Drosophila Sirt2/mammalian SIRT3 deacetylates ATP synthase β and regulates complex V activity

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Abstract

Adenosine triphosphate (ATP) synthase β, the catalytic subunit of mitochondrial complex V, synthesizes ATP. We show that ATP synthase β is deacetylated by a human nicotinamide adenine dinucleotide (NAD⁺)-dependent protein deacetylase, sirtuin 3, and its Drosophila melanogaster homologue, dSirt2. dSirt2 mutant flies displayed increased acetylation of specific Lys residues in ATP synthase β and decreased complex V activity. Overexpression of dSirt2 increased complex V activity. Substitution of Lys 259 and Lys 480 with Gln, mimicking acetylation, decreased activity. Mass spectrometry and proteomic experiments from wild-type and dsirt2 mitochondria identified the Drosophila mitochondrial acetylome and revealed dSirt2 as an important regulator of mitochondrial energy metabolism. Additionally, we unravel a ceramide–NAD⁺–sirtuin axis wherein increased ceramide, a sphingolipid known to induce stress responses, resulted in depletion of NAD⁺ and consequent decrease in sirtuin activity. These results provide insight into sirtuin-mediated regulation of complex V and reveal a novel link between ceramide and Drosophila acetylome.

Introduction

Mitochondria generate cellular energy in the form of ATP through oxidative phosphorylation (OXPHOS). During this process, four multiprotein complexes located in the inner mitochondrial membrane transfer electrons in a series of redox reactions that creates a proton electrochemical gradient across the membrane. Complex V (ATP synthase or FOF1-ATPase) is a fifth multisubunit complex, which generates ATP using the energy created by the proton electrochemical gradient. ATP synthesis and hydrolysis by complex V is a sophisticated process that depends on the coordinated expression of nuclear and mitochondrial genes and the assembly and function of several subunits as an active complex. As a result of the centrality of mitochondrial energy generation in all vital cellular functions, impairment of this process in the mitochondria is strongly associated with aging and pathology in neurodegenerative disorders, cardiovascular disease, and diabetes, and cancer (Wallace, 2005).

Protein acetylation is emerging as a major posttranslational modification, and it involves reversible acetylation of the ε-amino group of internal lysine residues, which is regulated by lysine acetyltransferases and lysine deacetylases (Kouzarides, 2000; Yang, 2004). In recent years, the class III histone deacytases, the sirtuins, have emerged as prominent deacetylases (Haigis and Sinclair, 2010; Zhao et al., 2010; Lombard et al., 2011; Newman et al., 2012; Xiong and Guan, 2012). Mammals contain seven sirtuins: SIRT1, SIRT6, and SIRT7 are nuclear; SIRT2 is predominantly cytoplasmic; and SIRT3, SIRT4, and SIRT5 localize to the mitochondria. There are five sirtuins in the mitochondrial acetylome. Sir2 (CG5216), Sirt2 (CG5085), Sirt4 (CG3187), Sirt6 (CG6284), and Sirt7 (CG11305). BLAST (Basic Local Alignment Search Tool) searches reveal that Drosophila Sir2 shares 42% sequence identity with human SIR2, dSirt2 shows 49% identity to SIRT2 and 50% identity to human SIRT3, dSirt4 shares 49% identity with human SIRT4, dSirt6

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shows 50% identity to human SIRT6, and dSirt7 shows 46% identity to human SIRT7. dSir2 is the most well characterized among the *Drosophila* sirtuins. It is an essential gene that is expressed during development, and its localization is thought to be both cytoplasmic and nuclear. Sir2 is required for heterochromatic gene silencing and euchromatic repression (Rosenberg and Parkhurst, 2002). Earlier studies have also demonstrated roles for *Drosophila* Sir2 in life span extension and regulation of cell death and survival (Wood et al., 2004; Griswold et al., 2008; Banerjee et al., 2012). Sir2 has also been identified as a negative regulator of fat storage in *Drosophila* larvae (Reis et al., 2010). A neuroprotective role has been suggested for Sir2 because its loss leads to rescue of photoreceptor death observed in *Drosophila* models of Huntington’s disease (Luthi-Carter et al., 2010). Sirtuin activity depends on NAD+, which suggests that their activity is linked to the energy status of the cell via the NAD+/NADH ratio (Imai et al., 2000; Houtkooper et al., 2010; Imai and Guarente, 2010).

Global proteomic surveys have shown that mitochondrial proteins are extensively modified by lysine acetylation (Kim et al., 2006; Lombard et al., 2007; Choudhary et al., 2009; Hebert et al., 2013; Rardin et al., 2013). SIRT3 appears to be the major mitochondrial deacetylase. SIRT3-deficient mice exhibit mitochondrial protein hyperacetylation, whereas no significant changes were observed in *SIRT4*−/− and *SIRT5*−/− mitochondria. Despite the increased acetylation of proteins, germline deletion of SIRT3 or deletion of SIRT3 in a muscle- or liver-specific manner does not result in overt metabolic phenotypes (Lombard et al., 2007; Fernandez-Marcos et al., 2012). However, under conditions of stress such as fasting or caloric restriction, SIRT3 has been shown to regulate fatty acid oxidation by activating long chain acyl-CoA (coenzyme A) dehydrogenase, ketone body production through 3-hydroxy-3-methylglutaryl CoA synthase 2, in mitigating reactive oxygen species (ROS) damage by deacetylating superoxide dismutase, and protecting mice from age-related hearing loss through activation of isocitrate dehydrogenase (Hirschey et al., 2010; Qiu et al., 2010; Shimazu et al., 2010; Someya et al., 2010; Tao et al., 2010; Chen et al., 2011). A role for SIRT3 has been implicated in regulating OXPHOS because germline *Sirt3*−/− mice show a decrease in ATP levels in different organs (Ahn et al., 2008; Cimen et al., 2010; Finley et al., 2011b; Shimmura et al., 2011; Wu et al., 2013). However, muscle- or liver-specific deletion of SIRT3 did not result in changes in ATP levels, suggesting that SIRT3 deletion in a tissue-specific manner does not affect cellular energy levels (Fernandez-Marcos et al., 2012). In this study, we have used *Drosophila* as a model and performed mass spectrometric analyses on wild-type and *dsir2* mutant flies to identify the *Drosophila* mitochondrial and dSirt2-regulated acetylome. Our proteomic experiments show *Drosophila* Sirt2 is an important regulator of mitochondrial function and is the functional homologue of mammalian SIRT3. These experiments also provide a comprehensive view of the impact of acetylation on OXPHOS and its regulation by dSirt2. We demonstrate that ATP synthase β, the catalytic subunit of complex V, is an acetylated protein, and it is a substrate of *Drosophila* Sirt2 and human SIRT3.

