Sirt6 regulates lifespan in *Drosophila melanogaster*

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Sirt6 is a multifunctional enzyme that regulates diverse cellular processes such as metabolism, DNA repair, and aging. Overexpressing Sirt6 extends lifespan in mice, but the underlying cellular mechanisms are unclear. *Drosophila melanogaster* are an excellent model to study genetic regulation of lifespan; however, despite extensive study in mammals, very little is known about Sirt6 function in flies. Here, we characterized the *Drosophila* ortholog of Sirt6, dSirt6, and examined its role in regulating longevity; dSirt6 is a nuclear and chromatin-associated protein with NAD^+-dependent histone deacetylase activity. *dSirt6* overexpression (OE) in flies produces robust lifespan extension in both sexes, while reducing *dSirt6* levels shortens lifespan. *dSirt6* OE flies have normal food consumption and fertility but increased resistance to oxidative stress and reduced protein synthesis rates. Transcriptomic analyses reveal that *dSirt6* OE reduces expression of genes involved in ribosome biogenesis, including many dMyc target genes. *dSirt6* OE partially rescues many effects of *dMyc* OE, including increased nuclear size, up-regulation of ribosome biogenesis genes, and lifespan shortening. Last, *dMyc* haploinsufficiency does not convey additional lifespan extension to *dSirt6* OE flies, suggesting *dSirt6* OE is upstream of *dMyc* in regulating lifespan. Our results provide insight into the mechanisms by which Sirt6 OE leads to longer lifespan.

Sirt6 | Myc | lifespan | aging | histone

Advanced age is the leading risk factor for many chronic diseases. Interventions which target the aging process itself may be useful to delay or prevent multiple age-related diseases simultaneously (1). At the cellular and molecular level, primary drivers of the aging process include genome instability, epigenetic alterations, telomere attrition, and loss of proteostasis (2). Enhancing the activity of genes which oppose these processes may therefore be an effective strategy to combat aging and age-related disease.

Sirtuins are a family of NAD^+-dependent protein deacylases well known for their role in regulating metabolism, genomic stability, and aging. Mammalian genomes contain seven sirtuins, and sirtuin overexpression (OE) has been shown to extend lifespan in yeast (3), worms (4), flies (5, 6), and mice (7).

Over the last 15 y, increasing evidence has defined Sirt6 as a key regulator of longevity with diverse antiaging functions across multiple cellular pathways (8). Most notably, Sirt6 OE extends lifespan in mice (9, 10) and ameliorates some aging phenotypes, while Sirt6 knockout mice are short lived and present an accelerated aging-like phenotype (11). Sirt6 is a nuclear-localized sirtuin that associates tightly with nucleosomes and chromatin (11, 12). In vitro, Sirt6 has weak deacetylase activity but is an efficient deacylase of long-chain fatty acids (13). Despite this, ample evidence suggests that Sirt6 functions in vivo as a transcriptional corepressor via deacetylation of H3K9ac near gene promoters (8). This discrepancy may be explained by the finding that Sirt6 deacetylase activity is enhanced by binding to free fatty acids (13), as well as association with nucleosomes (12). In addition, Sirt6 has mono-adenosine 5′-diphosphate (mono-ADP) ribosylation activity (14), which promotes DNA repair via PARP1 (15), retrotransposon (RTE) silencing via KAP1 (16), and activation of NRF2 target genes via BAF170 (17).

In mammalian systems, Sirt6 has been found to regulate an extensive list of cellular processes in a manner which may enhance longevity. Sirt6 plays a major role in DNA repair, rapidly localizing to damage sites and recruiting DNA repair factors and chromatin remodelers (15, 18, 19). In addition, Sirt6 enhances telomere stability via H3K9ac deacetylation (20). Sirt6 also opposes numerous proaging processes, including NF-κB signaling (21), RTE activation (16, 22), ribosome biogenesis (23), protein synthesis (24), and tumor growth (23). Although it is clear that Sirt6 possesses many functions relevant to aging, the precise mechanisms by which it is able to extend lifespan via OE are unclear.

Drosophila melanogaster represents an excellent model for investigating genetic regulation of longevity. *Drosophila* possess five sirtuin genes, orthologous to mammalian *Sirt1*, *Sirt2*, *Sirt4*, *Sirt6*, and *Sirt7*. Despite extensive study of *Sirt6* in mammals, very little is known about the function of the *Drosophila*

Significance

Sirt6 is well known for its role in regulating the aging process, particularly for its ability to extend lifespan in mice when overexpressed. However, the underlying molecular mechanisms responsible for lifespan regulation by Sirt6 are not well understood. Here, we characterized dSirt6 in fruit flies (*Drosophila melanogaster*). We found that dSirt6 functions very similarly to mammalian Sirt6 at the molecular and biochemical levels. Furthermore, overexpressing dSirt6 increased lifespan in flies. *dSirt6* overexpression extends lifespan, in part, by opposing the activity of Myc, a master regulator of protein synthesis, which is associated with decreased protein synthesis. These findings have relevance for the treatment of age-related disease by modulating Sirt6 activity.

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ortholog of *Sirt6* (herein called "*dSirt6*"). Here we characterize the *D. melanogaster* d*Sirt6* gene and investigate its role in regulating longevity.

Results

Drosophila Sirt6 Is a Nuclear Protein with NAD$^+$-Dependent Histone Deacetylase Function. To examine Sirt6 function in *Drosophila*, we cloned *dSirt6* into several different expression constructs and created transgenic flies with transgenes for wild-type *dSirt6* (*UAS-dSirt6*) and dSirt6 with a C-terminal GFP-fusion protein (*UAS-dSirt6-GFP*), under the control of UAS-regulatory sequences. Driving expression of *UAS-dSirt6-GFP* in the whole fly with *daughterless-GAL4* (*da*-GAL4), we observed a strong nuclear GFP signal in all tissues of larvae (salivary gland shown in Fig. 1*A*). In addition, dSirt6-GFP protein was strongly associated with salivary gland polytene chromosomes (Fig. 1*B*), as evident by immunofluorescent staining. We next quantified NAD$^+$-dependent deacetylase activity of dSirt6 using an in vitro deacetylase assay. dSirt6 deacetylated H3K9ac peptide substrate in an NAD$^+$-dependent fashion and at a rate very similar to human SIRT6 (Fig. 1*C*). Furthermore, we found that adult *dSirt6* OE flies have reduced levels of H3K9ac in vivo, at both young (10 d old) (Fig. 1*D*) and old (40 d old) (*SI Appendix*, Fig. S1) ages, compared to wild-type w^{1118} controls. The observed reduction of H3K9ac during *dSirt6* OE was consistent with previous findings (25). We also found reduced levels of H3K56ac in *dSirt6* OE flies (*SI Appendix*, Figs. S1 and S2). Overall, these results indicate that, like mammalian SIRT6, dSirt6 is a predominantly nuclear and chromatin-associated protein that functions as an NAD$^+$-dependent histone deacetylase.

