the long-lived ELAV-GAL4/*dSir2^{EP2300}* and tubulin-GAL4/*dSir2^{EP2300}* flies were placed on a diet of low-calorie food, no further increase in

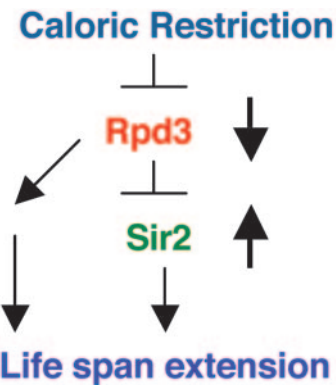


Fig. 3. Calorie-restriction/Rpd3/Sir2 pathway for life span extension in the fly. In this model a stimulus of calorie reduction results in a decrease in Rpd3 (16). The reduction in Rpd3 activity decreases the inhibition of Sir2, causing an increase in Sir2 (16). The increase in Sir2, in conjunction with the decrease in Rpd3, effects life span extension.

life span was seen with the ELAV-GAL4/*dSir2^{EP2300}* flies, whereas the tubulin-GAL4/*dSir2^{EP2300}* flies showed a reduction in life span toward normal (Fig. 2 c–f). Control flies for ELAV-GAL4/*dSir2^{EP2300}* placed on a diet of low-calorie food demonstrated a 28% and 22% increase in median life span for males and females, respectively (Fig. 2 c and d). The lack of a cumulative effect of calorie reduction and dSir2 overexpression on life span suggests that life span extensions are mediated through similar or related pathways.

dSir2 and Rpd3 Are in the Same Calorie-Restriction Pathway. The data presented here on dSir2, along with previous work on life span and calorie reduction of *rp3* mutants (16), indicate that the life-span-extending effects of nutrient reduction in the fly are mediated through dSir2 and Rpd3. The observation of an increase in dSir2 mRNA in long-lived *rp3* mutants (16) led us to postulate that the effect of Rpd3 may depend partially on an increase in dSir2. Together these data suggest a model for how calorie reduction extends life span in the fly (Fig. 3). In this model, the stimulus of calorie reduction triggers a decrease in Rpd3 activity and a subsequent increase in dSir2 activity. The increase in dSir2, either alone or in conjunction with additional changes initiated by the decrease in Rpd3 activity, results in life span extension.

Further confirmation that dSir2 and Rpd3 are in the same life-span-extending pathway was obtained by examining the life span of flies carrying a long-lived *rp3* mutation (*rp3^{def24}*) and a *dSir2* mutation (*dSir2¹⁷* or *dSir2^{EP2300}*). *dSir2* mutations do not reduce life span in otherwise normal flies (ref. 29 and data not shown). Therefore, if dSir2 were not in the same pathway as Rpd3, the life span would not be altered from the extended life span of *rp3* mutant flies. As predicted by our model, flies with both a *dSir2* mutation and *rp3^{def24}* mutation were not long-lived, whereas their counterparts, flies mutant for only *rp3^{def24}*, remained long-lived (Fig. 4). dSir2 and Rpd3, therefore, seem to be in the calorie-reduction life-span-extending pathway in flies. The finding that long-lived *rp3* mutations increase dSir2 levels (16) suggests that dSir2 is downstream of Rpd3.

Discussion

The availability and utilization of nutrients seems to be a central feature of a variety of environmental and genetic interventions that extend life span in many different organisms. Calorie restriction, in particular, is one of the most well conserved interventions known, extending life span in organisms as diverse as yeast and mammals (2, 4–12). Despite the nearly universal