In this study, we also reveal a novel connection between NAD metabolism, sirtuins, and the sphingolipid ceramide. Sphingolipids are an essential class of lipids that are building blocks for membranes and serve as transducers in signaling cascades that regulate cell growth and death (Hannun and Obeid, 2008). Ceramide, a central intermediate in sphingolipid metabolism, mediates many stress responses, and recent literature highlights that perturbations in ceramide levels can affect glucose and fat metabolism (Bikman and Summers, 2011). How ceramide and other sphingolipids affect cellular metabolism, what metabolic pathways they impinge on, and identification of the ensuing functional consequences are only beginning to be explored. We show that *Drosophila* mutants of sphingolipid metabolism, particularly, ceramide kinase mutants (*dcerk*), have increased levels of ceramide and decreased levels of NAD+. This results in reduced dSirt2 activity in *dcerk* mutants, leading to acetylation of several subunits of complex V, including ATP synthase β and reduced complex V activity. These experiments reveal a novel axis involving ceramide, NAD, and sirtuins.

**Results**

**Ceramide increase affects NAD+ level and sirtuin activity**

We performed metabolomic profiling on sphingolipid mutants that accumulate ceramide to gain insight into metabolic pathways that could be altered in these mutants. Our earlier study combined metabolomic profiling with genetic and biochemical approaches and demonstrated that *dcerk* mutants show an increased reliance on glycolysis, which leads to an increase in lactate to compensate for the decreased production of ATP through OXPHOS (Nirala et al., 2013). The increase in glycolytic flux is also observed in a mammalian model of ceramide increase, mice heterozygous for the ceramide transfer protein (Wang et al., 2009; Nirala et al., 2013). In addition to changes in glycolytic intermediates, metabolomic profiling revealed that *dcerk* mutants have a significantly decreased level of NAD+ compared with that in *w*+/+ (control) flies (Fig. 1 A). The NAD+ level is controlled by balancing synthesis, salvage, and consumption pathways (Fig. 1 B). Like in mammals, NAD+ can be synthesized in *Drosophila* from the salvage pathway from nicotinic acid, nicotinamide, and nicotinamide riboside (nicotinamide mononucleotide) and by the de novo pathway from tryptophan (Zhai et al., 2006; Campesan et al., 2011). We used mass spectrometry (MS) to measure the levels of intermediates in these pathways and related metabolites. The levels of key intermediates, such as nicotinamide riboside in the salvage pathway and hydroxykynurenine in the de novo pathway, of NAD+ synthesis in *dcerk* are increased compared with those in controls, suggesting that synthesis pathways do not appear to be compromised (Fig. 1 C). We then tested whether the NAD+ level is altered in the ceramidase mutant (*cdase*), another mutant of the sphingolipid pathway that accumulates ceramide (Acharya et al., 2008). The NAD+ level is also decreased in *cdase* (Fig. S1). Estimation of intermediates of the salvage and de novo pathways of NAD+ synthesis in *cdase* reveals a fivefold increase in 3-hydroxy kynurenine, which suggests a compensatory increase
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Figure 1. Increase in ceramide levels results in depletion of NAD⁺ and decrease in sirtuin activity leading to hyperacetylation of proteins in different cellular compartments. (A) dcerk¹ fly extracts show 65% reduction in NAD⁺ level compared with w¹¹¹ control. n = 3. (B) NAD synthesis and salvage pathways. TDO, tryptophan-2,3-dioxygenase; KMO, kynurenine 3-monooxygenase; QPRTase, quinolinate phosphoribosyltransferase; NaMNAT, nicotinic acid mononucleotide adenyltransferase; NADS, NAD synthetase; NMNAT, nicotinamide mononucleotide adenyltransferase; NAmPRTase, nicotinamide phosphoribosyltransferase; NDase, nicotinamidase; NαPRTase, nicotinamide phosphoribosyltransferase. (C) Mass spectrometric measurements of metabolites in the salvage and the de novo pathways for synthesis of NAD⁺. n = 3. (D) Soluble, mitochondrial, and nuclear extracts were prepared from w¹¹¹ and dcerk¹ mutant flies and separated by PAGE. Protein acetylation was monitored by Western blotting using an anti–acetyl-Lys antibody. The individual blots were probed with antibodies to actin, porin, and H2A as loading controls. dcerk¹ mutants show protein hyperacetylation in the different cellular compartments. Arrows indicate proteins that are hyperacetylated in dcerk¹ compared with w¹¹¹ control. MM, molecular mass. (E) Mitochondrial NAD⁺ levels are decreased in dcerk¹ compared with control. (F) d14 long chain base ceramides with different fatty acids were estimated by MS in sphingolipid-enriched fractions prepared from w¹¹¹ and dcerk¹ mitochondria. C denotes the carbon chain length of fatty acids in the different ceramides. The amount of ceramide is normalized to total carbon content, and the level in w¹¹¹ is taken as 100%. Many ceramides show significant increase in the mutant mitochondria compared with w¹¹¹. n = 3. Error bars represent SDs. *, P ≤ 0.05–0.01; **, P ≤ 0.01–0.001; ***, P ≤ 0.001–0.0001 in Student’s t test.
Figure 2.  

dcerk\textsuperscript{1} mutants show acetylation of many OXPHOS subunits and decrease in complex V activity, which is rescued by supplementing NAD\textsuperscript{+} and inhibited by nicotinamide. dSim2 regulates complex V activity in dcerk\textsuperscript{1} mutants. (A) BN-PAGE–separated bands from dcerk\textsuperscript{1} were digested with trypsin and subjected to LC-MS/MS to identify the different subunits of the complexes and the subunits that are acetylated. (B) dcerk\textsuperscript{1} mitochondria show a 40\% reduction in complex V activity. Supplementing with NAD\textsuperscript{+} restores complex V activity in dcerk\textsuperscript{1}. Complex V activity was normalized to the activity of

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in tryptophan metabolism in an attempt to maintain NAD⁺ levels. These results suggest a connection between ceramide and NAD metabolism.

