

Fat Body *dSir2* Regulates Muscle Mitochondrial Physiology and Energy Homeostasis Nonautonomously and Mimics the Autonomous Functions of *dSir2* in Muscles

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Sir2 is an evolutionarily conserved NAD⁺-dependent deacetylase which has been shown to play a critical role in glucose and fat metabolism. In this study, we have perturbed *Drosophila* Sir2 (*dSir2*) expression, bidirectionally, in muscles and the fat body. We report that *dSir2* plays a critical role in insulin signaling, glucose homeostasis, and mitochondrial functions. Importantly, we establish the nonautonomous functions of fat body *dSir2* in regulating mitochondrial physiology and insulin signaling in muscles. We have identified a novel interplay between *dSir2* and dFOXO at an organismal level, which involves *Drosophila* insulin-like peptide (dILP)-dependent insulin signaling. By genetic perturbations and metabolic rescue, we provide evidence to illustrate that fat body *dSir2* mediates its effects on the muscles via free fatty acids (FFA) and dILPs (from the insulin-producing cells [IPCs]). In summary, we show that fat body *dSir2* is a master regulator of organismal energy homeostasis and is required for maintaining the metabolic regulatory network across tissues.

Metabolic and energy homeostasis are tightly linked to insulin signaling and fat metabolism, which together become crucial determinants of organismal physiology (1, 2). From a clinical perspective, dysfunctions in insulin signaling and metabolic defects affect each other, as seen in obesity and type 2 diabetes (2, 3). Comorbidity of these conditions arises because abrogated fat metabolism has been implicated in insulin resistance and because alterations in insulin signaling are known to regulate the expression of fat metabolism genes. However, the interplay between insulin signaling, fat metabolism, and mitochondrial functions in the etiology of metabolic diseases is still unclear (4).

Recent reports in mammals and flies clearly show that *SIRT1/Sir2* plays an important role in fat metabolism (5–12) and affects starvation survival (5). Additionally, ablation of *SIRT1* in liver and muscles has been shown to result in an insulin resistance-like phenotype (12, 13). However, it is not clear if fat metabolism and systemic insulin signaling are regulated independently by *Sir2/SIRT1*. An important link between insulin signaling and fat metabolism is the FOXO family of transcription factors. FOXOs are typically associated with the transcription of genes downstream to insulin/insulin-like growth factor (IGF) signaling, which include lipogenic and lipolytic genes (14, 15). Although *SIRT1* regulates FOXO-dependent transcription (16), the importance of the SIRT1-FOXO cross talk in mediating the effects of insulin signaling at the organismal level is poorly addressed (17–19). In this respect, it is important to delineate the Sir2/SIRT1-dependent fat phenotype from insulin resistance in the liver and in peripheral tissues (muscles), in addition to investigating the role of FOXO in regulating these effects.

Impaired insulin signaling has been associated with mitochondrial dysfunctions and defects in energy homeostasis. Several reports highlight the role of SIRT1 in affecting the transcription of mitochondrial genes via *PGC1 α* (20–22). Although SIRT1-mediated transcriptional regulation is expected to affect mitochondrial functions and energy homeostasis, the physiological relevance, specifically at the organismal level, is still unclear. Given the piv-

otal functions of SIRT1 in the liver, investigating its ability to affect metabolic parameters in peripheral tissues becomes important.

The ability of an organism to maintain metabolic and energy homeostasis has been implicated as a major determinant of survival in response to acute and chronic dietary alterations. Although longevity is regulated by homeostatic mechanisms, the robustness of such metabolic adaptations (specifically, energy metabolism) is often measured as a function of starvation survival or resistance.

Using *Drosophila* as a model system, we have addressed the role of *dSir2* in maintaining tissue-specific as well as organismal energy homeostasis. Although previous reports have highlighted the role of *Sir2/SIRT1* in muscles (13, 23–25), we show that its overexpression in this tissue is sufficient to regulate glucose homeostasis. We also show that an absence of *dSir2* in the fat body leads to abrogated insulin signaling and impaired energy homeostasis in the muscles. Moreover, *dSir2* in the fat body mimics the effects of *dSir2* in the muscles, highlighting the similarity in the functions of *dSir2* in these two tissues. We report an increase in insulin signaling and, hence, reduced nuclear localization of *dFOXO* within the fat body of fat body-specific *dSir2* knockdown flies. Further, by simultaneous overexpression of a constitutively nuclear *dFOXO* (*dFOXO-TM*) and knockdown of *dSir2* in the fat body, we delineate the effects of fat body *dSir2* on fat metabolism from systemic insulin signaling. We have found that ablation of *dSir2* in the fat body leads to an imbalance in energy homeostasis and causes a

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“nutrient-dependent mitochondrial stress” condition in the organism. This is evident from the rescue of the signaling defects in the muscles of fat body-specific *dSir2* knockdown flies by the administration of L-carnitine. Finally, we report that although there are similarities in the metabolic functions of *dSir2* in the muscles and fat body, the ability to adapt to an acute metabolic stress like starvation is differentially regulated. In conclusion, we highlight the interaction between two key metabolic sensors in the fat body in establishing communication across tissues for maintaining energy homeostasis, and we identify a physiological mechanism underlying the nonautonomous effects of fat body *dSir2* on muscles.

MATERIALS AND METHODS

Fly strains. *P{Switch1}106-Gal4* (26–28), *Sir2^{EP2300}*, and *Sir2^{EP2384}* flies were obtained from Bloomington Stock Center (Indiana University). The *P{Switch tub-Gal4}* strain was a kind gift from Stephen Helfand. *yw⁺*; *UAS-dFOXO-TM* (*dFOXO^{AAA}*) and *yw⁺*; *UAS-dFOXO-WT* flies were kind gifts from Marc Tatar. The *UAS-dFOXO-GFP* strain was provided to us by Gaiti Hasan, National Centre for Biological Sciences (NCBS), Bangalore, India. The *rp²⁹⁸*; *P{Switch-MHC}-Gal4* (27) strain was obtained from NCBS, Bangalore, India. *dSir2^{RNAi}* (CG5216:23201/GD and 23199/GD) and *chico^{RNAi}* (*ch^{RNAi}*) (CG5685:1402/GD) flies were obtained from the Vienna *Drosophila* RNAi Center (VDRC). *mCherry^{RNAi}* flies were provided by Richa Rikhy from the Indian Institute of Science Education and Research (IISER), Pune, India. Flies were grown on normal food under noncrowding conditions at 25°C with a 12/12-h light/dark cycle. Age-matched virgin female flies 3 to 5 days old were used for all analyses.

Activation of inducible Gal4. The inducible Gal4 (*P{Switch}* lines) was activated by rearing flies on medium containing 200 μ M RU486 (mifepristone) (in ethanol). Flies reared on a diet containing only ethanol were used as controls. When two different upstream activation sequence (UAS) genes were activated simultaneously by the same Gal4, as in the case of *dSir2-dFOXO* and *dSir2-chico* stocks, 500 μ M RU486 (mifepristone) was used to activate the Gal4.

Mitochondrial DNA estimation. For mitochondrial DNA estimation, total genomic DNA was isolated using a Bangalore Genei genomic DNA isolation kit (catalog number 118729). The relative mitochondrial content was quantified by real-time PCR using the primers for *COX* subunit I and nuclear *Actin* (for normalization).

Tissue isolation. Indirect flight muscles and abdominal fat body were dissected in ice-cold phosphate-buffered saline (PBS) from anesthetized adult flies. Fresh tissue isolates were used for preparing protein lysates or for RNA extraction.

Hemolymph isolation. Hemolymph was isolated from 12 adult flies by capillary action. The hemolymph was collected in capillaries after a small puncture in the head capsule. Twenty microliters of the hemolymph was diluted in 180 μ l of PBS for further analyses.

RNA isolation and reverse transcription. Total RNA was isolated from eight flies (pooled together) using TRIzol (catalog number 15596-026; Invitrogen,) according to the manufacturer’s instructions. One microgram of RNA was used for cDNA synthesis using a SuperScript III reverse transcriptase kit (catalog number 18080-044; Invitrogen) per the manufacturer’s instructions.

Real-time PCR analyses. Quantitative PCR (qPCR) was performed using Quantifast SYBR green (catalog number 204054; Qiagen) and an Eppendorf Realplex instrument. The cycling conditions were as prescribed by the manufacturer. The primer pairs used for the assay are tabulated in Table S1 in the supplemental material. Various levels of actin (*actin5c*) and *rp49* were used for normalization.

Lipid and glucose measurement. Six (3- to 5-day-old) flies reared on a normal diet were snap-frozen in liquid nitrogen and homogenized in 600 μ l of PBS–0.05% Tween 20 (PBST). The homogenate was heated to 70°C for 10 min. Following centrifugation at 12,000 rpm for 15 min, the

supernatant was transferred to fresh tubes, which were assayed at a pathology laboratory (Shahbazker’s Diagnostic Center, Mumbai, India).

FFA level measurement. Hemolymph samples isolated from eight (3- to 5-day-old) flies were used to estimate free fatty acid (FFA) levels. Free fatty acid level measurement was done using a free fatty acid quantification kit from Abcam (catalog number ab65341) according to the manufacturer’s instructions.

oGTT. An oral glucose tolerance test (oGTT) was performed according to Haselton et al. (29) with modifications. Briefly, 3- to 5-day-old flies were starved for 16 h, and an oGTT was performed by shifting them to vials containing 10% glucose and 2% agar for 30 min before transferring them back to 2% agar. Following this, glucose levels were measured in the hemolymph at various time points.

