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 affects 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 antagonist 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 dSir224.5 and dSir225.26-null mutant lines were obtained from S. Smolik (Oregon Health & Science University, Portland); the dSir227.7-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 dSir2EP2300, dSir2EP2384, and dSir2KG00871 mutant lines were obtained from the Bloomington Drosophila Stock Center at Indiana University; the dSir2Y0602 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 dSir2EP2300/Cyo, dSir2EP2384/Cyo, or dSir2Y0602/Cyo females. To generate flies that ubiquitously overexpressed dSir2 at a lower level, >90 armadillo-GAL4 male flies were crossed to >100 virgin dSir2EP2300/+; tubulin-GAL4+/+, dSir2EP2384/+; tubulin-GAL4+/+, dSir2Y0602/+; tubulin-GAL4+/+, armadillo-GAL4/dSir2EP2380 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 males flies from each condition to obtain white-eyed flies lacking balancer chromosomes, the tubulin-GAL4 driver chromosome, or chromosomes containing dSir2EP2300, dSir2EP2384, or dSir2Y0602. 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 dSir2EP2300 line to drive overexpression of dSir2. A single cohort of F1 dSir2EP2300/+; 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 μM or food with only diluent from the first day after eclosion.

Flies heteroallelic for dSir2-null mutations, dSir224.5/dSir225.26 flies, were generated by crossing dSir224.5/Cyo to dSir225.26/Cyo. Flies heterozygous for the null allele of rpd3 (rpd35674) and the null allele dSir227 (28) or the hypomorphic allele dSir2EP2300 were crossed to Canton-S flies to generate a matched genetic background. dSir227/dSir22-; rpd35674/rpd35674 flies were generated by

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crossing dSir2 (CyO and rpd3/TM6Sb) flies. dSir2\textsuperscript{EP2300}/dSir2\textsuperscript{+}; rpd3\textsuperscript{+}\textsuperscript{25} rpd3\textsuperscript{+} flies possessing a copy of the dSir2-hypomorph allele dSir2\textsuperscript{EP2306} and a copy of the rpd3-null allele rpd3\textsuperscript{del24} were generated in the same way. Control flies, generated in a similar manner, were obtained by mating >50 F\textsubscript{1} CyO; Sb (without dSir2\textsuperscript{EP2300} or rpd3\textsuperscript{del24}) male flies and >50 F\textsubscript{1} virgin CyO; Sb (without dSir2\textsuperscript{EP2300} or rpd3\textsuperscript{del24}) 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\textsuperscript{EP2300}, dSir2\textsuperscript{EP384}, or dSir2\textsuperscript{Ye06602} 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\textsuperscript{EP2300} tubulin-GAL4/dSir2\textsuperscript{EP384} and tubulin-GAL4/dSir2\textsuperscript{Ye06602} flies, with an increase across all lines of 29% for females and 18% for males (Fig. 1a–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\textsuperscript{EP2300} chromosome. The armadillo-GAL4 driver is a weaker driver than the tubulin-GAL4 driver: compared with control flies, armadillo-GAL4/dSir2\textsuperscript{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 cells (Fig. 8, which is published as supporting information on the PNAS web site). The finding of a prominent expression of dSir2 mRNA in the experimental flies but which do not contain the Armadillo 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).

ELAV-GAL4 drives expression in embryos and larvae as well as adults. A different system for overexpressing dSir2 was used to test whether increased expression of dSir2 only in adult
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
animals from the same cohort, one group receiving RU-486,
expression restricted to neurons throughout life (ELAV-GAL4/
dSir2EP2300) compared with their genetically matched controls.
Number of flies in each life span; Tub, tubulin-GAL4 driver; ELAV,
ELAV-GS, ELAV-GeneSwitch driver. (For example, 
Tub/ru-486) is a fly that possesses only one copy of the tubulin-GAL4
and one copy of the dSir2EP2300 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
Table 1. Life span is extended when Sir2 expression is increased