One of the main NAD⁺-consuming pathways involves sirtuins because they are NAD⁺-dependent enzymes, and the availability of NAD⁺ is an important mechanism that regulates their activity (Imai et al., 2000). dcerk¹ had greater decreases in NAD⁺ levels compared with those in control; therefore, we investigated this mutant in more detail. As a readout for sirtuin activity in dcerk¹, we compared the acetylation status of proteins in extracts prepared from different cellular compartments by western analysis using a pan–acetyl-Lys antibody. Fig. 1 D shows that protein acetylation is increased in soluble, nuclear, and mitochondrial extracts of dcerk¹ compared with those in control extracts, suggesting a likely decrease in sirtuin deacetylase activity in dcerk¹. We decided to focus on the mitochondrial compartment because dcerk¹ exhibits phenotypes associated with mitochondrial dysfunction. These include decreased OXPHOS and decreased mitochondrial ATP level (Nirala et al., 2013). To test whether NAD⁺ level is altered in the mitochondria, we estimated its level in mitochondria isolated from w¹¹¹⁸ and dcerk¹ flies. Indeed, the mitochondrial NAD⁺ level is decreased in dcerk¹ (Fig. 1 E). We estimated different ceramides by MS in purified mitochondria isolated from dcerk¹ and w¹¹¹⁸ to test whether ceramide levels are increased in mutant mitochondria (Dasgupta et al., 2009). Many ceramides show significantly increased levels in dcerk¹ mitochondria compared with those in the control (Fig. 1 F). The experiments described in the following sections probe the correlation between dcerk¹, sirtuin function, the acetylation of mitochondrial proteins, and its impact on mitochondrial function.

Several OXPHOS proteins including those of complex V are acetylated in dcerk¹ mutants

To investigate the increase in mitochondrial Lys acetylation observed in dcerk¹, we decided to focus on OXPHOS because it plays a central role in mitochondrial function. We prepared mitochondria from control and dcerk¹ flies and resolved individual OXPHOS complexes by blue native (BN) PAGE (Fig. S2 A). BN-PAGE allows for separation of complexes in their native state, which enables assessment of both the amount and activity of complexes (Wittig et al., 2006). We confirmed the identity of each complex by in-gel activity staining. As seen from the Coomassie-stained gel, the amount of complexes is not different in control and mutant mitochondria, whereas activity staining suggested that activities of complexes II, III, IV, and V were reduced in dcerk¹ mutant flies. Each band (other than complex I, which could not be isolated in sufficient amounts to identify a majority of its 50 subunits) was subjected to proteolytic digestion and analyzed by liquid chromatography (LC)–MS/MS. The proteins identified in each complex in dcerk¹ and those that are acetylated are shown in Fig. 2 A. Acetylated proteins were identified in each of the four complexes, suggesting that it could be a prevalent modification among OXPHOS proteins. Of the four complexes, we chose complex V for detailed analyses because it showed the largest number of acetylated proteins and because it directly controls ATP synthesis and hydrolysis, thereby strongly influencing cellular ATP levels.

**Drosophila sirt2 mutants regulate complex V activity**

Complex V catalyzes both ATP synthesis and ATP hydrolysis coupled with transmembrane proton translocation in mitochondria (Boyer, 1997). The enzyme has two moieties—the water-soluble F1 portion, which contains the catalytic sites for ATP generation and hydrolysis, and the membrane-integrated F0 portion, which mediates proton translocation (Abrahams et al., 1994; Noji et al., 1997). The enzymatic complex consists of a catalytic headpiece (α₃β₃) that contains the three catalytic sites for ATP synthesis (one in each β subunit, a proton channel (α₃) and two stalks, the central rotor (γ, δ, and ε), and the peripheral stator (βF₆OSCP)).

dcerk¹ mutants display a 40% decrease in complex V ATPase activity compared with that of control (Fig. 2, B and C). Because this decrease in activity was accompanied by decreased NAD⁺ and increased acetylation of complex V subunits, we tested whether we could rescue complex V activity in dcerk¹ by supplementing with NAD⁺. We raised dcerk¹ flies for a short period of time in food supplemented with NAD⁺ and measured complex V activity. Supplementing with NAD⁺ rescued the ATPase activity in dcerk¹ (Fig. 2 B). Supplementing high concentrations of nicotinamide, an inhibitor of sirtuin, further decreases complex V activity in the mutants (Fig. 2 C).

We estimated NAD⁺ and nicotinamide levels in wild-type flies supplemented with NAD⁺ and measured complex V activity. Supplementing with NAD⁺ and nicotinamide in the fed flies as a result of feeding pharmacological amount of nicotinamide in these flies (Fig. S2 B). These results show that complex V activity can be modulated by activation of a sirtuin with NAD⁺ or inhibition of a sirtuin with nicotinamide.

To test whether any of the five *Drosophila* sirtuins could regulate complex V, we measured ATPase activity of the complex in mitochondria prepared from *sir2-*, *sir2-*, *sir4-*, and *sir5-*. *Sir2* expression was ubiquitously overexpressed using the actin-GAL4 driver in *dsir2* and *dcerk¹* mutants. The UAS-Sir2 transgenic and GAL4 driver in *dsir2* mutants show a further reduction in complex V activity compared with the single mutants. Supplementing with NAD⁺ does not alter this activity. n = 3. In *fas* flies, the wild-type Sir2 transgene was ubiquitously overexpressed by using the actin-GAL4 driver in *dsir2* and *dcerk¹* mutants. The UAS-Sir2 transgenic and GAL4 driver in each genetic background were additional controls. Mitochondria were prepared, and complex V activity was measured. The activity of w¹¹¹⁸ was taken as 100%. Overexpression of the Sir2 transgene significantly rescues complex V activity in the *dsir2* mutant and partially in the *dcerk¹* mutant. Error bars represent 95% confidence intervals.