Mitochondrial membrane potential. Mitochondrial membrane potential was detected using 1,1',3,3'-tetraethylbenzamidazolocarboxyanin iodide (JC-1) (Molecular Probes). A stock solution at 5 mg/ml was made in dimethyl sulfoxide (DMSO), as per the manufacturer’s instruction. The final staining solution having 5 μ M JC-1 was made in Schneider’s incomplete medium (GIBCO). Flies were anesthetized using ether, and indirect flight muscles were dissected into Schneider’s medium. Muscles were incubated in JC-1 solution for 20 min on a shaker. Samples were then washed with medium and finally with PBS. They were fixed with 4% paraformaldehyde (PFA) and mounted on slides for imaging on an LSM510 confocal microscope (Carl Zeiss) under a 63 \times objective. For quantitation, mean integrated densities from 30 sections from three flies per treatment or genotype were obtained using ImageJ software. The ratios of red and green intensities were used for depicting the change in membrane potential.

Western blotting. Total fly lysates were incubated on ice for 15 to 20 min in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 6 mM EGTA, 20 mM NaF, 1% Triton X-100, and protease inhibitors (catalog number 05-056-489001; Roche). The lysates were then centrifuged at 12,000 rpm for 15 min at 4°C to pellet the debris. Supernatants were used for protein estimations using a bicinchoninic acid (BCA) kit (catalog number BCA9643; Sigma) and resolved by 10% SDS-PAGE. Following electrotransfer onto polyvinylidene difluoride (PVDF) membranes, they were probed with appropriate antibodies according to standard procedures. Anti-Akt, anti-phospho-AKT (anti-p-Akt), and antiactin antibodies were purchased from Cell Signaling (catalog numbers 9273 and 4054) and Sigma-Aldrich (catalog number A1978), respectively. Chemiluminescence detection (catalog number 12-015-196001; Roche) was used to visualize the bands. For quantitation, mean integrated densities of Western blots from three independent lysates (each obtained from multiple flies, as indicated in the figure legends) per treatment or genotype were obtained using ImageJ software. The ratios of p-Akt/Akt and actin intensities were used for depicting the change in phosphorylation status of AKT.

ATP assay. Adult flies were snap-frozen and boiled in water for 15 min. The debris was pelleted by centrifugation at 12,000 rpm at 4°C for 6 min. The supernatant was used to determine total ATP levels using an ATP bioluminescent assay kit (FLAA-1KT; Sigma-Aldrich) and Berthold luminometer. ATP levels were normalized to total protein in the supernatant (determined by using Bradford’s reagent) (catalog number 500-0205; Bio-Rad). Normalized ATP levels indicate total ATP per unit of optical density (OD).

L-Carnitine and etomoxir treatments. For L-carnitine treatment, flies were reared for 24 h on a normal diet containing L-carnitine (catalog number C0283-5G; Sigma-Aldrich) at a concentration of 25 mg/ml in water. For etomoxir treatment, flies were reared for 12 h on a normal diet containing etomoxir (catalog number E1905; Sigma-Aldrich) at a concentration of 25 μ M in water.

Statistical analyses. SigmaPlot, version 12.0, was used for all statistical analyses. Student’s *t* test and analysis of variance (ANOVA) were used to analyze statistical significance of the data. A log rank Mantel-Cox test was used for calculating significance for survival assays.

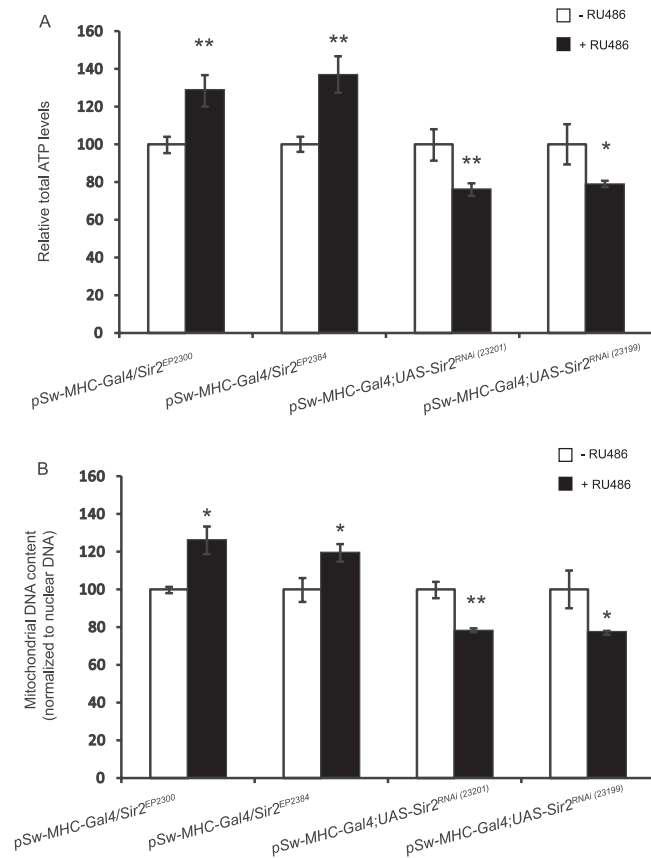


FIG 1 Muscle *dSir2* regulates mitochondrial functions and energy homeostasis. Total ATP and mitochondrial DNA content were measured in muscle-specific *dSir2* overexpression, *mdSir2*^{OE} (*pSw-MHC-Gal4/Sir2*^{EP2300} and *pSw-MHC-Gal4/Sir2*^{EP2384}), and knockdown, *mdSir2*^{KD} [*pSw-MHC-Gal4; Sir2*^{RNAi(23201)} and *pSw-MHC-Gal4; Sir2*^{RNAi(23199)}], flies. (A) Normalized total ATP levels in *mdSir2*^{OE} and *mdSir2*^{KD} flies. (B) Mitochondrial DNA content (normalized to nuclear DNA content) in *mdSir2*^{OE} and *mdSir2*^{KD} flies. All the assays were done on muscle samples. Sample sizes were 36 flies for the ATP assay and 24 flies for the measurement of mtDNA. Control, -RU486; overexpression/knockdown, +RU486 (200 μ M). All data are shown as means \pm standard errors of the means. *, $P < 0.05$; **, $P < 0.01$.

RESULTS

Muscle *dSir2* regulates mitochondrial functions and energy homeostasis autonomously and mimics the nonautonomous effects of fat body *dSir2*. To understand the link between *dSir2* and energy homeostasis in the muscles, we wanted to determine whether perturbing the expression of *dSir2* would affect mitochondrial functions. Muscle-specific *dSir2* overexpression (*mdSir2*^{OE}) (see Fig. S1 in the supplemental material) using two overexpression lines increased ATP levels and mitochondrial DNA (mtDNA) content autonomously (Fig. 1A and B). Muscle-specific *dSir2* knockdown (*mdSir2*^{KD}) (see Fig. S1) using two RNA interference (RNAi) lines led to a reduction in these parameters (Fig. 1A and B). In addition to muscles, fat body (equivalent to liver and adipocytes) is important for metabolic and energy homeostasis in flies. Fat body-specific *dSir2* knockdown (*fbdSir2*^{KD}) (see Fig. S1) and *dSir2* overexpression (*fbdSir2*^{OE}) (see Fig. S1) flies exhibited bidirectional changes in total ATP levels in the whole body (Fig. 2A). We checked if this was associated with changes in mitochondrial DNA content in *fbdSir2*^{KD} and