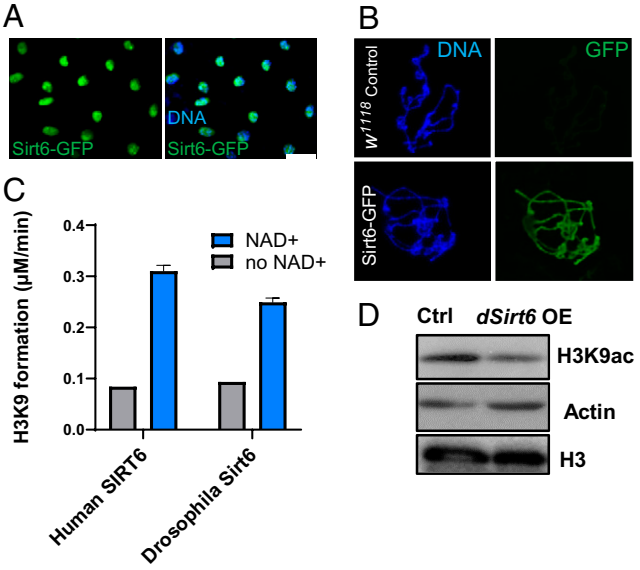

Fig. 1. *Drosophila Sirt6* is a chromatin associated protein with NAD$^+$-dependent histone deacetylase activity. (*A*) *da-GAL4>dSirt6-GFP* immunofluorescence (green) in live, whole-mount larvae salivary gland. (Scale bar, 50 μM.) (*B*) Polytene chromosome preparation from salivary glands of *da-GAL4>*w^{1118} (*Top*; "control") and *da-GAL4>UAS-dSirt6-GFP* (*Bottom*; "Sirt6-GFP") third instar larvae. As GFP fluorescence is quenched by polytene preparation, dSirt6-GFP was visualized by staining with fluorescent antibody to GFP (green). In both *A* and *B*, DNA was labeled by Hoechst dye and shown in blue. Images were taken at 20× magnification. (*C*) H3K9ac in vitro deacetylation assay with human SIRT6 and dSirt6, in the presence or absence of NAD$^+$. Rate of formation of the deacetylated H3K9 peptide is quantified; $n = 3$ independent replicates. Gray bars, without NAD$^+$; blue bars, with NAD$^+$. Error bars represent the SEM. (*D*) Western blot of H3K9ac from control (*tub-GAL4>*w^{1118}) and dSirt6 OE (*tub-GAL4>UAS-dSirt6*) eviscerated abdomen lysates from 10-d-old female flies ($n = 20$ flies per group). Histone H3 and actin were used as loading controls.

Modulating dSirt6 Levels Influences Organismal Lifespan. Having established the conserved cellular localization and histone deacetylase activity of dSirt6 in flies, we next tested whether its role in lifespan regulation was also conserved. We began by overexpressing *dSirt6* in the whole body using *tubulin-GAL4* (*tub-GAL4*) to drive *UAS-Sirt6*, and measuring lifespan. Strikingly, whole-body *dSirt6* OE increased median lifespan by 38% in females and 33% in males (Fig. 2 *A* and *B*), compared to *tub-GAL4>*w^{1118} controls. We repeated these experiments twice more and consistently found that *dSirt6* OE extends lifespan, ranging from 25 to 38% in females, and 10 to 33% in males (Dataset S1). A second *UAS-dSirt6* line, created using site-specific integration of the *UAS-dSirt6* transgene into an *attP* landing site, also reproducibly extended lifespan when crossed to *tub-GAL4* flies, compared to controls (*SI Appendix*, Fig. S3 and Dataset S1). Whole-body OE of the native *dSirt6* gene, achieved using an *EP-dSirt6* line (which contains a *GAL4* activation site inserted ∼250 bp upstream of the *dSirt6* 5′ untranslated region), also extended lifespan, by 14% in males and 17% in females (Fig. 2 *C* and *D*). In the experiments in Fig. 2 *C* and *D*, we activated *EP-dSirt6* using the mifepristone (RU486) inducible *tubulin-GeneSwitch* (*tubGS*) driver, which activates the UAS-transgene upon flies being fed RU486. Flies fed diluent (EtOH) control serve as genetically identical controls from the same cohort (26). Together, these results indicate that OE of both transgenic and the native *dSirt6* gene extends lifespan in both male and female flies. Conversely, reducing *dSirt6* expression levels by ∼40% by RNA interference (RNAi) in adult flies (*SI Appendix*, Fig. S4) using the *tubGS* system shortened median lifespan by 12% and 10% in males and females, respectively (Fig. 2 *E* and *F*). As an additional control, we found that expressing *mCherry* RNAi in this same system did not shorten lifespan in either sex (*SI Appendix*, Fig. S3 and Dataset S1). Last, we found that overexpressing *dSirt6* in the fat body alone, using the fat body–specific GeneSwitch driver *S106*, was sufficient to extend lifespan by 8% in male and 14% female flies (Fig. 2 *G* and *H*).

Longevity-Associated Phenotypes in dSirt6 OE Flies. We next asked whether lifespan extension in *dSirt6* OE flies was associated with reduced nutrient intake or reduced fertility, as either of these phenotypes can lead to increased longevity (27, 28). Food consumption was not altered in *dSirt6* OE flies, as measured by Capillary Feeder (CAFE) assay (*SI Appendix*, Fig. S5). Similarly, we did not observe any difference in the fertility of *dSirt6* OE females (*SI Appendix*, Fig. S6), compared to controls. We then examined phenotypes previously shown to be impacted by Sirt6 levels in mammalian systems that can also impact longevity, specifically, oxidative stress resistance (15) and protein synthesis rate (29). *dSirt6* OE flies survived longer than controls when treated with the oxidative stress-inducing agent paraquat (Fig. 3 *A*–*D*), particularly in old (40 d old) flies (Fig. 3 *B* and *D*), indicating *dSirt6* OE conveys increased resistance to oxidative stress. In addition, we found that protein synthesis is reduced by ∼35% in *dSirt6* OE flies compared to controls, as measured by surface sensing of translation (SUnSET) assay (30, 31) (Fig. 3 *E* and *F*). Given the reduction in protein synthesis, we also examined body weight and found that both male and female dSirt6 OE flies weigh ∼5% less than genetically matched controls (Fig. 3*G*). Finally, we also examined the impact of dSirt6 OE on physical function in aged flies by measuring their climbing ability. As expected, control flies showed a significant decline in climbing performance from day 10 to day 30 (*SI Appendix*, Fig. S7); however, dSirt6 OE flies had preserved climbing ability at day 30. Together, these data indicate that *dSirt6* OE may extend lifespan and health span in flies via increased oxidative stress resistance and/or reduced protein synthesis.