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*Citrate synthase, a mitochondrial marker. The ATPase activity of untreated w¹¹¹⁸ was taken as 100%. (C) Nicotinamide treatment further inhibits complex V activity in dcerk¹. The ATPase activity of untreated w¹¹¹⁸ was taken as 100%. n = 3. (D) Mitochondria were isolated from different sir2-null mutants, and complex V activity was measured. Complex V activity was normalized to the activity of citrate synthase. The ATPase activity of w¹¹¹⁸ was taken as 100%. *Sirt2* mutants show 30% reduction in activity. n = 3. (E) Mitochondria were isolated from w¹¹¹⁸, dcerk¹, dsir2, dcerk¹.dsir2, and dcerk¹.dsir2 raised on food supplemented with NAD⁺, and complex V activity was measured. The ATPase activity of w¹¹¹⁸ was taken as 100%. dcerk¹.dsir2 mutants show a further reduction in complex V activity compared with the single mutants. Supplementing with NAD⁺ does not alter this activity. n = 3. (F) The wild-type Sir2 transgene was ubiquitously overexpressed using the actin-GAL4 driver in *dsir2* and *dcerk¹* mutants. The UAS-Sir2 transgenic and GAL4 driver in each genetic background were additional controls. Mitochondria were prepared, and complex V activity was measured. The activity of w¹¹¹⁸ was taken as 100%. Overexpression of the Sir2 transgene significantly rescues complex V activity in the *dsir2* mutant and partially in the *dcerk¹* mutant. Error bars represent SDs. * P ≤ 0.05–0.01; ** P ≤ 0.01–0.001; *** P ≤ 0.001–0.0001 in Student’s t test.*
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sirt7-null mutants (Xie and Golic, 2004). Because Sirt6-null mutants are not available, Sirt6 knockdown flies were used, and this did not result in a significant reduction of complex V activity (unpublished data). Fig. 2 D shows that sirt2 mutant mitochondria display ~30% reduction in ATPase activity compared with control. We then generated dcerk1.sirt2 double mutants and assessed complex V activity. As seen in Fig. 2 E, there is a further reduction in complex V activity of dcerk1 in the absence of sirt2. Furthermore, feeding NAD+ does not rescue complex V activity of dcerk1 mutants in the absence of sirt2 (Fig. 2 E). In addition, the double mutants are semilethal, whereas individual mutants are viable, supporting a genetic interaction between these two mutants. Ubiquitous overexpression of a wild-type copy of the Sirt2 transgene (using the actin-Gal4 driver) in the sirt2 mutant results in a significant increase in complex V activity (Fig. 2 F). Overexpression of wild-type Sirt2 in the dcerk1 mutant results in partial rescue. Overexpressed Sirt2 could compete for the limited NAD+ in dcerk1 and result in better deacetylation of its substrates, including complex V, thereby leading to partial rescue (Fig. 2 F).

We also measured the ATP synthase activity in dcerk1 and dsiirt2 single and dcerk1.dsiirt2 double mutant flies. In intact mitochondria, the amount of oxygen consumption reflects the amount of ATP synthesis, and inhibition of ATP synthase or other OXPHOS complexes can cause a decrease in oxygen consumption. We measured state 3 respiration (in the presence of added ADP) in freshly isolated mitochondria from the different flies. The dcerk1 and dsiirt2 mitochondria displayed decreased
oxygen consumption and decreased ADP responsiveness compared with that in control, suggesting that the rate of ATP synthesis via OXPHOS was lower in the mutants compared with that in the control (Fig. 3 A). Absence of sirt2 further decreases the rate in dcerk as observed in dcerk dsirt2 double mutant flies (Fig. 3 A). We measured the ATP level in mitochondria isolated from w1118, dcerk1, and dsirt2 single mutants and dcerk1 dsirt2 double mutants. Indeed, dcerk1 and dsirt2 show a 40% reduction in ATP levels compared with w1118, whereas there is a further decrease in the double mutants (Fig. 3 B). These results suggest that Drosophila Sirt2 is a primary regulator of complex V activity in the dcerk1 mutant. Because absence of Sirt2 exacerbates complex V activity and ATP level of dcerk1, we also tested whether loss of Sirt2 would further increase the acetylation of mitochondrial proteins observed in dcerk1. Indeed, mitochondrial proteins from dcerk1 dsirt2 double mutants show increased acetylation compared with the single mutants (Fig. 3 C). We then tested how dsirt2 mutant flies respond to conditions such as starvation stress, which increase ATP demand. dsirt2 mutants succumb to starvation stress more rapidly than wild-type flies (Fig. 3 D). The decreased ATP synthetic capacity in the mutants likely exacerbates the starvation survival response. dcerk1 mutants, however, do not show increased sensitivity to starvation because of AKT/FOXO-mediated up-regulation of novel triglyceride lipases (Nirala et al., 2013). A further indication of mitochondrial dysfunction in the dsirt2 mutant is the increased ROS level (Fig. S3).

Drosophila mitochondrial protein acetylome and dsirt2-regulated acetylome

To begin to understand how Dsirt2 influences mitochondrial protein acetylation, we characterized the mitochondrial acetylome in wild-type and dsirt2 flies by quantitative MS. Previously, one proteome-wide mapping of the Drosophila acetylome has been performed from embryonically derived SL2 tissue-culture cells (Weinert et al., 2011). However, no proteomic investigations of either the total or mitochondrial acetylome have been performed in flies. The strategy we used for identification of the Drosophila mitochondrial acetylome and the dsirt2-regulated acetylome is outlined in Fig. S4. We identified 1,178 unique acetyl-Lys sites in 530 proteins. Of these, 652 unique acetyl-Lys sites in 214 proteins were identified as mitochondrial using FlyBase and MitoDrome, a database of Drosophila nuclear genes coding for mitochondrial proteins (Table S1; Sardiello et al., 2003). The identification of a large set of acetylated proteins enabled a systematic analysis of these proteins.

Pathway analysis of the Drosophila mitochondrial protein acetylome reveals widespread acetylation of OXPHOS proteins and other metabolic pathways

We performed functional annotation to determine the gene ontology (GO) terms associated with the acetylated proteins in wild-type control flies. The cellular component ontology, which describes protein location at the substructural level, shows a significant enrichment of mitochondrial-associated terms (Fig. 4 A). Analysis of the distribution of the number of acetyl-Lys sites detected per mitochondrial protein shows that 43% of the proteins have one identifiable site, whereas the remainder have two or more sites, which suggests multiple points of regulation for a given protein (Fig. 4 B).

To gain insight into the biological functions of the acetylated proteins, we performed a pathway enrichment analysis of the mitochondrial acetylated proteins. Fig. 4 C shows that there is significant enrichment of proteins involved in the respiratory electron transport chain and enzymes involved in pyruvate metabolism, TCA cycle, and amino acid metabolism. Other pathways identified include β-oxidation of fatty acids, branched-chain amino acid catabolism, ketone body metabolism, and antioxidant metabolism. In addition, our study identifies acetylated proteins in Lys catabolism (lysine ketoglutarate reductase), β-oxidation of pristanoyl CoA (pristanoyl CoA oxidase), and ω fatty acid metabolism. To visualize probable consensus patterns around the acetyl-Lys sites, we compared the amino acid sequences of all acetylated sites using iceLogo (Colaert et al., 2009). A preference for Leu or Tyr is observed at the +1 position, and a preference for Asp is observed at the −1 and −3 positions, whereas positively charged residues are excluded at these positions (Fig. 4 D).