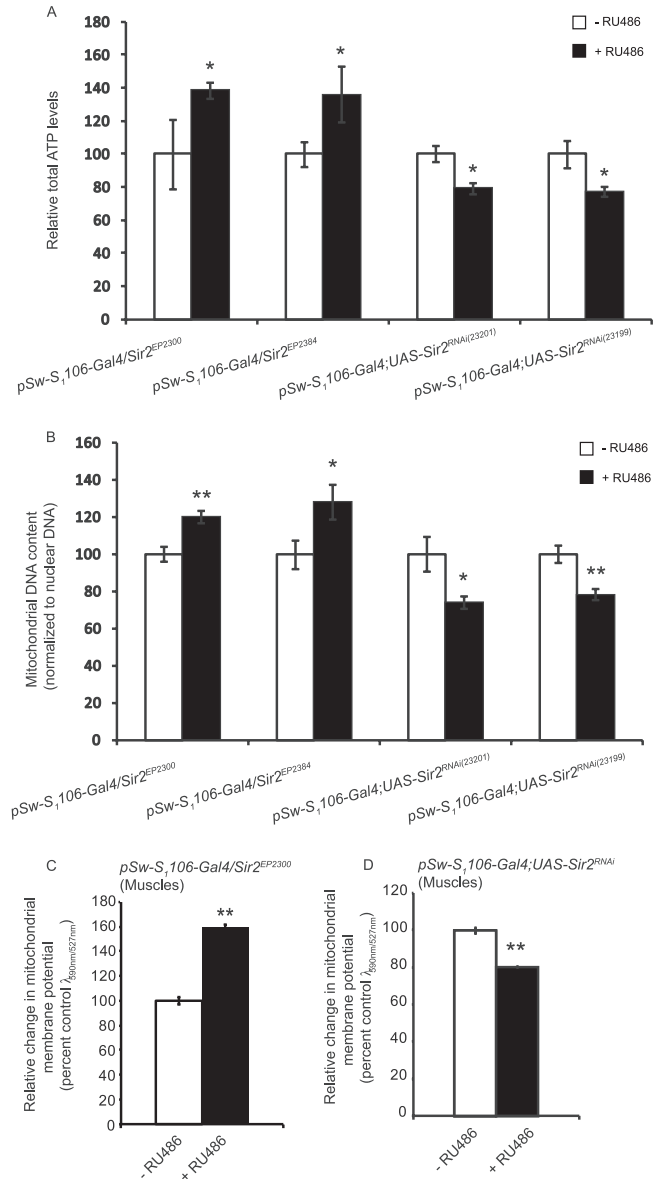


FIG 2 Fat body *dSir2* nonautonomously regulates muscle mitochondrial functions and energy homeostasis. Total ATP, mitochondrial DNA content, and mitochondrial membrane potential were measured in fat body-specific *dSir2* overexpression, *fbdSir2*^{OE} (*pSw-S,106-Gal4/Sir2*^{EP2300} and *pSw-S,106-Gal4/Sir2*^{EP2384}), and knockdown, *fbdSir2*^{KD} [*pSw-S,106-Gal4; UAS-Sir2*^{RNAi(23201)} and *pSw-S,106-Gal4; UAS-Sir2*^{RNAi(23199)}], flies. (A) Normalized total ATP levels in *fbdSir2*^{OE} and *fbdSir2*^{KD} flies. (B) Mitochondrial DNA content (normalized to nuclear DNA content) in *fbdSir2*^{OE} and *fbdSir2*^{KD} flies. (C) Mitochondrial membrane potential as measured using JC-1 staining and confocal microscopy as detailed in Materials and Methods in *fbdSir2*^{OE} and *fbdSir2*^{KD} flies. All the assays were done on muscle samples. Sample sizes were 36 flies for the ATP assay and 24 flies for the measurement of mtDNA. For measurement of mitochondrial membrane potential and microscopy, four flies were used, and a total of 90 stacks were imaged and quantified using ImageJ. Control, -RU486; overexpression/knockdown, +RU486 (200 μ M). All data are shown as means \pm standard errors of the means. *, $P < 0.05$; **, $P < 0.01$.

fbdSir2^{OE} flies. Consistent with the ATP results, *dSir2*^{KD} and *dSir2*^{OE} in the fat body led to similar alterations in mtDNA content in the muscles (Fig. 2B). The electrochemical gradient across the inner mitochondrial membrane is the major driver for mito-

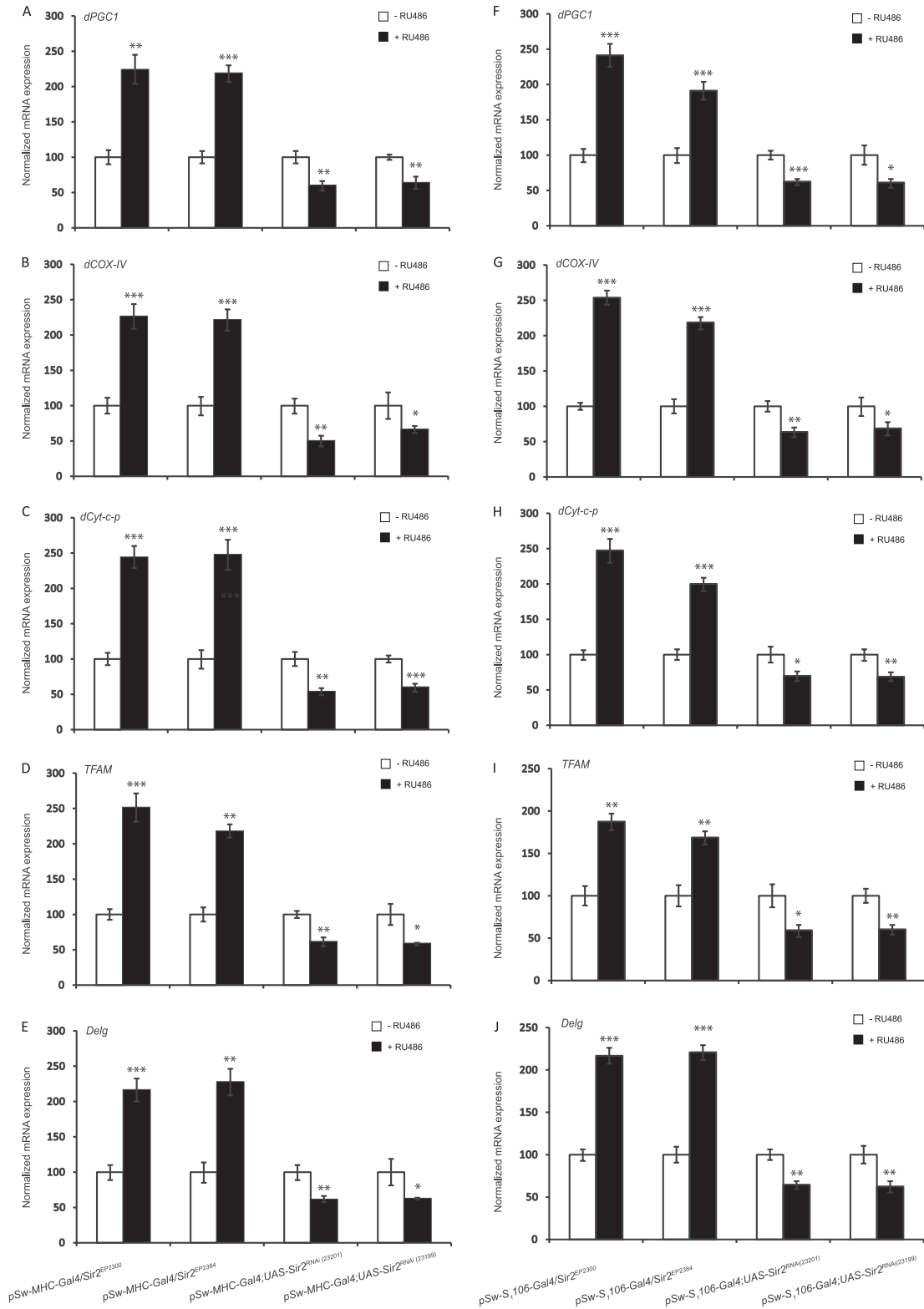
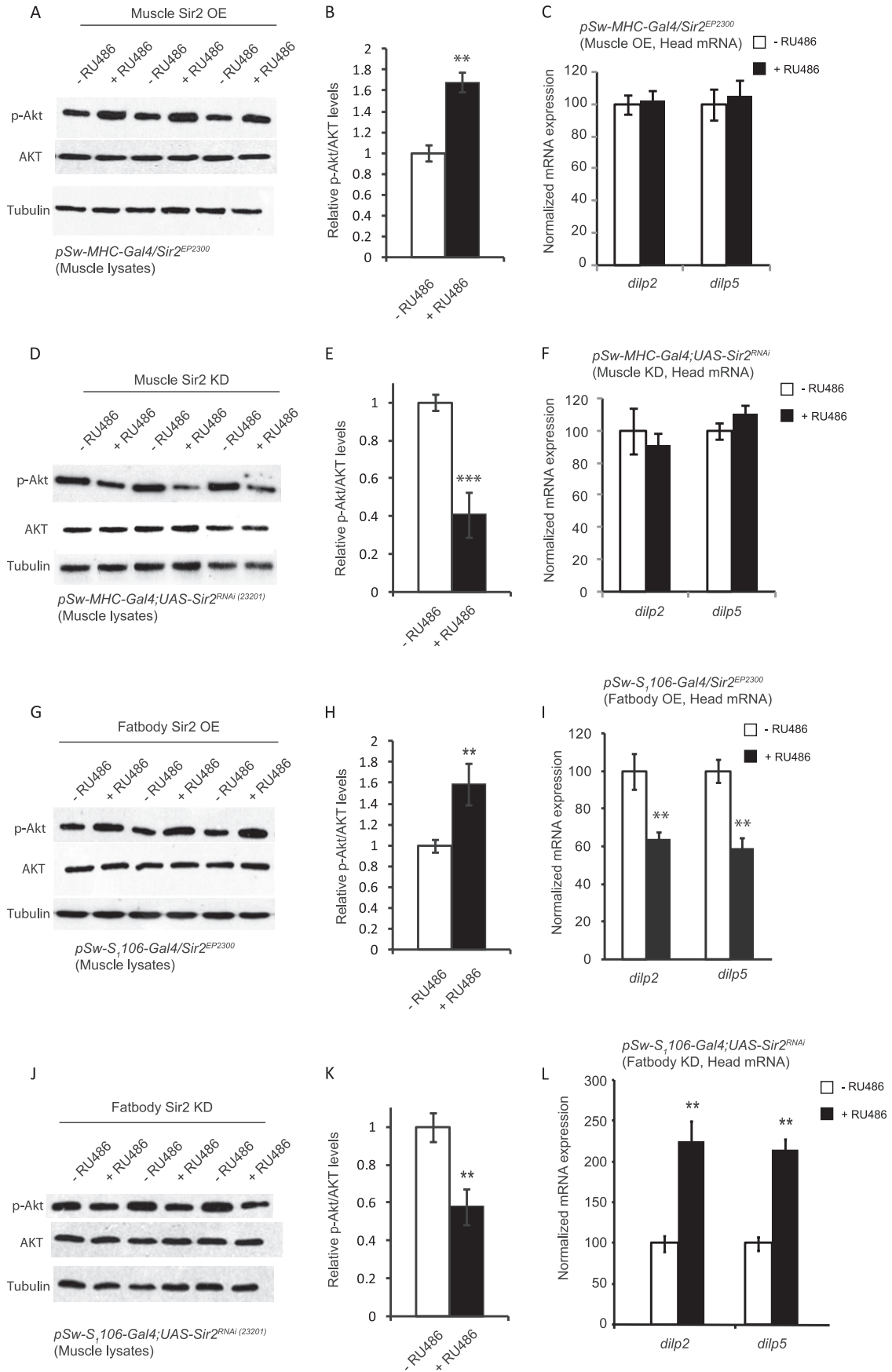