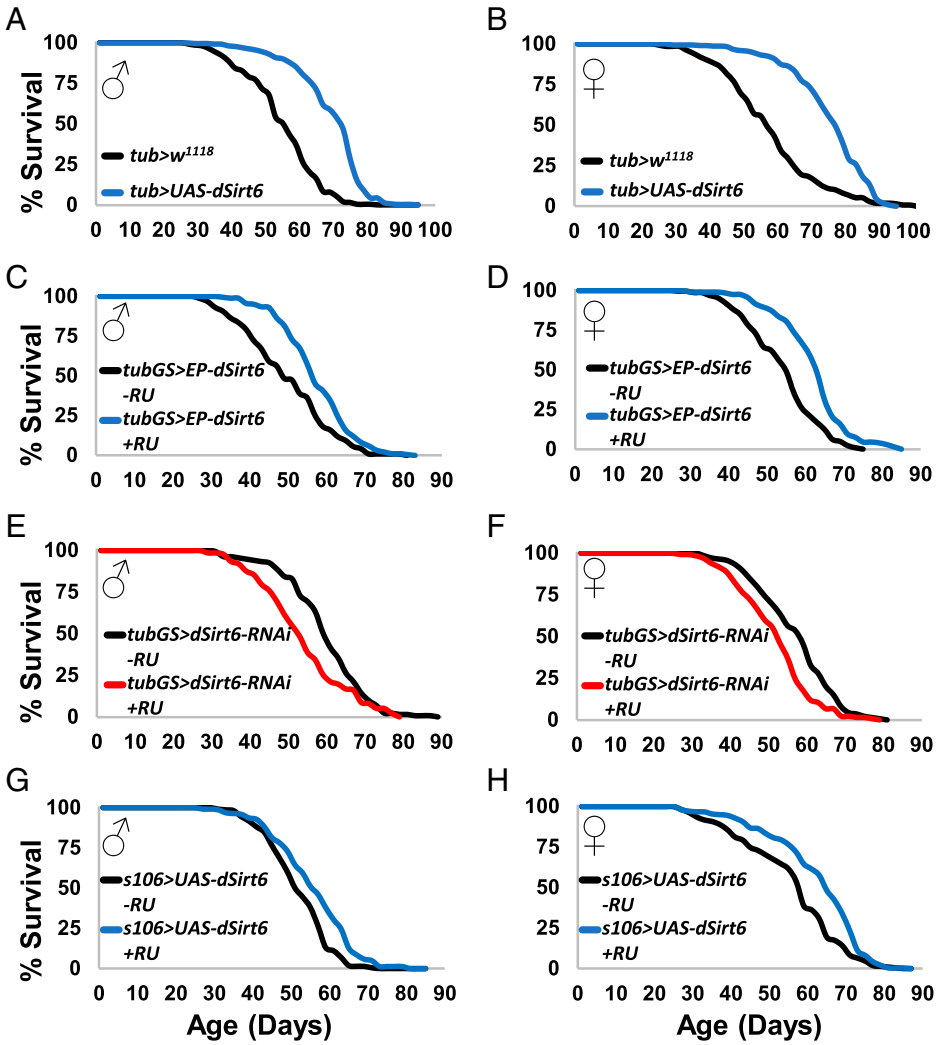

Fig. 2. Effect of dSirt6 on lifespan. Flies overexpressing *dSirt6* in whole body (*tub-GAL4>UAS-dSirt6*; blue lines) are long-lived compared with *tub-GAL4>w*^{1118} controls (black lines), showing a 33% median lifespan increase in males (*A*) and a 38% increase in females (*B*). OE of the native dSirt6 gene using *tubGS>EP-dSirt6* fed RU486 (blue lines) extends median lifespan by 14% in males (*C*) and 17% in females (*D*), compared to diluent-fed controls (*tubGS>EP-dSirt6* -RU; black lines). Knockdown of *dSirt6* by RNAi (*tubGS>dSirt6-RNAi* +RU486; red lines) shortens median lifespan by 12% in males (*E*) and 10% in females (*F*), compared to diluent-fed controls (*tubGS>dSirt6-RNAi* -RU; black lines). Fat body–specific *dSirt6* OE (*S106>UAS-dSirt6* +RU486; blue lines) extends median lifespan by 8% in males (*G*) and 14% in females (*H*), compared to diluent-fed controls (*S106>UAS-dSirt6* -RU; black lines). Log-rank *P* value < 0.0001 for all lifespans is shown. Full statistics, including number of individuals assayed and median lifespan values for all experiments, are presented in Dataset S1.

***dSirt6* OE Reduces Expression of dMyc Target Genes.** Given Sirt6's role as a histone deacetylase and major regulator of gene expression in mammals, we next sought to determine whether *dSirt6* OE altered expression of genes involved in stress resistance and/or growth and protein synthesis. To address this, we performed RNA sequencing (RNA-seq) of fat body tissue from young (10 d old) and old (40 d old) control and *dSirt6* OE flies. *dSirt6* OE had a significant impact on gene expression, resulting in 130 up- and 242 down-regulated genes in 10-d-old flies, and 413 up- and 204 down-regulated genes in 40-d-old flies (false discovery rate [FDR] <0.05 and $\log_2$ fold change [FC] > 0.585 or < −0.585 [1.5× FC]), versus age-matched controls (Fig. 4 *A* and *B* and Dataset S2). Pathway analysis of genes significantly down-regulated by d*Sirt6* OE revealed a strong enrichment for endoplasmic reticulum and protein processing genes, as well as previously described functions of mammalian Sirt6, such as negative regulation of ribosomal and glycolysis genes (Dataset S3). Although many genes were also up-regulated in *dSirt6 OE* flies, these genes showed minimal enrichment for specific pathways in young animals (Dataset S3 and *SI Appendix*, Fig. S8). Old *dSirt6* OE flies had increased expression of immune response genes as well as lipid metabolism genes (Dataset S3).