Analysis of the dSirt2-regulated acetylome identifies substrates in OXPHOS and metabolic pathways

A comparison of the wild-type Drosophila mitochondrial acetylome to that of dsirt2 mitochondria identifies that 204 acetylation sites in 116 proteins increased >1.5-fold in the mutant (Table S2). The GO cellular component analysis showed a significant enrichment of mitochondrial terms (Fig. 4 E). Pathways enriched in the dsirt2 mutant included TCA cycle, amino acid metabolism, and electron transport chain (Fig. 4 F). Previously validated substrates of mouse Sirt3, such as succinate dehydrogenase A, isocitrate dehydrogenase 2, and long chain acyl-CoA dehydrogenase, are identified in our study. These results suggest that Drosophila Sirt2 could serve as the functional homologue of mammalian SIRT3. In addition, mammalian SIRT3 shows highest homology (50% identity and 64% similarity) to Drosophila Sirt2.

Analyses of flanking sequence preferences in acetylated proteins that are increased in dsirt2 suggest a preference for Arg at the +1 site and exclusion of positive charge at the −1 position (Fig. 4 G). The molecular function and biological process components of GO reveal significant enrichment of different complexes of the electron transport chain, with complex I being most significant followed by complex V in the wild-type mitochondrial acetylome (Fig. 5 A). The distribution of acetyl-Lys sites among the electron transport chain complexes suggests that 30% of the acetylated subunits have one Lys site, whereas 70% have more than one site (Fig. 5 B). GO shows that both complex I and complex V feature prominently in the Sirt2 mutant acetylome (Fig. 5 C). Fig. 5 D shows a list of complex V subunits with site-specific acetyl-Lys identified earlier in dcerk and those that change 1.5-fold or more in dsirt2. To understand how complex V activity could be influenced by reversible acetylation, we focused on ATP synthase β, as it is the catalytic subunit of the complex. We performed subsequent experiments in mammalian...
Figure 4. Analyses of the *Drosophila* mitochondrial acetylome and dsir2 acetylome reveal extensive acetylation of proteins engaged in OXPHOS and metabolic pathways involved in energy production. (A) GO analysis (cellular component) of the acetylome shows significant enrichment of mitochondria-related terms. (B) Distribution of acetyl-Lys sites identified per protein in the mitochondrial acetylome. (C) Pathway analysis of the mitochondrial acetylome with the number of proteins identified per pathway indicated. (D) Consensus sequence logo plot for acetylation sites, ±6 amino acids from all acetyl-Lys identified in the mitochondrial acetylome. (E) GO analysis (cellular component) of the acetylated proteins that increase in the dsir2 mutant. (F) Pathway analysis of the acetylated proteins that increase in dsir2 with the number of proteins identified per pathway indicated. (G) Consensus sequence logo plot for acetylation sites, ±6 amino acids from all acetyl-Lys identified in proteins that increase in dsir2.
Human ATP synthase β is an acetylated protein, and SIRT3 regulates its deacetylation and complex V activity

We evaluated whether mammalian ATP synthase β is an acetylated protein. An expression vector encoding DDK-tagged human ATP synthase β or vector alone was transfected into HEK293T cells. After immunoprecipitation with the DDK tag antibody, acetylation level was determined by Western blotting with the acetyl-Lys antibody. ATP synthase β is clearly an acetylated protein (Fig. 6A). Based on our results from the experiments in Drosophila described in the previous sections, we decided to test whether human SIRT3 can modulate the reversible acetylation of ATP synthase β. Knockdown of endogenous SIRT3 by siRNA increased the acetylation of ATP synthase β in the mammalian system. The mammalian experiments also benefited from the availability of reagents and tools that are lacking in Drosophila.
β (Fig. 6 B). Conversely, overexpression of SIRT3 leads to increased deacetylation of ATP synthase β (Fig. 6 C). To ascertain whether ATP synthase β is a specific target of SIRT3, we knocked down or overexpressed two other mitochondrial sirtuins—SIRT4 and SIRT5. Knockdown of endogenous SIRT4 or SIRT5 by siRNA does not affect acetylation status of ATP synthase β (Fig. 6, D and F). Overexpression of SIRT4 and SIRT5 also does not affect acetylation of ATP synthase β (Fig. 6, E and G). Additionally, knockdown or overexpression of a nuclear sirtuin, SIRT1, also does not impact acetylation of ATP synthase β (Fig. 6, H and I). To determine whether the acetylation state of ATP synthase β altered complex V activity, we measured complex V activity in mitochondria prepared from cells treated with SIRT3 siRNA and scrambled siRNA. Knockdown of SIRT3 results in ~40% decrease in complex V activity (Fig. 6 J). We tested whether SIRT3 could directly interact with ATP synthase β. We immunoprecipitated endogenous ATP synthase β from HEK293T cells overexpressing SIRT3 and found that SIRT3 could communoprecipitate with ATP synthase β (Fig. 6 K). These results collectively suggest that mammalian SIRT3, like Drosophila Sirt2, can influence complex V activity by regulating the acetylation status of ATP synthase β.

Conserved Lys residues in ATP synthase β regulate complex V activity

We identified which Lys residues on ATP synthase β were important for regulating complex V activity. MS analysis of mitochondrial proteins shows that ATP synthase β is hyperacetylated at Lys 236 and Lys 457 in the absence of Drosophila Sirt2 (in dsirt2). Both these Lys residues were also identified in ATP synthase β in dceerk1. The equivalent residues in human ATP synthase β are Lys 259 and Lys 480. Before assessing the functional significance of these site-specific Lys residues, we down-regulated endogenous ATP synthase β level in HEK293T cells by siRNA to minimize any contribution from the endogenous protein. We generated an siRNA-resistant ATP synthase β construct by altering the codons without changing the amino acid residues in the siRNA target sequences. Fig. 7 A shows that robust expression of this construct is observed in the presence of siRNA compared with that of the nonresistant construct. We generated siRNA-resistant Lys 259 and Lys 480 mutants by changing the individual Lys to Arg or Glu. The Lys to Arg mutation is considered to mimic the deacetylated state, whereas the Lys to Glu mutation abolishes the positive charge and mimics the acetylated state (Schwer et al., 2006; Tao et al., 2010). Because of the limited amount of mitochondria obtained from the transfected cells, we used a commercially available assay for measuring complex V activity described in detail in the Materials and methods section. Using this assay, we determined the activity of the Lys 259 and Lys 480 variants. Fig. 7 B shows that K259R and K480R mutants have a 70–80% increase in activity, whereas K259Q and K480Q have an ~40% decrease in activity compared with that of the control. We also generated double mutants that mimic the deacetylated state (K259/480R), which show a further increase (150%) in activity, whereas double mutants that mimic the acetylated state (K259/480Q) show a 75–80% decrease in activity compared with that of the control. These results indicate that complex V activity correlates with the acetylation state of ATP synthase β. These results are consistent with a model proposing that SIRT3 regulates this process by deacetylating Lys 259 and Lys 480.