FIG 3 Muscle and fat body *dSir2* regulate the expression of nuclear genes for mitochondrial proteins in the muscles in an autonomous and nonautonomous manner, respectively. As indicated, relative mRNA expression levels of *dPGC1*, *dCyt.C-p*, *dCOX-IV*, *TFAM*, and *Delg* in the muscle samples of *mdSir2*^{OE} (*pSw-MHC-Gal4/Sir2*^{EP2300} and *pSw-MHC-Gal4/Sir2*^{EP2384}) and *mdSir2*^{KD} [*pSw-MHC-Gal4; UAS-Sir2*^{RNAi(23201)} and *pSw-MHC-Gal4; UAS-Sir2*^{RNAi(23199)}] flies (A to E) and of *fbdSir2*^{OE} (*pSw-S₁106-Gal4/Sir2*^{EP2300} and *pSw-S₁106-Gal4/Sir2*^{EP2384}) and *fbdSir2*^{KD} [*pSw-S₁106-Gal4; UAS-Sir2*^{RNAi(23201)} and *pSw-S₁106-Gal4; UAS-Sir2*^{RNAi(23199)}] (F to J) flies. Sample size, 60 flies. Control, -RU486; overexpression/knockdown, +RU486 (200 μM). All data are shown as means ± standard errors of the means. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.



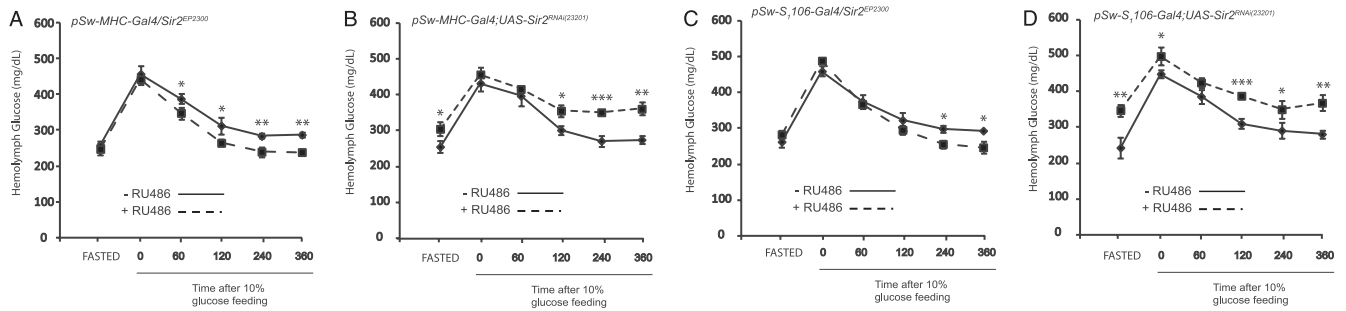


FIG 5 *dSir2* in the fat body and muscles regulates glucose homeostasis. An oral glucose tolerance test (oGTT) was performed using hemolymph isolated from *pSw-MHC-Gal4/Sir2^{EP2300}* (*mdSir2^{OE}*) (A), *pSw-MHC-Gal4;UAS-Sir2^{RNAi}* (*mdSir2^{KD}*) (B), *pSw-S,106-Gal4/Sir2^{EP2300}* (*fbdSir2^{OE}*) (C), and *pSw-S,106-Gal4;UAS-Sir2^{RNAi}* (*fbdSir2^{KD}*) (D) flies. For every time point, 5 samples from hemolymph isolated from 12 flies each were used. Control, -RU486; overexpression/knockdown, +RU486 (200 μ M). All data are shown as means \pm standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

chondrial ATP synthesis and is an indicator of mitochondrial activity. We measured mitochondrial membrane potential in the muscles of *fbdSir2^{OE}* and *fbdSir2^{KD}* flies. *fbdSir2^{OE}* flies showed a significant increase in mitochondrial membrane potential in the muscles while *fbdSir2^{KD}* flies showed a decrease (Fig. 2C and D).

We wanted to investigate if the changes in mitochondrial parameters (Fig. 1 and 2) were also associated with changes in the expression of mitochondrial proteins due to changes in the nuclear genes encoding them. As shown in Fig. 3A to E, *mdSir2^{OE}* flies showed a significant increase in *dPGC1*, *dCyt.C-p*, *dCOX-IV*, *TFAM*, and *Delg (NRF2)* in the muscles, while there was downregulation in the expression of these genes in *mdSir2^{KD}* flies (Fig. 3; see also Fig. S2 and S5 in the supplemental material). The transcript levels in the muscles of *fbdSir2^{OE}* and *fbdSir2^{KD}* flies mimicked those of *mdSir2^{OE}* and *mdSir2^{KD}* flies (Fig. 3; see also Fig. S3 and S5 in the supplemental material). Overall, these results clearly demonstrated that both *mdSir2* and *fbdSir2* regulate mitochondrial functions and energy homeostasis in the muscles in an autonomous and nonautonomous manner, respectively. Additionally, these results also highlight the evolutionary conservation of the role of Sir2 and SIRT1 in the muscles to regulate energy homeostasis (30).

Insulin signaling in the muscles is regulated autonomously as well as nonautonomously by *dSir2*. Aberrations in mitochondrial functions are often associated with impaired insulin signaling. Our previous work shows that *dSir2* affects insulin signaling by regulating the expression of the *Drosophila* insulin-like peptides (dILP2 and -5 and not the other dILPs) in a diet-dependent manner (5). To investigate if the altered mitochondrial functions observed in the results shown in Fig. 1 to 3 were also associated with changes in insulin signaling, we measured phospho-AKT levels and the expression of *dilp-2* and *dilp-5* in both muscles and fat body *dSir2^{OE}* and *dSir2^{KD}* flies. While perturbing *dSir2* in the muscles affected phospho-AKT levels (Fig. 4A and D; and see also Fig. S4 and S5 in the supplemental material), the expression of

both *dilp-2* and -5 remained unaltered (Fig. 4B and F; see also Fig. S4 and S5). Interestingly, *fbdSir2^{OE}* led to a significant downregulation in the expression of both *dilp-2* and -5, while *fbdSir2^{KD}* flies showed an increase in their expression (Fig. 4I and L; see also Fig. S4 and S5). The phospho-AKT levels increased in the muscles of *fbdSir2^{OE}* flies while there was a reduction in phospho-AKT levels in the muscles of *fbdSir2^{KD}* flies (Fig. 4G, H, K, and L; see also Fig. S4 and S5). These results suggested that *dSir2* is a critical regulator of insulin signaling in the muscles.

Insulin signaling governs the efficiency of glucose utilization in organisms. We performed an oral glucose tolerance test (oGTT) (29) in muscle and fat body *dSir2^{OE}* and *dSir2^{KD}* flies to check the physiological output of the effect of *dSir2* on insulin signaling in the muscles. Overexpression of *dSir2* in the muscles improved glucose clearance, while a muscle-specific knockdown of *dSir2* hampered it (Fig. 5A and B). Similar to the results of overexpression in muscles, overexpression of *dSir2* in the fat body improved the oGTT response while knocking it down worsened the oGTT response (Fig. 5C and D). These results clearly highlight that the role of Sir2 and SIRT1 in maintaining glucose homeostasis is evolutionarily conserved in both invertebrates and vertebrates (23, 31).

Fat body *dSir2* mediates its effects on dFOXO-dependent fat metabolism via dILPs and insulin/IGF signaling (IIS). We have earlier shown that fat body *dSir2* regulates systemic insulin signaling and affects fat metabolism (5). To determine if the aberrations in insulin signaling arose due to defects in fat metabolism, we looked at triglyceride levels, phospho-AKT levels, and expression of insulin signaling components in the fat body of *fbdSir2^{KD}* flies. Knockdown of *dSir2* in the fat body led to an increase in total triglyceride levels within the fat body (see Fig. S6 in the supplemental material). Unlike in the muscles, knocking down *dSir2* in the fat body led to an increase in the phospho-AKT levels in the fat body (Fig. 6A and B), indicating an increase in insulin signaling. Increased insulin signaling reduces nuclear localization of FOXO and transcription of lipolytic genes (32, 33). To check if the in-

FIG 4 Insulin signaling in the muscles is regulated autonomously as well as nonautonomously by *dSir2*. (A to C) Muscle-specific *dSir2* overexpression (*pSw-MHC-Gal4/Sir2^{EP2300}*, or *mdSir2^{OE}*): phospho-AKT levels in muscle samples (A), ratio of p-Akt/Akt in these samples (B), and relative mRNA expression levels of *dilp-2* and -5 in head samples (C). (D to F) Muscle-specific *dSir2* knockdown (*pSw-MHC-Gal4; Sir2^{RNAi}*, or *mdSir2^{KD}*): phospho-AKT levels in muscle samples (D), ratio of p-Akt/Akt in these samples (E), and relative mRNA expression levels of *dilp-2* and -5 in head samples (F). (G to I) Fat body-specific *dSir2* overexpression (*pSw-S,106-Gal4/Sir2^{EP2300}*, or *fbdSir2^{OE}*): phospho-AKT levels in muscle samples (G), ratio of p-Akt/Akt in these samples (H), and relative mRNA expression levels of *dilp-2* and -5 in head samples (I). (J to L) Fat body-specific *dSir2* knockdown (*S,106-Gal4; UAS-Sir2^{RNAi}*, or *fbdSir2^{KD}*): phospho-AKT levels in muscle samples (J), ratio of p-Akt/Akt in these samples (K), and relative mRNA expression levels of *dilp-2* and -5 in head samples (L). Sample sizes were 16 flies for phospho-AKT, Akt, and actin levels per loading and 24 flies for measurement of *dilp* mRNA expression. Control, -RU486; overexpression/knockdown, +RU486 (200 μ M). All data are shown as means \pm standard errors of the means. **, $P < 0.01$; ***, $P < 0.001$.