We also conducted gene set enrichment analysis (GSEA) (32) to identify pathways affected by *dSirt6* OE. GSEA identified pathways similar to those described above (Fig. 4 *C* and *D*), as well as several additional pathways. Notably, *dSirt6* OE in young flies was associated with decreased expression of genes in multiple annotated datasets related to ribosome biogenesis and protein synthesis, including "transfer RNA (tRNA) metabolic processes," "ribosome biogenesis," "noncoding RNA metabolic processes," and "nucleolus" (Fig. 4*C*). In old flies, *dSirt6* OE was associated with decreased expression of cytosolic ribosomal protein genes and translation genes, as well as decreased expression of mitochondrial matrix and oxidative phosphorylation genes, compared to age-matched controls (Fig. 4*D*).

dMyc [the *Drosophila* ortholog of mammalian Myc genes (33)] is a master regulator of ribosome biogenesis genes in

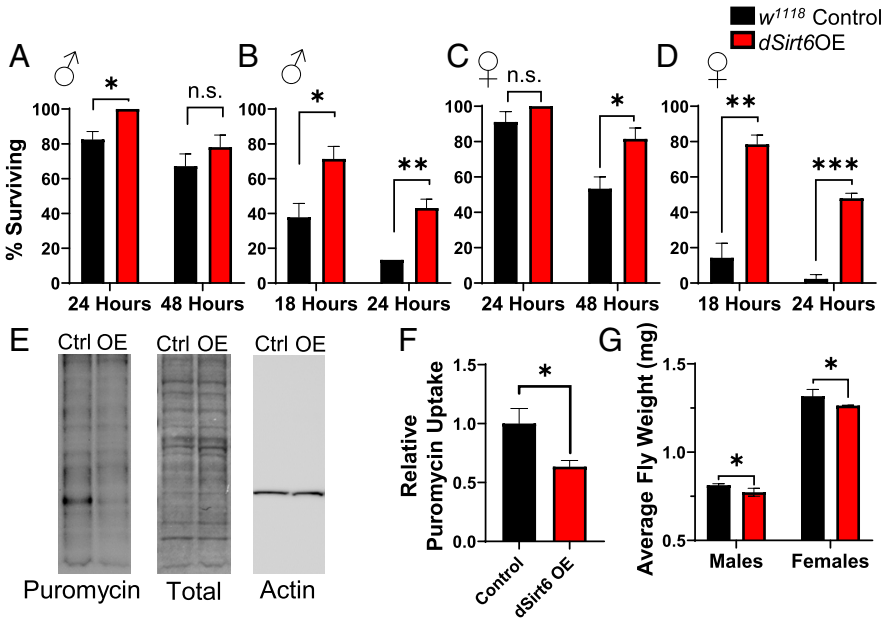

Fig. 3. *dSirt6* OE Flies have increased resistance to oxidative stress, decreased protein synthesis, and reduced body weight. (*A–D*) Survival of w^{1118} control (black bars) and *dSirt6* OE (red bars) flies maintained on 20 mM paraquat. *dSirt6* OE improves survival in young (10 d old) male (*A*) and female flies (*C*), and strongly improves survival in old (40 d old) male (*B*) and female (*D*) flies. (*E*) Western blot analysis depicting reduced puromycin incorporation into eviscerated abdomen tissue (enriched for fat body), a measurement of protein synthesis rate (SUnSET assay), in female *dSirt6* OE flies compared to controls. Total protein and actin were used as loading controls. (*F*) Quantification of SUnSET assay results, normalized to total protein; $n = 3$ replicates of 10 flies each. (*G*) *dSirt6* OE flies have reduced body weight compared to controls; $n = 3$ replicates of 10 pooled flies each. In all experiments, the genotype of control flies was *tub-GAL4>*w^{1118}, and the genotype of *dSirt6* OE flies was *tub-GAL4>UAS-dSirt6*. n.s., not significant; $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, unpaired two-tailed *t* test. Error bars represent SEM.

Drosophila, and, in mammals, Sirt6 acts as a negative regulator of Myc-induced transcriptional activation of ribosomal protein genes (23, 34). Our RNA-seq results, along with these previous findings, led us to ask whether *dSirt6* OE may be acting as a negative regulator of dMyc target genes. To test this, we created two custom gene sets for GSEA: one of genes up-regulated by dMyc OE in *Drosophila* ["dMyc Induced" (35)], and the other of genes with high-confidence dMyc binding sites near their TSS (determined by chromatin immunoprecipitation sequencing [ChIP-seq]) and whose expression is also reduced by *dMyc* RNAi ["dMyc Bound" (36)] (Dataset S4), and examined whether expression of these genes was altered by *dSirt6* OE. In young flies, the "dMyc Induced" gene set showed the strongest enrichment (based on normalized enrichment score [NES]) of any gene set for down-regulation by *dSirt6* OE (Fig. 4 *C* and *E*), and was also among the top gene sets down-regulated by *dSirt6* OE in old flies (Fig. 4 *D* and *F*). The "dMyc Bound" gene set was also among the top sets of genes down-regulated by *dSirt6* OE in both young and old flies (Fig. 4 *C–F*). Expression of *dMyc* itself was moderately elevated in young *dSirt6* OE flies (1.58 FC, FDR = 0.007) vs. control flies, possibly reflecting a cellular compensation for reduced expression of *dMyc* target genes, but did not differ vs. controls in old *dSirt6* OE flies. Together, these data strongly indicate that *dSirt6* OE reduces the expression of dMyc target genes, which are primarily involved in ribosome biogenesis.

Mammalian Sirt6 has been previously shown to repress target genes via deacetylation of H3K9ac at promoter-proximal regions and gene bodies (34). We examined whether dMyc target genes with reduced transcript levels in *dSirt6* OE flies also had lower levels of H3K9 acetylation. Using ChIP-qPCR on chromatin from fat body tissue, we found that H3K9ac was strongly enriched near the transcription start site (TSS) of selected dMyc target genes in control flies (*SI Appendix*, Fig. S9). These regions had reduced levels of H3K9ac in *dSirt6* OE flies, suggesting epigenetic repression of these dMyc target genes by dSirt6.

dSirt6 and dMyc Are Epistatic. Reducing Myc expression extends lifespan in both mice (37) (*c-Myc*) and flies (38) (*dMyc*). Given the connection between reduced Myc activity and longevity, along with our results that *dSirt6* OE reduces expression of dMyc target genes, we sought to further explore the role of dSirt6 in repressing dMyc function. We used a well-characterized *UAS-dMyc* line (39) to OE *dMyc* in adulthood ("*dMyc* OE"), and tested whether co-OE of *dSirt6* could alter or reverse *dMyc*-induced phenotypes. *dMyc* OE in *Drosophila* causes endoreplication and increased transcription of nucleolar and ribosome biogenesis genes, leading to increased nuclear and nucleolar size (35). Thus, we asked whether co-OE of *dSirt6* together with *dMyc* ("*dMyc+dSirt6* co-OE") could also attenuate the increase in nuclear size caused by *dMyc* OE. *dMyc* OE led to a 2.2-fold increase in median nuclear cross-sectional area in the adult fat body (24.4 μM^2 in controls vs. 53.3 μM^2 in dMyc OE, $P < 0.0001$) (Fig. 5 *A* and *B*), a size increase consistent with previous results in larvae (40). Co-OE of *dSirt6* together with *dMyc* attenuated this size increase by 0.59-fold (median size: 39.3 μM^2 in *dMyc+dSirt6* co-OE vs. 53.3 μM^2 in *dMyc* single-OE, $P < 0.0001$ [Fig. 5 *A* and *B*]). In addition to increased nuclear size, *dMyc* OE is also associated with increased protein synthesis. Since we had already observed that *dSirt6* OE flies have reduced protein synthesis, we also tested whether *dSirt6* OE could block increased protein synthesis caused by *dMyc* OE. As expected, we observed increased protein synthesis rates (approximately twofold) in *dMyc* OE flies, compared to controls. However, co-OE of *dSirt6* almost completely blocked this increase (*SI Appendix*, Fig. S10). Together, these data suggest *dSirt6* OE directly counteracts the effects of *dMyc* OE at the cellular level.