To understand how these Lys residues could affect the catalytic activity of ATP synthase β, we examined the crystal structure of the bovine F1–stator complex in different conformational states: nucleotide free or bound to adenylyl imidodiphosphate, ADP, or ADP with the transition state mimic aluminum fluoride (Abrahams et al., 1994; Menz et al., 2001; Rees et al., 2009). Lys 259 (Lys 206, based on crystal structure labeling) identified in our study is located in a surface-exposed loop in all three states (Fig. 7 C). This loop connects to helix C, which follows the loop containing β–Glu 188 and β–Arg 189, and contacts α–Arg 373. All three residues are essential for catalysis (Menz et al., 2001). β–Glu 188 directly interacts with the nucleophilic water molecule that attacks the terminal phosphate of ATP during hydrolysis. β–Arg 189 is involved in the direct binding of the γ phosphate, and α–Arg 373 in the α chain is a critical residue, termed an arginine finger, which contributes to catalysis by accelerating the rate of ATP cleavage by stabilizing the transition state of ATP hydrolysis. Thus, acetylation of Lys 206 could potentially lead to conformational changes in the active site region and affect the positioning of β–Glu 188, β–Arg 189, and α–Arg 373.

Inverse correlation between acetylation of ATP synthase β and complex V activity in human cancer cell lines

We finally assessed the pathophysiological implications of acetylation of ATP synthase β. The prevalence of acetyl modifications in mitochondrial proteins that affect energy metabolism suggests that altered acetylation could potentially contribute to diseases such as cancer and cardiac dysfunction, which exhibit recognizable changes in energy metabolism. For these experiments, we chose three human breast cancer cell lines with different invasive potential: T47D, MDA-MB-435, and MDA-MB-231. T47D cells are more differentiated, weakly invasive, and rely less on aerobic glycolysis for energy compared with MDA-MB-231 cells, which are less differentiated, strongly invasive, and have increased reliance on glycolysis for energy generation. We immunoprecipitated endogenous ATP synthase β from these cells and probed them with the acetyl-Lys antibody. ATP synthase β is less acetylated in T47D cells compared with those of...
Figure 6. Human ATP synthase is an acetylated protein, and its deacetylation is regulated by SIRT3. (A) pCMV vector or ATP synthase β (DDK tagged) was transfected in HEK293T cells, immunoprecipitated using an antibody to DDK tag, and probed with an antibody to acetyl-Lys [Ac-Lys]. (B) HEK293T cells were cotransfected with ATP synthase β (ATP syn β) and either SIRT3 siRNA or scrambled siRNA. ATP synthase β was immunoprecipitated, and its acetylation status was assessed. The bottom blot shows reduction of SIRT3 protein upon siRNA treatment. Knockdown of SIRT3 increases acetylation of ATP synthase β. (C) Expression vector for wild-type SIRT3 was cotransfected in HEK293T cells with ATP synthase β, and its acetylation status was assessed after immunoprecipitation. Overexpression of SIRT3 decreases acetylation of ATP synthase β. (D) HEK293T cells were cotransfected with ATP synthase β and either SIRT4 siRNA or scrambled siRNA. SIRT4 knockdown does not affect acetylation of ATP synthase β. (E) Wild-type SIRT4 expression vector was cotransfected in HEK293T cells with ATP synthase β, and its acetylation status was assessed after immunoprecipitation. SIRT4 overexpression does not affect acetylation of ATP synthase β. (F) HEK293T cells were cotransfected with ATP synthase β and either SIRT5 siRNA or scrambled siRNA. SIRT5 knockdown does not affect acetylation of ATP synthase β. (G) Wild-type SIRT5 expression vector was cotransfected in HEK293T cells with ATP synthase β, and its acetylation status was assessed after immunoprecipitation. SIRT5 overexpression does not affect acetylation of ATP synthase β. (H) HEK293T cells were cotransfected with ATP synthase β and either SIRT1 siRNA or scrambled siRNA. SIRT1 knockdown does not affect acetylation of ATP synthase β. (I) Wild-type SIRT1 expression vector was cotransfected in HEK293T cells with ATP synthase β, and its acetylation status was assessed after immunoprecipitation. SIRT1 overexpression does not affect acetylation of ATP synthase β. (J) Mitochondria were prepared from SIRT3 siRNA–treated or scrambled siRNA–treated cells, and complex V activity was measured. The activity of mitochondria from scrambled siRNA treatment was taken as 100%. SIRT3 knockdown results in an ~40% decrease in complex V activity. n = 3; error bars represent SDs. **, P ≤ 0.01–0.001 in Student’s t test. (K) Endogenous ATP synthase β was immunoprecipitated from HEK293T cells overexpressing SIRT3, and the immunoprecipitate was probed with antibodies to ATP synthase β and SIRT3. SIRT3 can coimmunoprecipitate with ATP synthase β. IP, immunoprecipitation; WB, Western blot.
Figure 7. Acetylation of ATP synthase β at Lys 259 and Lys 480 regulates complex V activity. (A) Nondegradable (non-deg) ATP synthase β [ATP syn β] is resistant to targeted siRNA-mediated degradation. (B) siRNA-resistant versions of ATP synthase β wherein Lys 259 or Lys 480 either individually or together were substituted with Arg or Gln and cotransfected with siRNA to ATP synthase β. Mitochondria were prepared, and complex V activity was measured using an immunocapture assay followed by the amount of ATP synthase β in the same samples. The activity of siRNA-resistant ATP synthase β is taken as 100%. Substitution of either Lys or both with Arg results in increased activity, whereas substitution with Gln results in decreased complex V activity. **, P ≤ 0.01–0.001; ***, P ≤ 0.001–0.0001. (C) An overview of the crystal structure of bovine mitochondrial F1–stator complex is shown on the left in ribbon representation. The F1 domain contains 3α (green), 3β (purple), and a single subunit of γ (pink). The stator complex shows portions of subunit b (teal), oligomycin sensitivity-conferring protein (orange), and F6 (yellowish green). The right shows a closer view of the region around the active site (marked by the black box in the left image). The Lys residues are shown as spheres, and the active site amino acids are shown as stick models. Acetylation of Lys 259 (Lys 206 in the crystal structure) and Lys 480 (430 in the crystal structure) could affect protein conformation near the active site.
Our proteomic study using mitochondria from wild-type and mice (Sol et al., 2012; Hebert et al., 2013; Rardin et al., 2013). Complex V activity. Error bars represent SDs. IP, immunoprecipitation; WB, Western blot.