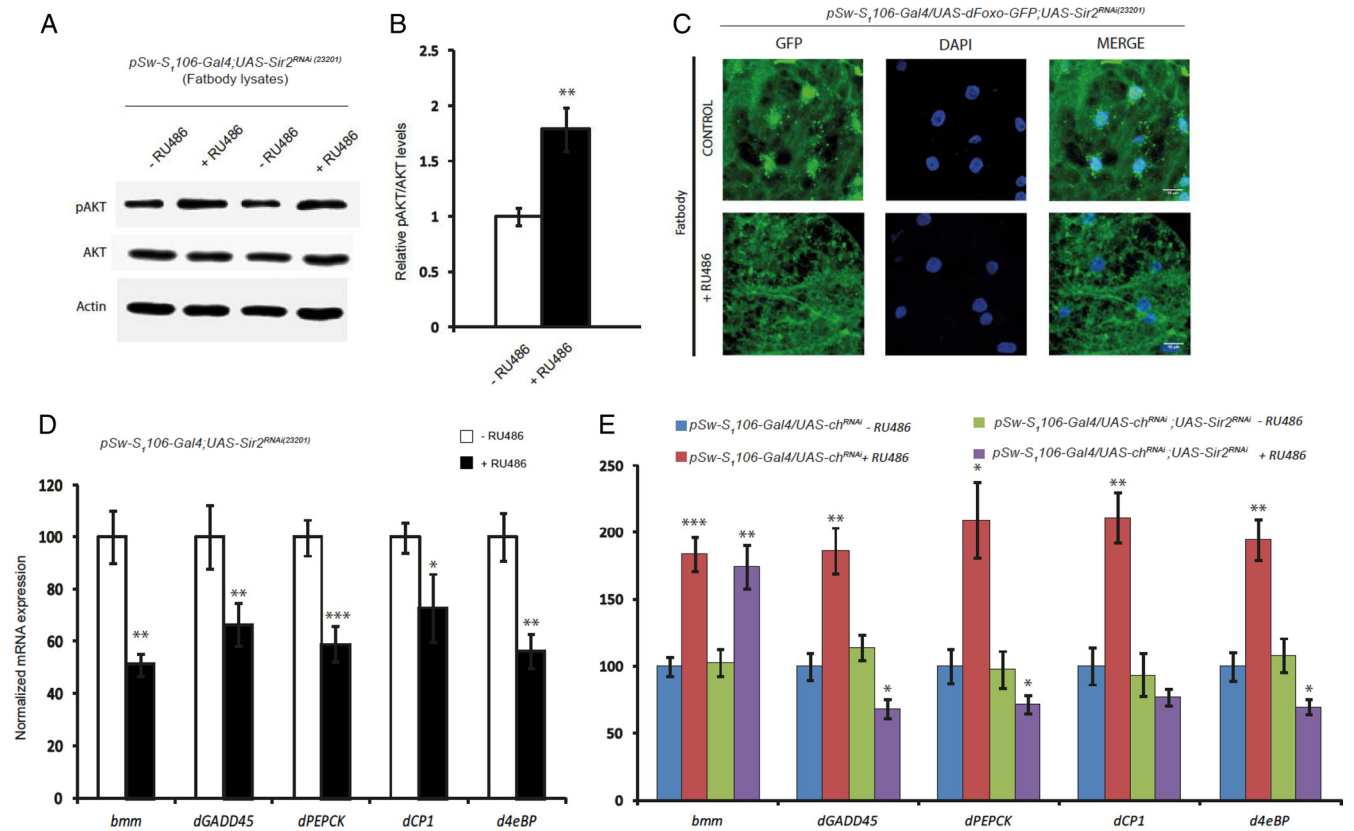


FIG 6 Fat body *dSir2* regulates *dFOXO* localization and activity in an insulin-dependent manner. Phospho-AKT levels in fat body samples of *pSw-S1,106-Gal4/UAS-Sir2^{RNAi}* (*fbdSir2^{KD}*) flies (A) and the ratio of p-Akt/Akt in these samples are shown (B). (C) *dFOXO-GFP* localization in the fat bodies of control and *fbdSir2^{KD}* flies. (D and E) Relative mRNA expression levels of *brummer* (*bmm*), *dGADD45*, *dPEPCK*, *Cathepsin-L* (*dCPI*), and *d4eBP* in the fat bodies of *pSw-S1,106-Gal4/UAS-Sir2^{RNAi}*, *pSw-S1,106-Gal4/UAS-Ch^{RNAi}*, and *pSw-S1,106-Gal4/UAS-ch^{RNAi}; UAS-Sir2^{RNAi}* flies with relevant controls ($n = 24$ flies for measurement of mRNA expression). Control, -RU486; overexpression/knockdown, +RU486 (200 μ M). All data are shown as means \pm standard errors of the means. Images were captured using a Zeiss 510 metaconfocal microscope at a magnification of $\times 63$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

crease in insulin signaling in the fat body of *fbdSir2^{KD}* flies led to a reduction in the nuclear localization of dFOXO, we monitored the localization of dFOXO-green fluorescent protein (GFP) in the fat body of control and *fbdSir2^{KD}* flies. As shown in Fig. 6C, knockdown of *dSir2* in the fat body significantly reduces the nuclear localization of dFOXO-GFP (Fig. 6C). This in addition to an increase in *dilp-2* and *dilp-5* (Fig. 4) was interpreted as an increase in insulin signaling within the fat body in *fbdSir2^{KD}* flies.

Decreased nuclear localization of FOXO is associated with a reduction in its transcriptional activity and a consequential downregulation of its target genes (14). In addition to a reduction in the nuclear localization of dFOXO-GFP (Fig. 6C), increased insulin signaling in the fat body of *fbdSir2^{KD}* flies led to a downregulation of the expression of FOXO target genes (Fig. 6D). *chico*, the *Drosophila* homolog of mammalian *IRS-1* is a critical component of insulin signaling, and abrogating *chico* expression leads to reduced insulin signaling (34). We wanted to check if reducing insulin signaling, using *chico^{RNAi}*, in *fbdSir2^{KD}* flies restores FOXO-dependent transcription in the fat body. We compared the expression of FOXO target genes in the fat bodies of *pSw-S1,106-Gal4/ch^{RNAi}* and *pSw-S1,106/UAS-ch^{RNAi}; UAS-Sir2^{RNAi}* flies. It is interesting that knocking down *chico* in the fat bodies of *fbdSir2^{KD}* flies (*pSw-S1,106/UAS-ch^{RNAi}; UAS-Sir2^{RNAi}*) rescued the expression of the dFOXO target *brummer* lipase (*bmm*) (35, 36) but not that of the other FOXO target genes compared to *fbdSir2^{KD}*

(Fig. 6E). The rescue in the expression of *bmm* alone indicated that FOXO-dependent regulation of fat metabolism is probably independent of a direct interplay between *dSir2* and FOXO in the fat body.

However, it was not clear if the defects in fat metabolism resulted in abrogated signaling or if increased insulin signaling led to fat accumulation in the absence of fat body *dSir2*. To dissect the interplay between insulin signaling and fat metabolism in *fbdSir2^{KD}* flies, we overexpressed both wild-type and constitutively nuclear dFOXO (*dFOXO-TM*) (37). Overexpression of phosphorylation-defective FOXO has been shown to act independent of alterations in insulin signaling in both mammals and flies (33, 37). Interestingly, while overexpression of *dFOXO-TM* alone in the fat body did not alter total triglycerides (TAGs), it brought down the TAGs to basal levels in fat body *dSir2* knockdown flies, thereby rescuing the fat phenotype (Fig. 7A). This rescue was associated with a 4-fold increase in the expression of *bmm* in *fbdSir2^{KD} + FOXO-TM^{OE}* compared to *fbdSir2^{KD}* flies (Fig. 7B). In contrast, flies overexpressing only wild-type dFOXO (*fbdFOXO-WT^{OE}*) and in the background of fat body *dSir2^{KD}* (*fbdSir2^{KD}; FOXO-WT^{OE}*) showed TAGs and *bmm* mRNA levels similar to those of fat body *dSir2^{KD}* flies (see Fig. S7 and S8 in the supplemental material).