<table>
<thead>
<tr>
<th>Gender</th>
<th>Genotype</th>
<th>n</th>
<th>Median life span (% change)</th>
<th>Maximal life span (% change)</th>
</tr>
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<tbody>
<tr>
<td>F</td>
<td>Tub/dSir2EP2300</td>
<td>196</td>
<td>58 (57)</td>
<td>139.754</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>171</td>
<td>37</td>
<td>62.1</td>
</tr>
<tr>
<td>M</td>
<td>Tub/dSir2EP2300</td>
<td>194</td>
<td>54 (32)</td>
<td>37.024</td>
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<tr>
<td></td>
<td>Control</td>
<td>179</td>
<td>41</td>
<td>65.43</td>
</tr>
<tr>
<td>F</td>
<td>Tub/dSir2EP2300</td>
<td>185</td>
<td>52 (44)</td>
<td>84.391</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>159</td>
<td>36</td>
<td>62.75</td>
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<tr>
<td>M</td>
<td>Tub/dSir2EP2300</td>
<td>180</td>
<td>50 (14)</td>
<td>24.597</td>
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<tr>
<td></td>
<td>Control</td>
<td>149</td>
<td>44</td>
<td>67.53</td>
</tr>
<tr>
<td>F</td>
<td>ELAV/dSir2EP2300</td>
<td>208</td>
<td>59 (52)</td>
<td>135.190</td>
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<tr>
<td></td>
<td>Control</td>
<td>191</td>
<td>39</td>
<td>70 (19)</td>
</tr>
<tr>
<td>M</td>
<td>ELAV/dSir2EP2300</td>
<td>179</td>
<td>61 (20)</td>
<td>28.347</td>
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<tr>
<td></td>
<td>Control</td>
<td>202</td>
<td>51</td>
<td>75.29</td>
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<tr>
<td>F</td>
<td>Tub/dSir2EYO3602</td>
<td>193</td>
<td>41 (−15)</td>
<td>37.803</td>
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<td></td>
<td>Control</td>
<td>183</td>
<td>47</td>
<td>55.25 (−11)</td>
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<tr>
<td>M</td>
<td>Tub/dSir2EYO3602</td>
<td>184</td>
<td>47 (9)</td>
<td>62.24</td>
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<td></td>
<td>Control</td>
<td>161</td>
<td>43</td>
<td>58.79</td>
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<tr>
<td>F</td>
<td>ELAV-GS/dSir2EP2300</td>
<td>232</td>
<td>56 (12)</td>
<td>19.10</td>
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<tr>
<td></td>
<td>Control</td>
<td>202</td>
<td>51</td>
<td>84 (9)</td>
</tr>
<tr>
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<td>ELAV-GS/dSir2EP2300</td>
<td>234</td>
<td>60 (−5)</td>
<td>0.319*</td>
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<tr>
<td></td>
<td>Control</td>
<td>187</td>
<td>63</td>
<td>86 (−1)</td>
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<tr>
<td>F</td>
<td>ELAV-GS/dSir2EP2300</td>
<td>186</td>
<td>60 (5)</td>
<td>6.260**</td>
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<tr>
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<td>Control</td>
<td>200</td>
<td>56</td>
<td>88 (16)</td>
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<tr>
<td>M</td>
<td>ELAV-GS/dSir2EP2300</td>
<td>187</td>
<td>62 (−3)</td>
<td>2.659***</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>185</td>
<td>64</td>
<td>84 (11)</td>
</tr>
</tbody>
</table>

The median and maximal life spans of male (M) and female (F) flies with increased expression of dSir2 ubiquitously (tubulin-GAL4/ dSir2EP2300), tubulin-GAL4/dSir2EP2300, and tubulin-GAL4/dSir2EP2300, expression restricted to neurons throughout life (ELAV-GAL4/ dSir2EP2300), or expression restricted to adult neurons (ELAV-GeneSwitch/dSir2EP2300) compared with their genetically matched controls. Number of flies in each life span; Tub, tubulin-GAL4 driver; ELAV, ELAV-GAL4 driver; ELAV-GS, ELAV-GeneSwitch driver. (For example, Tub/ru-486) is a fly that possesses only one copy of the tubulin-GAL4 driver and one copy of the dSir2EP2300 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 = 0.57; **, P = 0.01; ***, P = 0.10.

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.,
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 interventions known, extending life span in organisms as diverse as yeast and mammals (2, 4–12).
mutations in dSir2. Flies labeled as “rpd3” are heterozygous for the mutants. The extension in life span seen with rpd3 additional cross to remove the null allele of dSir2. Flies labeled as “dSir2 and rpd3” possess one copy of the dSir2-null (28) or dSir2 knock-out homomorphyc allele. (a) dSir2. Flies labeled “rpd3,” “dSir2 and rpd3,” and “control” were each backcrossed to a Canton-S background. (b) dSir2 and dSir2 and rpd3, “rpd3,” “dSir2 and rpd3,” and “control” were collected from the offspring of crosses between the null allele, rpd3null, and dSir2null. Flies labeled “control” were derived from an additional cross to remove the rpd3 and dSir2null alleles and any balancer chromosomes. Each life span included at least 200 male and 200 female flies (16).

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 GAL4 drivers (tubulin, ELAV, armadillo, ELAV-Gene-Switch, 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 in flies 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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