protein acetylation/deacetylation or the effects of sphingolipids depicted in Fig. 7 G. During the course of this study, we identified the Drosophila mitochondrial acetylome and determined potential substrates for dSirt2.

Although sphingolipids have been extensively studied, a connection between enzymes and metabolites of this pathway and protein acetylation/deacetylation or the effects of sphingolipids on NAD+ metabolism and sirtuins are largely unexplored. Our observations in dcker mutants set the stage to further explore the sphingolipid–NAD+-sirtuin axis and delineate links between sphingolipid metabolites and NAD metabolism. Although the reason for depletion of NAD+ is not clear, the increased glycolysis and decreased OXPHOS observed in dcker would accentuate this decrease. NAD+ has been proposed as an attractive target in the management of various pathologies, particularly in the prevention of aging and related disorders, such as diabetes, obesity, and cancer (Yoshino et al., 2011; Houtkooper and Auwerx, 2012). Many sphingolipids, including ceramide, are altered in obesity, diabetes, and aging (Russo et al., 2013). Further studies should help us decipher whether changes in the sphingolipid–NAD+ axis contribute to stress-associated pathologies observed in these conditions.

Recent global proteomic surveys involving mitochondrial acetylation have focused on liver tissue from wild-type and Sirt3–/– mice and embryonic fibroblasts derived from these mice (Sol et al., 2012; Hebert et al., 2013; Rardin et al., 2013). Our proteomic study using mitochondria from wild-type and dsirt2 flies provides the first inventory of acetylated proteins and sites in Drosophila mitochondria. In addition to complementing the mouse studies, the availability of the Drosophila data will enable the use of the Drosophila model for evaluation of several site-specific Lys variants in different proteins. It will facilitate studies of tissue-specific expression of constitutively acetylated or deacetylated mutants, and the phenotypic consequences observed in these studies would lead to an understanding of the role of site-specific modifications in vivo. Enzymes involved in the TCA cycle, OXPHOS, β-oxidation of fatty acids, and branched-chain amino acid catabolism, which are enriched in the mouse acetylome, are also enriched in the Drosophila acetylome. These results indicate a high degree of conservation of mitochondrial acetylation. Analyses of the sirt2 acetylome reveal that many proteins that are hyperacetylated in dsirt2 mutants are also hyperacetylated in liver from Sirt3–/– mice, and some of these candidates have been validated as substrates of SIRT3. These results along with phenotypes, related to mitochondrial dysfunction, observed in the dsirt2 mutants (increased ROS levels, decreased oxygen consumption, decreased ATP level, and increased sensitivity to starvation) strengthen the idea that dSirt2 serves as a functional homologue of mammalian SIRT3. For any organism, tight regulation of ATP synthase activity is crucial to meet physiological energy demands in quickly changing nutritional or environmental conditions. Sirtuins regulate reversible acetylation under stress conditions. It is conceivable that acetylation-mediated regulation of complex V could constitute part of an elaborate control system.

Cancer cells generate a greater proportion of ATP via glycolysis instead of OXPHOS, a phenomenon called the Warburg effect (Warburg, 1956). Recent studies show that SIRT3 dysfunction could be an important factor in this metabolic reprogramming (Kim et al., 2010; Finley et al., 2011a). Thus, alterations in mitochondrial acetylation states could contribute to the preference for aerobic glycolysis observed in cancer. Our results with human breast cancer cell lines show that ATP synthase β is more acetylated in MDA-MB-231 cells (which are less differentiated, strongly invasive, and more glycolytic) compared with that in T47D cells (which are more differentiated, less invasive, and less reliant on aerobic glycolysis). It would be interesting to address whether the acetylation status of ATP synthase β could contribute to the pathological shift in energy generation observed in cancer cells. In conclusion, our study has identified a ceramide–NAD+-sirtuin connection in Drosophila and has shown that deacetylation of the ATP synthase β subunit mediated by Drosophila Sirt2/mammalian SIRT3 adds a new layer of regulation of mitochondrial complex V activity.
Materials and methods

Fly stocks

Drosophila stocks were raised on standard corn meal agar and maintained at 25°C. dckerk is a severe hypomorphic allele generated by P element excision and characterized as previously described (Dasgupta et al., 2009). dslr2, sirt2, sirt4, and sirt7 mutant flies and the actin-GAL4 driver were obtained from Bloomington Stock Center (Indiana University). The upstream activation sequence (UAS)–dSirt2 transgene was a gift from K. Chang (University of Southern California, Los Angeles, CA). The UAS RNAi line for dSirt6 was obtained from the Vienna Drosophila RNAi Center. dckerk/dsrlr2 double mutants were generated by meiotic recombination, loss of ceramide kinase was confirmed by Western blotting, and absence of Sirt2 was confirmed by quantitative PCR. For rescue experiments, dckerk mutants were reconstructed to UAS-dSirt2 transgenic flies, and dsrlr2 mutants were also recombined to UAS-dSirt2 transgenic flies.

Isolation of mitochondria

Approximately 1,000 flies were homogenized in mitochondrial isolation buffer containing 250 mM sucrose, 10 mM Tris, pH 7.4, and 0.15 mM MgCl2 with a pestle and mortar. The homogenate was centrifuged at 16,000 g for 15 min at 4°C and then passed through an acrodisc syringe filter (0.45-μm pore size). The supernatant was centrifuged at 16,000 g for 30 min to obtain a mitochondria-enriched pellet. The supernatant was re-centrifuged at 16,000 g for 15 min, and the pellets were pooled, washed, and resuspended in isolation buffer for activity measurements. Mitochondrial enrichment was assessed by Western blotting the extract with an antibody to porin, a mitochondrial marker.