Hyperlipidemia in mammals is associated with severe inflammatory responses (38, 39). We investigated if the hyperlipidemia

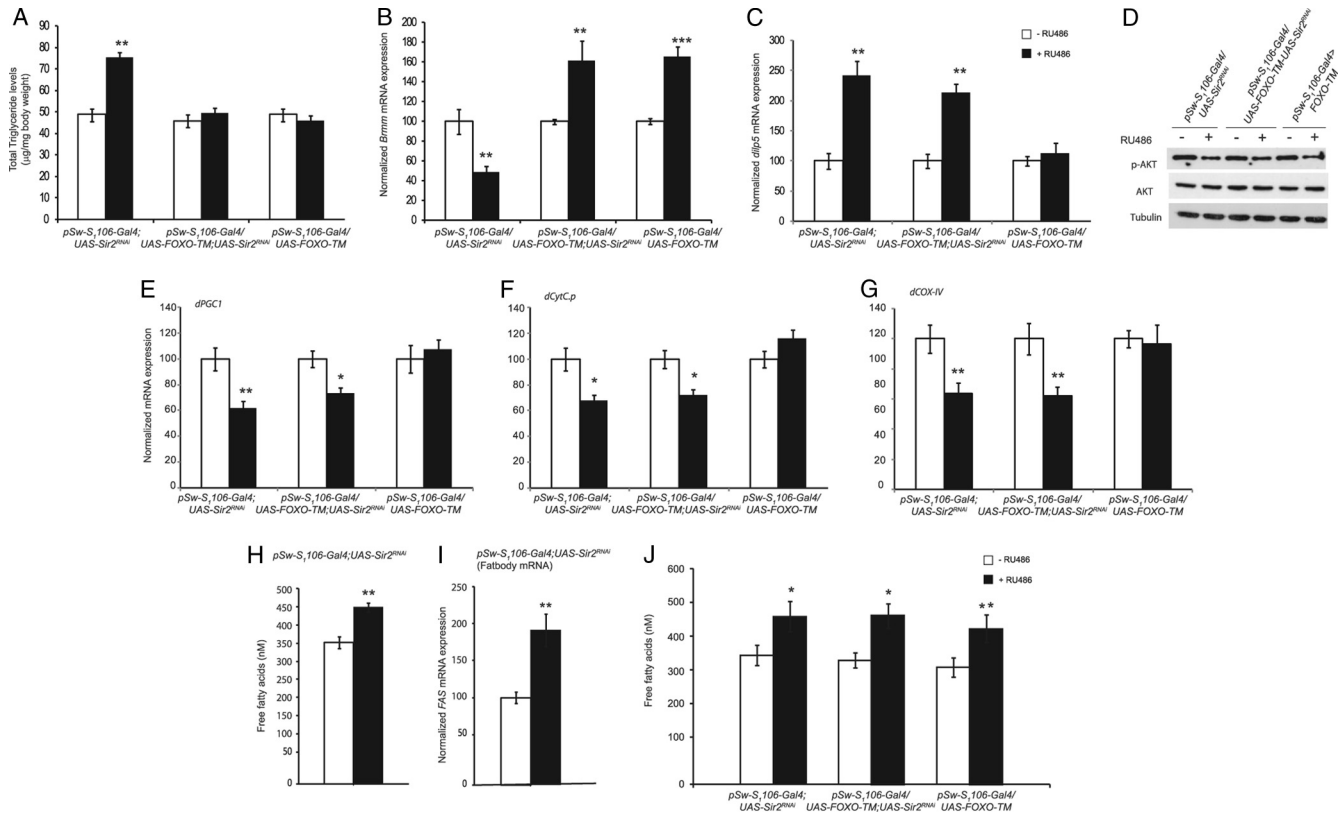


FIG 7 Fat body *dFOXO-TM* overexpression in the absence of *dSir2* rescues triglyceride levels but not muscle insulin signaling defects. As indicated on the figure, data are for fat body-specific *dSir2* knockdown (*pSw-S,106-Gal4; UAS-Sir2^{RNAi}*), fat body-specific overexpression of *dFOXO-TM* (*pSw-S,106-Gal4/UAS-dFOXO-TM*), and simultaneous fat body-specific knockdown and overexpression of *dSir2* and *dFOXO-TM* (*pSw-S,106-Gal4/UAS-FOXO-TM; UAS-Sir2^{RNAi}*). (A) Total triglyceride levels. (B) Expression of *brummer* lipase. (C) Relative expression of *dilp-5* in head sample. (D) p-Akt levels in muscle samples. Relative mRNA expression levels of *dPGC1* (E), *dCyt.C-p* (F), and *dCOX-IV* (G) in muscle samples are shown. (H and J) Circulating free fatty acid levels. (I) Relative mRNA expression of fatty acid synthase in the fat body. Sample sizes were 16 flies for phospho-AKT levels and 60 flies for analysis of the expression levels of genes. For free fatty acid measurement eight sets with five flies were used. Control, -RU486; overexpression/knockdown, +RU486 (200 μM or 400 μM, for driving single or double UAS genes). All data are shown as means ± standard errors of the means. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

in the *fbdSir2^{KD}* flies led to an increase in the inflammatory response. As shown in Fig. S9 in the supplemental material, *fbdSir2^{KD}* flies showed a significant increase in *18w* (interleukin-2 [IL-2]) and *egr* (tumor necrosis factor alpha [TNF-α]) in the fat body, which indicated the evolutionary conservation of the functions of Sir2 and SIRT1 (40, 41).

Nonautonomous effect of fat body *dSir2* on insulin signaling in the muscles is mediated by free fatty acids. Based on the results presented above, we wanted to assess the contributions of hyperlipidemic and hyperinsulinemic effects on insulin signaling in the muscles of *fbdSir2^{KD}* flies. There was a reduction in the p-Akt levels in the muscles of *fbdSir2^{KD}; FOXO-TM^{OE}* flies similar to that in the *fbdSir2^{KD}* flies (Fig. 7D). Additionally, the decreased p-Akt levels in the muscles of these flies were associated with increased *dilp-2* and *dilp-5* expression (Fig. 7C; see also Fig. S10 in the supplemental material). There was also a significant reduction in the expression of *dPGC1*, *dCyt.C-p*, and *dCOX-IV* (Fig. 7E to G) in the muscles of *fbdSir2^{KD}; FOXO-TM^{OE}* flies which mimicked that in *fbdSir2^{KD}* flies.

In addition to TAGs, circulating free fatty acids have been shown to affect insulin signaling and lead to insulin resistance (42, 43). To examine the effect of *fbdSir2* on free fatty acids, we measured circulating free fatty acid levels in the hemolymph.

As shown in Fig. 7H, *fbdSir2^{KD}* led to a significant increase in circulating free fatty acid levels. Additionally, these flies also exhibited a marked increase in the expression of fatty acid synthase (*FAS*) gene (Fig. 7I; see Fig. S5 in the supplemental material). These results indicate that elevated free fatty acid levels, which are associated with abrogated fat metabolism in the fat body, might be contributing to the reduced insulin signaling in the muscles of these flies. Intriguingly, free fatty acid levels remained elevated in *fbdSir2^{KD}* with *FOXO-TM^{OE}* and *fbFOXO-TM^{OE}* flies (Fig. 7J).

To address the role of free fatty acids in the manifestation of the metabolic defects associated with *fbdSir2^{KD}*, we tried to rescue the phenotype by administering L-carnitine. As shown in Fig. 8A, administering L-carnitine not only reduced circulating free fatty acid levels in control flies but also decreased the elevated free fatty acid levels that were observed in *fbdSir2^{KD}* flies. L-Carnitine increases beta-oxidation by enhancing the mitochondrial uptake of fatty acids via carnitine palmitoyl transferase 1 (CPT1). Inhibition of CPT1 activity using etomoxir abrogated the L-carnitine-mediated decrease in circulating free fatty acids in both control and *fbdSir2^{KD}* flies (Fig. 8A).

Next, we wanted to check if the L-carnitine-mediated reduction in free fatty acid levels also rescued reduced insulin signaling