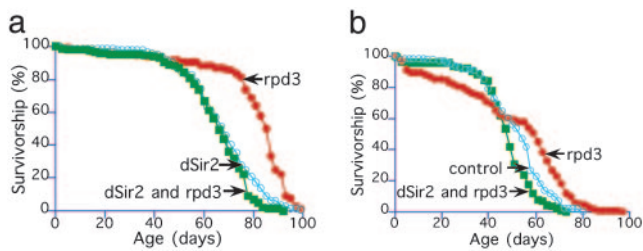


Fig. 4. Mutations in *dSir2* block the life-span-extending effect of *rpd3* mutants. The extension in life span seen with *rpd3* mutation was prevented by mutations in *dSir2*. Flies labeled as “*rpd3*” are heterozygous for the *rpd3*^{def24}-null allele of *rpd3*, whereas flies labeled as “*dSir2* and *rpd3*” possess one copy of the life-span-extending *rpd3*-null mutation and one copy of either the *dSir2*¹⁷-null (28) or *dSir2*^{EP2300}-hypomorphic allele. (a) *dSir2*¹⁷. Flies labeled “*rpd3*,” “*dSir2* and *rpd3*,” and “control” were each backcrossed to a Canton-5 background. (b) *dSir2*^{EP2300}. Flies labeled “*rpd3*,” “*dSir2* and *rpd3*,” and “control” were collected from the offspring of crosses between the *rpd3*-null allele, *rpd3*^{def24}, and *dSir2*^{EP2300}. Flies labeled “control” were derived from an additional cross to remove the *rpd3* and *dSir2*^{EP2300} alleles and any balancer chromosomes. Each life span included at least 200 male and 200 female flies (16).

effect on life span extension of calorie restriction, little is understood molecularly about how a decrease in nutrient availability triggers an extension in life span. Model organisms have proven useful for understanding the molecular and cellular mechanisms of other complex biological phenomena such as development. Organisms such as yeast, nematodes, and flies can rapidly provide information about causal relationships through the combined use of genetic and molecular approaches.

Calorie Restriction Extends Life Span in Flies by Increasing *dSir2*. The search for elements that extend life span in metazoans has identified the involvement of the insulin-signaling, nutrient-sensing, and Sir2 pathways (13, 14, 17–19, 34). Although the Sir2 pathway has been linked to calorie availability in yeast, it has not been shown to function in the calorie restriction pathway in metazoans (22). The data we present here demonstrate a direct link between the life-span-extending effects of *dSir2* and calorie restriction in the fly. We used five different GAL-4 drivers (tubulin, ELAV, armadillo, ELAV-GeneSwitch, and D42-motoneuron) to drive expression of endogenous *dSir2* genes with three separate nearby insertions of UAS elements. In four strains in which *dSir2* expression was increased substantially, either ubiquitously or in neuronal cells, the life span of the flies was extended substantially, up to 57% when *dSir2* mRNA expression was increased 4-fold. Conversely, in two other similarly constructed strains in which *dSir2* expression was not elevated or was only marginally elevated, life span was not altered relative to that of control flies. Thus, in six fly strains constructed by using different combinations of drivers and *dSir2* responders, increased longevity correlated very well with elevation of *dSir2*. Furthermore, we show here that life span cannot be extended by calorie restriction in flies that lack *dSir2* activity, nor can life span be further increased by calorie restriction in flies in which

dSir2 activity is already raised. The recent findings that a Sir2 agonist, resveratrol (shown to increase the activity of yeast, nematode, fly, and human Sir2) extends life span in yeast, nematodes, and flies in a manner that is Sir2-dependent and associated with calorie restriction provide additional evidence for a primary role of Sir2 activity in determining life span in metazoans (24, 25). Together, these observations make a strong case that calorie restriction extends life span by increasing *dSir2* activity.

***dSir2* Seems to Operate After Rpd3 in the Pathway Mediating Life Span Extension by Calorie Restriction.** The data presented here, in conjunction with our previous work on Rpd3 (16), show that *dSir2* and Rpd3 are important components in the calorie-restriction life-span-extending pathway of flies. A decrease in *dSir2* prevents the life-span-extending effect of calorie restriction, and the life-span-extending effect of calorie restriction is not cumulative with the life-span-extending effect of increased *dSir2*. Similarly, the life-span-extending effect of Rpd3 mutations is not cumulative with the effect of calorie restriction (16). We reported previously (16) that long-lived flies with reduced Rpd3 activity have elevated *dSir2* mRNA. We now show that, in flies with decreases in both Rpd3 and *dSir2* activity, life span is not extended, indicating that an increase in *dSir2* activity in response to a decrease in Rpd3 activity is necessary for life span extension. Together these data suggest that *dSir2* is downstream of Rpd3 in the calorie-restriction life-span-extending pathway in flies. This model provides a useful framework and testable model for examining the relationship of Sir2, calorie reduction, and longevity by using genetic, molecular, and pharmaceutical approaches (Fig. 3). The documentation of a molecular genetic pathway responsible for effecting calorie-restriction-related life span extension will be useful for identifying biochemical mediators and drug interventions that can mimic calorie restriction. Given the conservation of elements of the calorie restriction/Rpd3/Sir2 pathway in extending life span in yeast and now flies, agents that stimulate the activity of Sir2 are potential tools for extending life span in metazoans (24, 25).

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