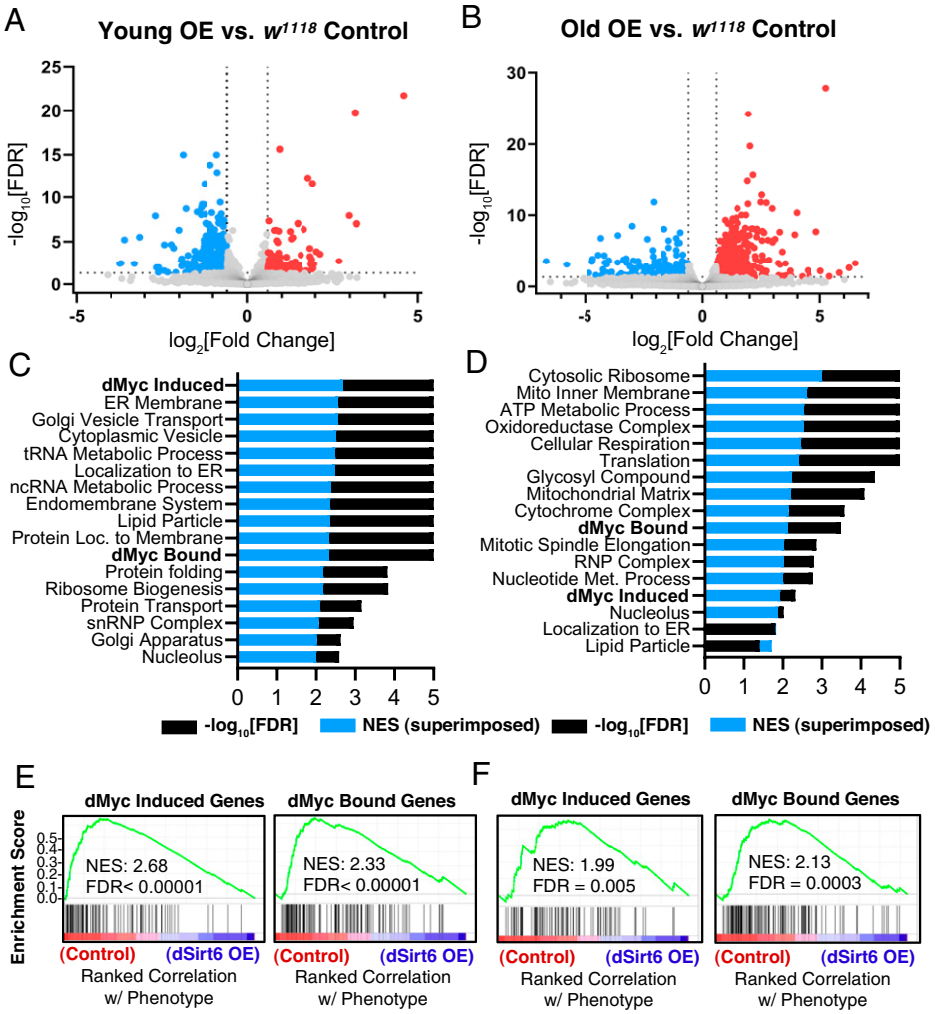

Fig. 4. *dSirt6* OE reduces ribosome biogenesis and expression of Myc target genes. (*A* and *B*) Volcano plots showing genes significantly up-regulated (red) and down-regulated (blue) by *dSirt6* OE in young, 10-d-old (*A*) and 40-d-old (*B*) fat body tissue, as determined by RNA-seq. Dotted lines indicate FDR cutoff of <0.05 (*y* axis) and $\log_2$ FC cutoff of ±0.585 (1.5× FC) (*x* axis). Genes that did not meet FDR and FC cutoff are colored gray. (*C* and *D*) Top GSEA terms identified in genes down-regulated by *dSirt6* OE in young (*C*) and old (*D*) flies; "dMyc Induced Genes" is a custom gene set of previously published genes up-regulated upon *UAS-dMyc* activation in *Drosophila* (35); "dMyc Bound Genes" is a custom gene set of previously published genes bound by dMyc in ChIP-seq experiments in *Drosophila* (36). GSEA reports FDR values < 0.00001 as zero, thus a –log10[FDR] of five means FDR ≤ 0.00001. NES reflects the degree to which a gene set is overrepresented, and is superimposed on top of −log10[FDR]. (*E* and *F*) Representative GSEA plots showing that most dMyc-induced and dMyc-bound genes exhibit higher expression in control genotype in young (*E*) and old (*F*) flies, compared to *dSirt6* OE flies (i.e., expression is lower in *dSirt6* OE flies). Green lines indicate enrichment score. Female flies were used for RNA-seq experiments, the genotype of control flies was *tub-GAL4>w*^{1118}, and the genotype of *dSirt6* OE flies was *tub-GAL4>UAS-dSirt6*.

Next, we performed RNA-seq on fat body tissue from control, *dMyc* OE, and *dMyc+dSirt6* co-OE flies (Fig. 5*C* and Dataset S5). We used a stringent FC and FDR cutoff (FDR < 0.01, FC > 2) to select the genes most strongly up-regulated by *dMyc* OE alone, and asked whether their expression was lower when *dMyc* OE was accompanied by *dSirt6* OE. Of the 627 genes up-regulated by *dMyc* OE alone under these significance criteria, 398 (64%) had considerably lower expression in *dMyc+dSirt6* co-OE flies (FDR 0.05, $\log_2$ FC < −0.263; median $\log_2$ FC = −0.59). Gene ontology analysis of dMyc-induced genes which are significantly down-regulated by *dSirt6* co-OE revealed that most of these genes are involved with various aspects of ribosome biogenesis (Fig. 5*D*), in agreement with these groups of genes being induced by dMyc (35, 36) and also in agreement with our previous results (Fig. 4 *C* and *D*) that *dSirt6* OE reduces ribosome biogenesis genes. Interestingly, the 229 dMyc-induced genes that were not statistically significantly attenuated by *dSirt6* co-OE were enriched for DNA replication and DNA repair genes (Fig. 5*E*); these two categories were not enriched in the 398 dMyc-induced genes reduced by *dSirt6* co-OE. Importantly, dMyc protein levels are not decreased by *dSirt6* co-OE, compared to *dMyc* OE alone (*SI Appendix*, Fig. S11). Finally, we used the 627 genes up-regulated by dMyc OE in adult fat body to create a custom gene set, and used this to run GSEA on our previous RNA-seq data from control and *dSirt6* OE flies. This gene set had significantly lower expression in *dSirt6* OE flies versus controls (FDR = 0.0027, NES = 2.05) (*SI Appendix*, Fig. S12), further supporting the idea that *dSirt6* OE represses dMyc target genes.