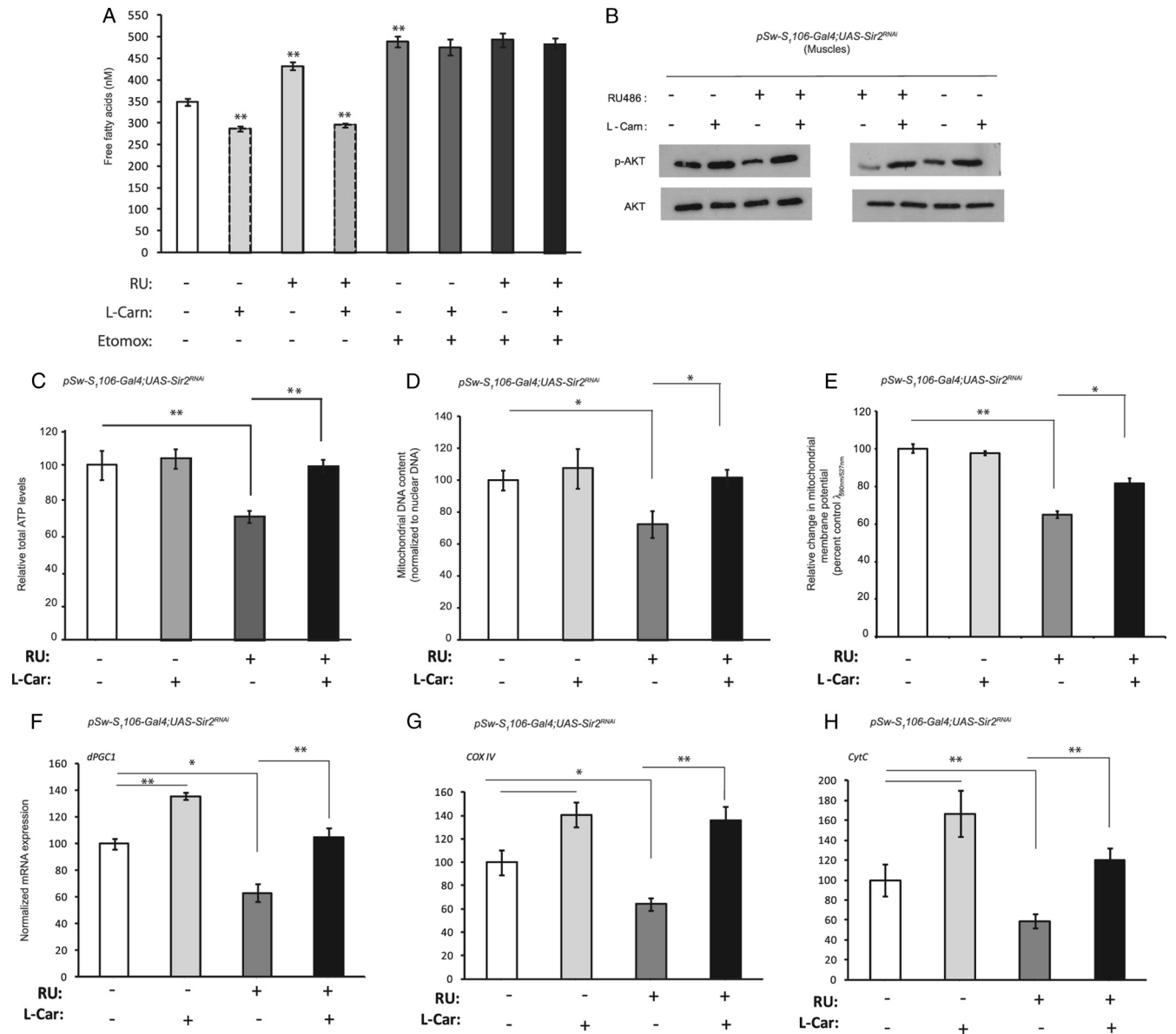


FIG 8 L-Carnitine rescues the defects in muscle physiology associated with fat body-specific knockdown of *dSir2*. Fat body-specific *dSir2* knockdown (*pSw-S,106-Gal4; UAS-Sir2^{RNAi}* or *fbdSir2^{KD}*) flies were treated or not treated with 25 mg/ml L-carnitine for 24 h and with 25 μ M etomoxir for 12 h. Circulating free fatty acid levels (A), phospho-AKT levels (B), total ATP levels (C), mtDNA content (D), and mitochondrial membrane potential (E) were determined. (F to H) Relative mRNA expression levels of *dPGC1*, *dCOX-IV*, and *dCyt.C-p*, as indicated. Sample size, eight sets each with 5 flies for free fatty acid measurement, 24 flies for measurement of phospho-AKT levels, and 36 flies for total ATP levels. Mitochondrial membrane potential was measured using JC-1 staining using four flies. In total, 90 stacks were imaged and quantified by ImageJ. Control, -RU486; overexpression/knockdown, +RU486 (200 μ M). All data are shown as means \pm standard errors of the means. *, $P < 0.05$; **, $P < 0.01$.

in the muscles of *fbdSir2^{KD}* flies. As expected, we found that L-carnitine administration rescued the reduced phospho-AKT levels in the muscles of *fbdSir2^{KD}* flies, and levels were comparable to those of control flies (Fig. 8B).

L-Carnitine rescues the defects in muscle mitochondrial physiology associated with fat body-specific knockdown of *dSir2*. Results shown in Fig. 2 and 3 demonstrate that *fbdSir2^{KD}* flies showed severe defects in mitochondrial functions. To investigate the contribution of free fatty acids in the mitochondrial dysfunctions associated with *fbdSir2^{KD}* flies, we tried to rescue the mitochondrial defects by L-carnitine. In response to L-carnitine

treatment, *fbdSir2^{KD}* flies showed a rescue in ATP levels (Fig. 8C), mitochondrial DNA content (Fig. 8D), and mitochondrial activity (Fig. 8E) as well as the expression of nuclear genes *dPGC1*, *dCOX IV*, and *dCyt.C-p* encoding mitochondrial proteins (Fig. 8F to H).

Overexpression of *dSir2* in the fat body but not muscles increases starvation survival. Based on our earlier report and the results presented above, we wanted to determine if overexpression of *dSir2* in the fat body and muscles influenced starvation survival. As shown in Fig. 9A, *fbdSir2^{OE}* led to a significant increase in starvation survival, and *fbdSir2^{KD}* reduced starvation resistance, consistent with our earlier findings (5). Corroborating our previ-

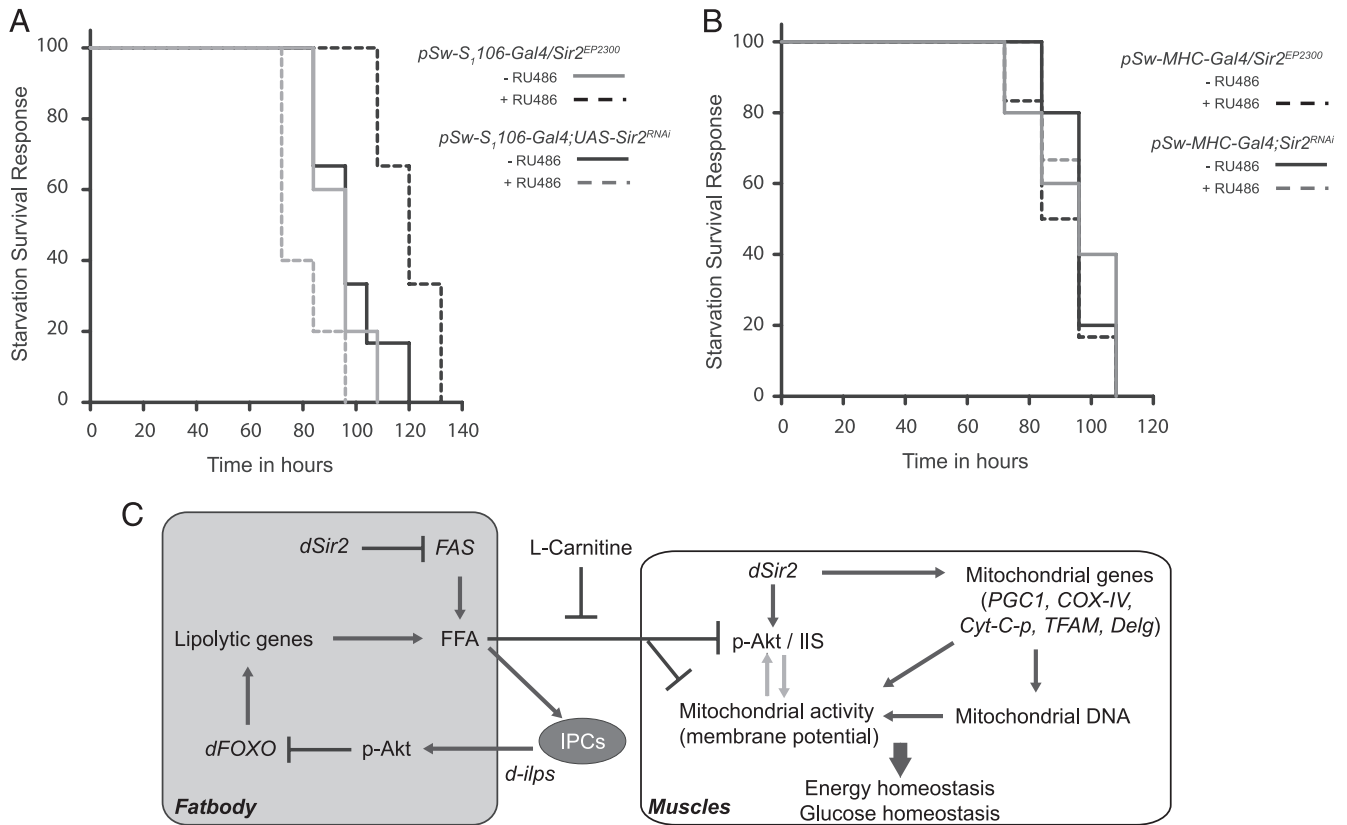


FIG 9 *dSir2* in the fat body is a key factor in maintaining metabolic regulatory network in the organism with consequences on survival. (A and B) Starvation survival of fat body-specific *dSir2* overexpression, *fbdSir2^{OE}* (*pSw-S₁106-Gal4/Sir2^{EP2300}*), and knockdown, *fbdSir2^{KD}* (*pSw-S₁106-Gal4; UAS-Sir2^{RNAi}*), flies (A) and muscle-specific *dSir2* overexpression, *mdSir2^{OE}* (*pSw-MHC-Gal4/Sir2^{EP2300}*), and knockdown, *mdSir2^{KD}* (*pSw-MHC-Gal4; UAS-Sir2^{RNAi}*), flies (B). Control, -RU486; overexpression/knockdown, +RU486 (200 μ M). For survival analyses a Mantel-Cox log rank test was used, and the statistical significance was as follows: $P = 0.0039$ for *fbdSir2^{OE}* and the control, $P = 0.049$ for *fbdSir2^{KD}* and the control, and P was nonsignificant for both *mdSir2^{OE}* and the control and for *mdSir2^{KD}* and the control. (C) Schematic indicating the functions of *dSir2* on organismal physiology. *dSir2* in the muscle is sufficient to regulate mitochondrial functions and glucose homeostasis. The ability of *dSir2* to maintain organismal physiology is elicited by its functions in the fat body (liver-equivalent tissue). Fat body *dSir2*-dependent changes in free fatty acids (FFA) might influence *dilp* production in the IPCs, which impinges on insulin signaling. *dilp*/IIS-dependent regulation of *dFOXO* activity affects lipid homeostasis in the fat body. We hypothesize that an absence of *dSir2* in the fat body leads to elevated FFA, thus resulting in a metabolic stress condition in muscles. This manifests as a prediabetic state, which is associated with reduced insulin signaling and mitochondrial dysfunctions in the muscle. Metabolic intervention using L-carnitine rescues this phenotype and supports the mechanistic insights into *dSir2*-dependent alterations in energy homeostasis.

ous report and highlighting the importance of fat body *dSir2*, overexpression and knockdown of *dSir2* in the muscles did not have any effect on starvation survival (Fig. 9B).

DISCUSSION

An inability to synchronize metabolic adaptation across tissues leads to physiological changes that are detrimental to the organism. Factors that mediate cellular responses to altered dietary inputs have also been implicated in establishing regulatory networks between metabolic tissues. In this study, we report the central role that *dSir2* plays in mediating organismal physiology. We also describe its metabolic functions in the fat body, which impinge upon muscle mitochondrial physiology and insulin signaling in a non-autonomous manner.

The importance of SIRT1 in insulin signaling has been reported by studies that have overexpressed or knocked down *SIRT1* (12, 13, 31, 44, 45). Using multiple lines to knock down and overexpress *dSir2* in the fat body (liver and adipocyte equivalent) and muscles, we show the evolutionary conservation of the role of Sir2 and SIRT1.

dSir2 enhancer trap lines (*EP2300* and *EP2384*), which contain an enhancer inserted in the upstream regulatory region of the *dSir2* gene, have been extensively used to overexpress the native protein (46–48). Due to the proximity of this *P*-element to the adjacent *DNAJ-H* gene, there are concerns about a simultaneous overexpression of *DNAJ-H* and *dSir2* in a Gal4-dependent manner. However, similar to an earlier report (46), we found that inducible overexpression of *dSir2* using various concentrations of RU486 did not alter the expression of *DNAJ-H* (see Fig. S11 in the supplemental material).

In this report, we show for the first time that overexpressing *Sir2* (*dSir2*) in the muscles improves insulin signaling and glucose homeostasis. Our results clearly point out the functional similarities between *dSir2* in the fat body and muscles in maintaining glucose homeostasis (Fig. 5). We show that muscle *dSir2* affects insulin signaling in an autonomous manner (Fig. 4), which is associated with alterations in *dInR* expression (see Fig. S12 in the supplemental material). It remains to be seen if muscle *dSir2* regulates other components of insulin signaling as in the case of mammalian *SIRT1* (13, 31, 49). Interestingly, the nonautono-

mous effects of fat body *dSir2* on muscle insulin signaling involved *dilp-2* and *dilp-5* and free fatty acids (Fig. 4 and 7). The increase in p-Akt levels in the muscles of *fbdSir2^{OE}* (Fig. 4) indicated enhanced insulin signaling.

We have earlier shown that ablating *dSir2* in the fat body results in defective fat metabolism (5). In this study, we have found that fat body *dSir2*-mediated changes affect lipid homeostasis, influence *dilp* production in the IPCs, and lead to an increase in insulin signaling in the fat body (Fig. 4, 6, and 7). In order to elucidate the complex interactions between abrogated fat metabolism and insulin signaling in the fat body, we have addressed the role of *dFOXO*. Alterations in *dFOXO*-GFP localization and changes in the expression of *dFOXO* target genes in *fbdSir2^{KD}* flies (Fig. 6) indicated that *dSir2* influenced *dFOXO* activity in an insulin-signaling-dependent manner. Simultaneous knockdown of *dSir2* and *chico* clearly demonstrated the involvement of insulin signaling in *dSir2*-dependent changes in *dFOXO* activity (Fig. 6). However, it was interesting that the expression of *brummer* lipase was independent of a direct interplay between *dSir2*-*dFOXO* in the fat body, unlike the other *dFOXO* target genes such as *GADD45*, *PEPCK*, *dCPL1*, and *d4eBP* (Fig. 6). These results indicated a novel interplay between *dSir2* and *dFOXO*, which is mediated by insulin signaling.

We have further explored the interplay between *dFOXO* and *dSir2* by overexpressing a constitutively nuclear form of *dFOXO* (*dFOXO-TM*). Interestingly, while TAG levels are brought back to control levels (Fig. 7), *fbdSir2^{KD}; dFOXO-TM^{OE}* flies have high circulating free fatty acids and elevated *dilp* levels and reduced p-Akt in the muscles (Fig. 7). Importantly, by comparing the phenotypes of *fbdSir2^{KD}; dFOXO-TM^{OE}* and *fbdSir2^{KD}; dFOXO-WT^{OE}* flies, it becomes obvious that in the absence of *fbdSir2*, a *dilp*-mediated increase in insulin signaling in the fat body impairs *dFOXO*-dependent transcription of lipolytic genes. It should also be noted that there is an increase in fatty acid synthase expression in the fat body of *fbdSir2^{KD}* flies (Fig. 7). A very recent report by Koh et al. has shown that an autonomous *dSir2*-*dFOXO* interaction is important for modulating mitochondrial functions in the background of a *PINK* mutation (47). However, our report is one of the first studies which describe the significance of *dSir2*-*dFOXO* interactions at the organismal level, specifically from a metabolic perspective. Moreover, our findings also point out that *dSir2* has a nonautonomous top-down control on *dFOXO* (and lipolytic effects) via *dilp*-IIS.

It is important that abrogating *dSir2* in the fat body leads to hyperlipidemic and hyperinsulinemic conditions, which are associated with insulin resistance in the muscles. Since these flies mimicked a prediabetic state, we wanted to investigate if *fbdSir2^{KD}* led to compromised mitochondrial functions and energy deficiency in the muscles. Interestingly, we found that both overexpression and knockdown of *dSir2* in the fat body resulted in an increase and decrease, respectively, of ATP in the muscles (Fig. 2). This was associated with changes in muscle mitochondrial DNA content and activity, which were positively correlated with *dSir2* expression in the fat body (Fig. 2). Further, the nonautonomous effects of *dSir2* on muscle mitochondrial physiology were phenocopied by flies that had *dSir2* perturbations (overexpression and knockdown) in the muscle (Fig. 1).

Obesity and diabetes are intrinsically characterized by elevated TAG/free fatty acid levels, impaired insulin signaling, and mitochondrial dysfunctions. Although several hypotheses have been

proposed to explain the causal relationship between fat metabolism, insulin signaling, and mitochondrial functions, a clear mechanistic insight is still lacking (4). We have examined whether defects in insulin signaling and mitochondrial functions originate due to the hyperlipidemic condition of *fbdSir2^{KD}* flies. We show that absence of fat body *dSir2* results in aberrant energy metabolism, which leads to an “energy-excess condition” with excessive accumulation of triglycerides (Fig. 7). This energy-excess condition leads to nutrient-induced mitochondrial stress, which is a major cause of insulin signaling defects. More importantly, nutrient-induced stress is associated with improper transport of fatty acids and impaired fatty acid oxidation in the mitochondria. A considerable body of work shows that L-carnitine improves the transport of fatty acids into the mitochondria and activates fatty acid oxidation (50–52). Additionally, L-carnitine has been previously used to rescue fat-dependent metabolic defects in diabetic and obese conditions (53, 54). In order to identify the primary cause of the phenotype associated with *fbdSir2^{KD}*, we attempted a metabolic rescue using L-carnitine. L-Carnitine administration led to a significant decrease in the fatty acids in *fbdSir2^{KD}* flies (Fig. 8). However, in the presence of etomoxir, which is an inhibitor of CPT1 (55, 56), L-carnitine administration failed to reduce the circulating free fatty acid levels (Fig. 8). This indicates that the ability of L-carnitine to reduce free fatty acids involves CPT1-dependent fatty acid oxidation in the mitochondria. Moreover, the L-carnitine-mediated rescue in free fatty acid levels was associated with the restoration of insulin signaling defects and mitochondrial functions in the muscles of these flies (Fig. 8). The metabolic rescue observed in these flies highlights the importance of elevated free fatty acids in manifestation of the metabolic defects associated with *fbdSir2^{KD}* flies (Fig. 8).

A recent report in mammals indicated that the ability of SIRT1 in the liver to regulate insulin signaling in the muscles and adipose tissue arises due to alterations in gluconeogenesis and glycemia (12). However, our findings in *fbdSir2^{KD}* flies indicate that aberrant fat metabolism and a systemic accumulation of free fatty acids are the primary cause of abrogated insulin signaling in the whole organism.

Our previous report and the results presented above highlight the importance of fat body *dSir2* in affecting inflammatory, metabolic, and energy parameters that affect organismal survival. Our results show that overexpression of *dSir2* in the fat body, but not muscle, increases starvation survival (Fig. 9). Although the functions of *fbdSir2* and *mdSir2* in regulating muscle-specific metabolic and glucose homeostasis are similar, the differential effects on organismal survival exemplify the importance of *fbdSir2* on organismal physiology.

In summary, we establish the central role that *dSir2* plays in maintaining organismal physiology. By comparing and contrasting its functions in the muscles and the fat body, we delineate the role of *dSir2* on fat metabolism from insulin signaling (Fig. 7). Our results also point out the top-down control that fat body *dSir2* exerts on insulin signaling, mitochondrial functions, and energy homeostasis in peripheral tissues, such as muscles (Fig. 9). In addition to describing the significance of the *dSir2*-*dFOXO* interactions at the organismal level, we provide mechanistic insights into *dSir2*-dependent cross talk across metabolic tissues, which is vital for physiological homeostasis (Fig. 9). Importantly, we show that overexpression of *dSir2* in fat body and muscles leads to better metabolic and energy homeostasis, which is phenocopied by flies

that have been treated with L-carnitine (Fig. 8 and 9). By demonstrating a metabolic rescue of the phenotype exhibited by fat body *dSir2* knockdown flies, we not only highlight its role in fat metabolism but also emphasize the clinical implications of Sir2 orthologs in diseases such as type 2 diabetes and obesity.

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