Having established that *dSirt6* OE opposes *dMyc* activity at the cellular and molecular levels, we next examined the interplay between these two genes in regulating lifespan. We first measured lifespan in the same three groups used above: *control*, *dMyc* OE, and *dMyc+dSirt6* co-OE. As expected based on previous work (38), *dMyc* OE shortened lifespan considerably, by 16% in male (Fig. 5*F*) and 36% in female (Fig. 5*G*) flies.

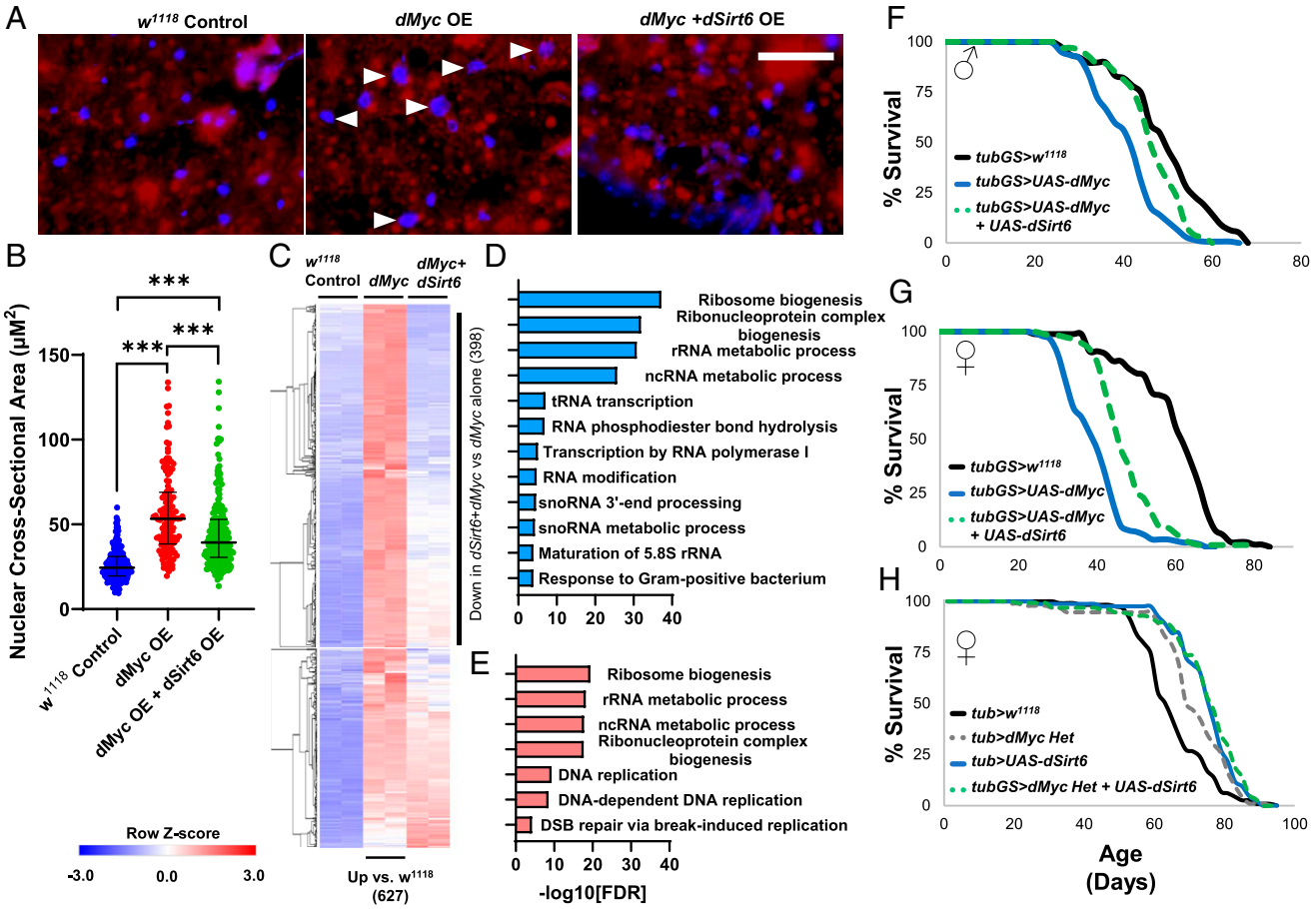

Fig. 5. *dSirt6* OE attenuates the effects of dMyc OE. (*A*) Representative images of fat body nuclei from abdominal cross-sections of adult control flies (*tubGS>w*^{1118} +RU486, "*w*^{1118} Control") and flies overexpressing *dMyc* (*tubGS>UAS-Myc* +RU486, "*dMyc* OE") or flies overexpressing both *dMyc* and *dSirt6* (*tubGS>UAS-Myc*; *UAS-dSirt6* +RU486, "*dMyc* + *dSirt6* OE"). Fat body tissue was identified by peripheral location in abdomen and presence of lipid droplets stained by Nile Red (red); nuclei are stained with Hoechst (blue). White arrowheads indicate enlarged nuclei induced by *dMyc* OE. (Scale bar, 50 μM.) (*B*) Quantification of nuclei cross-sectional area shown in *A*; n = 200, 137, and 224 individual nuclei for control, *dMyc* OE, and *dMyc*+*dSirt6* co-OE, respectively. Dots represent individual nuclei, bars are interquartile range, *** = adjusted *P* value < 0.0001, one-way ANOVA with Tukey's multiple comparison test. (*C*) RNA-seq heatmap of 627 genes most strongly up-regulated (FC > 2, FDR < 0.01) in fat body of adult *dMyc* OE flies (*Middle*) compared to controls (*Left*), and relative expression (Z score) of these same genes in *dMyc*+*dSirt6* co-OE flies (*Right*). The top portion of the heatmap represents 398 genes which were statistically significantly lower in *dMyc*+*dSirt6* co-OE flies vs. *dMyc* OE alone; the bottom portion represents 229 genes that were not statistically significantly lower in double-OE flies vs. *dMyc* OE alone; *n* = 2 replicates of 10 fat bodies each per group. (*D*) Top pathways (FDR < 0.0005) identified by gene ontology (GO) term analysis of 398 genes significantly lower in double-OE flies vs. *dMyc* OE alone; (*E*) top pathways identified by GO term analysis of 229 genes that were not significantly lower in double-OE flies vs. *dMyc* OE alone. Genotypes for RNA-seq experiment are the same as listed in *A*. (*F* and *G*) Lifespan curves of control (solid black line), *dMyc* OE (solid blue line), and *dMyc*+*dSirt6* co-OE (dashed green line) male (*F*) and female (*G*) flies, using same genotypes listed in *A*. (*H*) Lifespan curve for control (*tub-GAL4>w*^{1118}; solid black line), *dSirt6* OE (*tub-GAL4>UAS-dSirt6*; solid blue line), *dMyc* heterozygote (*tub-GAL4>w*^{1118}*;dMyc*^{4} dashed gray line), and *dSirt6 OE* + *dMyc* Heterozygote (*tub-GAL4>UAS-dSirt6;Myc*^{4}; dashed green line) flies. Log-rank adjusted *P* value < 0.05 for all comparisons in *H*, except for *dSirt6* OE vs. *dSirt6* OE + *dMyc* Heterozygote (adjusted *P* value > 0.05, with Bonferroni correction). Full statistics, including *n* (number of individuals assayed), median, mean, and maximum lifespan values for all experiments, are presented in Dataset S1.

dSirt6 co-OE partially rescued this effect, with *dMyc+dSirt6* co-OE flies having a median lifespan 10% longer than *dMyc* OE flies in both males (Fig. 5*F*) and females (Fig. 5*G*) (*P* < 0.005).

Given our findings that *dSirt6* OE reduces *dMyc* activity, and previous results indicating that reducing *dMyc* expression extends lifespan, we next tested whether lifespan extension by *dSirt6* OE was mediated by decreased dMyc activity. In agreement with previous findings, *dMyc* heterozygous flies, containing a null mutation in a single copy of dMyc ("*dMyc*^{4}") (38), were long-lived (median lifespan: 63 d vs. 71 d; 13% increase, adjusted [adj] *P* value < 0.00001) compared to *dMyc* homozygous sibling controls. *dSirt6* OE also extended median lifespan at levels consistent with our earlier findings (median lifespan: 63 d vs. 75 d; 19% increase, adj *P* value < 0.00001). However, overexpressing *dSirt6* in *dMyc* heterozygous flies conveyed only a very mild additional lifespan benefit (median lifespan: 75 d vs. 78 d; 4% increase, adj *P* value > 0.05), versus *dSirt6* OE alone. These findings suggest that *dSirt6* OE and *dMyc* haploinsufficiency may act within the same lifespan-extending pathway and that reducing dMyc activity is sufficient to account for a significant portion of the lifespan extension gained by *dSirt6* OE.

Discussion

Mammalian SIRT6/Sirt6 has been extensively studied and is appreciated for its important roles in multiple cellular

functions, disease, and longevity. Despite this, less is known about the *Drosophila* ortholog, dSirt6. We have performed an initial characterization of dSirt6 in *Drosophila*, particularly in regard to its molecular function and role in aging and longevity. We found that the ability of *Sirt6* OE to extend lifespan is conserved in lower eukaryotes, and we identified repression of *dMyc* transcriptional activity as a key mechanism for lifespan extension by *dSirt6* OE (*SI Appendix*, Fig. S13).

Like mammalian SIRT6, we found that dSirt6 is a chromatin-associated protein with NAD^+-dependent histone deacetylase activity. Specifically, dSirt6 exhibits in vitro deacetylase activity toward H3K9ac, and *dSirt6* OE leads to a strong in vivo reduction of H3K9ac. This reduction was observed both at the total protein level and near the TSS of dMyc target genes.

One of the most exciting discoveries made about Sirt6 is that increasing its expression levels extends lifespan in mice (9, 10). The current study provides important confirmation that Sirt6 OE extends organismal lifespan in other species. Additionally, by using the GeneSwitch-GAL4 system, we have demonstrated that *dSirt6* OE starting in adult life (i.e., not during development) is sufficient to extend lifespan. A recent report found that OE of mouse and beaver Sirt6 using the GeneSwitch system also extended lifespan in flies (41), supporting the notion that adult-specific OE is sufficient for lifespan extension. Finally, fat body–specific *dSirt6* OE was sufficient to convey some lifespan extension. *Drosophila* fat body is analogous to adipose, liver, and immune tissues of mammals. Interestingly, long-lived Sirt6 OE mice have reduced AKT phosphorylation in liver and white adipose tissue (9). A recent report also found that Sirt6 OE protects against age-related decline in hepatic glucose output and homeostasis, in part by enhancing glycerol release from adipose tissue (10). Together, these findings implicate the importance of these tissues in mediating lifespan extension by Sirt6 OE.

A major goal of our studies was to better understand the molecular mechanisms by which dSirt6 OE extends lifespan. Our transcriptomic analysis indicates that dSirt6 is a strong negative regulator of ribosome biogenesis and protein synthesis genes, which are also dMyc target genes. dMyc has been well studied in the context of transcriptional activation, with the core set of dMyc transcriptional targets consistently including ribosomal protein, ribosomal RNA processing, tRNA processing, and translation factor genes (35, 36). Importantly, our GSEA results showed that *dSirt6* OE led to decreased expression of two independent dMyc target gene sets, which are largely distinct from one another (only 16 common genes between 230 genes total; Dataset S4). This result was observed in both young and old flies. Mammalian Sirt6 was previously reported to act as a repressor of Myc transcriptional activity, although primarily in tumor cells (23). Our findings suggest that this function of Sirt6 is conserved in flies, and is a function of dSirt6 in healthy tissue. Myc does not appear to be deacetylated by Sirt6. Rather, the current model proposes that Sirt6 represses glycolysis and ribosomal protein gene transcription by binding to Pol II and blocking the recruitment of transcription elongation factors such as Myc, and deacetylating H3K9ac and H3K56ac, thereby promoting transcriptional pausing (34). Consistent with this, we found reduced ribosomal protein and glycolysis gene expression and reduced levels of H3K9ac in *dSirt6* OE flies, both at the protein level and specifically at the promoter and/or gene body of dMyc target genes that had reduced transcript levels in *dSirt6* OE flies. The requirement of dSirt6 deacetylase function for lifespan extension is still an important question and should be addressed in future studies.

Expression levels of Myc and ribosome biogenesis genes are major determinants of protein synthesis rates (29, 31, 37). Our initial phenotypic characterization of *dSirt6* OE flies revealed that they have reduced protein synthesis, a phenotype strongly associated with increased longevity (29, 42). A recent report demonstrated that protein synthesis is significantly elevated in mice with reduced Sirt6 levels ($Sirt6^{+/-}$ mice) (24). Importantly, this study also found that Sirt6 OE reduces protein synthesis in cell culture. Our in vivo studies in flies corroborate these results indicating that dSirt6 is a negative regulator of protein synthesis and suggest possible conservation of this function in mammals.

Inhibition of mTOR is a well-established means to extend lifespan. Similar to *dSirt6* OE, inhibition of mTOR by rapamycin treatment also reduces protein synthesis and extends lifespan in *Drosophila* (43), raising the question of whether there may be overlap in these pathways. In particular, it would be interesting, in future studies, to examine the combined effects of *dSirt6* OE and rapamycin treatment on lifespan and protein synthesis.

Our lifespan epistasis experiments indicate that the magnitude of lifespan extension granted by *dSirt6* OE alone receives little if any additional benefit from reducing *dMyc* expression, suggesting that lifespan extension by *dSirt6* OE is mediated, in part, through reducing dMyc activity. We found that *dSirt6 OE + dMyc* heterozygote flies lived longer than *dMyc* heterozygote flies, suggesting that dSirt6 OE extends lifespan through additional mechanisms beyond reducing dMyc activity. A likely possibility is that dSirt6 OE flies have enhanced DNA repair and/or reduced RTE expression, two additional functions of Sirt6 that are highly connected to aging and longevity. Our observation that dSirt6 OE improves survival in flies treated with paraquat is in line with previous reports that SIRT6 OE enhances DNA double-strand break repair in human fibroblasts treated with paraquat (15).

Several small-molecule activators of Sirt6 have been identified in recent years (44, 45), and our results highlight the potential for these compounds for treating age-related disease and extending health span in humans. *Drosophila* may offer a useful model to study these compounds on the aging process and age-related diseases.

Materials and Methods

Fly Stocks and Husbandry. *dSirt6* lines were made by cloning the coding DNA sequence of *Drosophila Sirt6* (CG6284) into the following constructs: a pUASt-based pTW vector (*Drosophila* Genomics Resource Center) for "*UAS-Sirt6*," pTGW for "*UAS-Sirt6-GFP*," and pUASg.attB for "*UAS-Sirt6-3R*." *UAS-Sirt6* and *UAS-Sirt6-GFP* were injected (Best Gene) into w^{1118} flies to create transgenic lines, with insertions on the second chromosome, while *UAS-Sirt6-3R* was injected into *M{3xP3-RFP.attP}ZH-86Fb* flies (Bloomington *Drosophila* Stock Center [BDSC] #24749) to generate a site-specific insertion on the third chromosome. The following additional stocks were obtained from the BDSC: *UAS-dMyc* (BDSC #9674), $dMyc^4$ (BDSC #64769), *tubulin-GAL4* (BDSC #5138), *EP-Sirt6* (BDSC #30115), *UAS-Sirt6-RNAi* (BDSC #34530), *s106* (BDSC #8151), and *daughterless-GAL4* (BDSC #55850). The *tubulinGeneSwitch* ("*tubGS*") was a kind gift from S. Pletcher, University of Michigan, Ann Arbor, MI.

For all experiments, flies were aged and maintained on 15% dextrose/15% yeast/2% agar food at 25 °C on a 12-h light/dark cycle at 60% relative humidity. For experiments using GeneSwitch drivers, either RU486 dissolved in EtOH at the indicated concentration (Dataset S1) or EtOH alone (for controls) was added to the food at 20 mL/L.

Lifespan Assays. For all experiments comparing a GAL4-driven UAS transgene to a w^{1118} control line (i.e., all except $^{+/-}$RU486 experiments), the UAS line was backcrossed to the w^{1118} line for at least eight generations. Flies were sorted under brief CO_2 anesthesia and placed in food vials at a density of 15 males and 15 females per vial, with a total of at least 150 flies per sex for most conditions. Flies were passed to fresh food every other day, and dead flies were scored and counted. Lifespan statistics and log-rank *P* values were determined using the web-based Online Application for Survival Analysis (OASIS) tool (46).

Western Blotting and SUnSET Assay. SUnSET assay was performed as described (31) using eviscerated adult abdomens. Histone Western blotting was performed as described (47). The following antibodies were used: anti-puromycin (Developmental Studies Hybridoma Bank, monoclonal [PMY-2A4]),

anti-Actin (Abcam, ab3280, monoclonal [C4]), anti-H3K9ac (Active Motif, polyclonal [39917]), anti-H3K56ac (Epitomics, 2134-1, monoclonal [EPR996Y]), anti-histone H3 (Abcam, ab1791, polyclonal), and anti-dMyc (DSHB, monoclonal [P4C4-B10]). Total protein for SUnSET assay was visualized using the Pierce Reversible Protein Stain Kit for PVDF Membranes (Thermo Fisher).

Deacetylase Assay. Deacetylation assay was performed as described (45). Full details are provided in *SI Appendix, Supplementary Methods*.

Imaging. Polytene chromosomes preparations were performed as described (48) and stained with anti-GFP (Thermo Fisher Scientific, A-11120, monoclonal [3E6]) and Hoechst 33342 (Fisher Scientific). For the nuclear size assay, female flies were sectioned at 10 μM on a cryostat and stained and imaged as described (49) with Nile Red (Invitrogen, N1142) and Hoechst 33342 (Fisher Scientific). Fat body cells were identified based on positive Nile Red staining, peripheral location, and large cytoplasm. All images were taken on a Zeiss AxioImager.Z1 ApoTome epifluorescence microscope and processed, and the cross-sectional area was analyzed using ZEN software (Zeiss) with an experimenter blinded to sample identities.

RNA-seq and Bioinformatics. For all groups, total RNA was collected from ∼10 dissected female fat bodies, using the RNaqueous Micro Kit with DNase digestions step (Ambion). For *dSirt6* OE and controls, RNA libraries were made using the Ovation Universal RNA-seq kit (Tecan Genomics). For *dMyc* OE rescue experiments, RNA libraries were made using the TruSeq RNA Library Prep Kit v2. Libraries were sequenced by GENEWIZ on an Illumina HiSeq, in 2 × 150 bp mode.

Transcript abundance was quantified using Salmon (50), and differential expression was calculated using the DESeq2 function in DEBrowser (51) using default parameters. Genes were considered differentially expressed with an FDR of < 0.05 and $\log_2$ FC of ± 0.585 (1.5×-fold). Gene ontology and pathway analysis were done with Flymine (52). GSEA (32) was performed using normalized count data. Redundant terms were removed using REVIGO (53).

Egg Lay Assay. Female flies were allowed to mate in groups of 10 males and 10 females for 3 d, then transferred as single flies to individual food vials, which were passed daily. The number of eggs laid by each individual fly over a 24-h period was quantified at day 7 and day 14. Egg lays from 10 individual females were quantified.

CAFE Assay. Cafe assay was performed as described (54, 55), with single flies placed in each CAFE vial. Food solution consisted of 5% sucrose. After a 24-h acclimation period, amount of liquid food consumed in a 24-h period was measured for each fly. Food consumption of 10 flies per condition was quantified.

Data Availability. RNA-seq FASTQ files have been deposited in GEO Omnibus (GSE191320) and can be accessed at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE191320 (56).

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