Assay of complex V (ATPase) activity

The assay relies on linking the ATPase activity to NADH oxidation via the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase and then pyruvate to lactate by lactate dehydrogenase. The reaction buffer consisted of 250 mM sucrose, 50 mM KCl, 2 mM MgCl2, and 20 mM Tris-HCl, pH 7.5. Before the test, 0.25 mM NADH, 1 mM phosphoenol pyruvate, 2.5 U/ml lactate dehydrogenase, and 2 U/ml pyruvate kinase were added to the reaction buffer. The reaction was started by adding 40 μg Drosophila mitochondria, and the change in absorbance was recorded over 3 min at 340 nm. To determine the oligomycin-sensitive activity, the experiment was repeated with 6 μg/ml oligomycin. Complex V activity was calculated by using the extinction coefficient 6.22 mM⁻¹ cm⁻¹.

Metabolic profiling

For measurement of NAD⁺ and related metabolites, dckerk and w¹¹¹B (100 flies each, in triplicate) were collected and frozen. The samples were prepared and analyzed by LC-MS, LC-MS/MS, and gas chromatography–MS platforms by Metabolon.

Feeding experiments

For feeding experiments, 1-d-old w¹¹¹B or dckerk flies were transferred to fly food containing 50 mM nicotinamide or 10 mM NAD⁺. 1,000 flies were used (40 flies per vial) in each feeding experiment. After 24 h, the flies were transferred to vials containing fresh nicotinamide or NAD⁺. The flies were collected after 48 h, and mitochondria were prepared in the presence of nicotinamide or NAD⁺ and assayed for mitochondrial complex V activity.

Mitochondrial oxygen consumption

The rate of oxygen consumption was measured using a Clark-type electrode. Freshly isolated mitochondria (0.5 mg/ml) were incubated in assay medium (120 mM KCl, 5 mM KH₂PO₄, 3 mM Hepes, 1 mM EGTA, 1 mM MgCl₂, and 0.2% bovine serum albumin, pH 7.2) supplemented with a mixture of 20 mM sodium pyruvate and 20 mM proline as a substrate. State 3 rates were measured after the addition of 2 mM ADP.

Mitochondrial ROS production

The rate of mitochondrial H₂O₂ production was assayed fluorometrically by measuring the increase in fluorescence (excitation at 312 nm and emission at 420 nm) as a result of oxidation of hemoglobin acid by H₂O₂ in the presence of HRP. Freshly isolated mitochondria (0.2 mg/ml) were incubated in 2 ml assay medium containing 0.1 mM hemoglobin acid and 6 μM HRP. After a steady signal was obtained, substrate was added: either 5 mM pyruvate + 5 mM proline or 20 mM sodium glycerol 3-phosphate followed by 5 μM retorne.

BN-PAGE

Mitochondria were prepared from flies in the presence of 10 mM nicotinamide and 500 nM trichostatin A and resuspended in buffer containing 20 mM Bis-Tris, pH 7.0, 50 mM NaCl, 2 mM 6-aminohexanoic acid, and 1 mM EDTA. 400 μg mitochondria was solubilized by adding 20% digitonin corresponding to digitonin/protein ratios ranging from 4 to 6 g/g. The samples were incubated for 30 min at 4°C and then centrifuged for 20 min at 16,000 g. The supernatant was separated by BN-PAGE at room temperature after addition of 5 μl of 50% glycerol and 3 μl Coomassie blue G-250 dye from a 5% suspension in 500 mM 6-aminohexanoic acid (Wittig et al., 2006). 4–12% gradient acrylamide gels were used for separation of the digitonin-solubilized respiratory complexes. The cathode buffer was 50 mM tricine, 15 mM Bis-Tris, pH 7.0, and 0.02% Serva blue G-250 (w/v) and the anode buffer was 50 mM Bis-Tris, pH 7.0. The gels were stained with Coomassie brilliant blue R-250 followed by destaining in a solution containing 10% methanol and 8% acetic acid, or in-gel activity assays were performed for mitochondrial protein complexes I–V. In-gel activity staining of OXPHOS complexes was performed as follows: for complex II staining, the gel strip was incubated in 20 ml of 5-MM Tris-HCl, pH 7.4, containing 0.5 M sodium succinate, 21.5 mM phenazine methosulfate, and 20 mg nitrotetrazolium blue. Staining of complex III was achieved by incubating the gel strip in 50 ml complex III assay buffer containing 50 mM potassium phosphate buffer, pH 7.4, and 20 mg DAB. After the color developed (~6 h), the gel was scanned and then put back in the assay buffer, and 50 mg cytochrome c was added to start the complex IV assay and stained for ~1 h. For complex V staining, the gel strip was incubated overnight in a 50ml solution containing 35 mM Tris-HCl, pH 8.0, 270 mM glycine, 14 mM MgSO₄, 8 mM ATP, and 0.3% [wt/vol] Pb(NO₃)₂ with slow agitation. All steps were conducted at room temperature, and the reactions were stopped after the color was developed by fixing the gel for 30 min in a solution containing 50% methanol (vol/vol) and 10% acetic acid (vol/vol).

Sample preparation, MS, and data analysis

Searches were performed against the most recent D. melanogaster database with a mass accuracy of ±50 ppm from a 5% suspension in 500 mM 6-aminohexanoic acid (Wittig et al., 2006). 4–12% gradient acrylamide gels were used for separation of the digitonin-solubilized respiratory complexes. The cathode buffer was 50 mM tricine, 15 mM Bis-Tris, pH 7.0, and 0.02% Serva blue G-250 (w/v) and the anode buffer was 50 mM Bis-Tris, pH 7.0. The gels were stained with Coomassie brilliant blue R-250 followed by destaining in a solution containing 10% methanol and 8% acetic acid, or in-gel activity assays were performed for mitochondrial protein complexes I–V. In-gel activity staining of OXPHOS complexes was performed as follows: for complex II staining, the gel strip was incubated in 20 ml of 5-MM Tris-HCl, pH 7.4, containing 0.5 M sodium succinate, 21.5 mM phenazine methosulfate, and 20 mg nitrotetrazolium blue. Staining of complex III was achieved by incubating the gel strip in 50 ml complex III assay buffer containing 50 mM potassium phosphate buffer, pH 7.4, and 20 mg DAB. After the color developed (~6 h), the gel was scanned and then put back in the assay buffer, and 50 mg cytochrome c was added to start the complex IV assay and stained for ~1 h. For complex V staining, the gel strip was incubated overnight in a 50ml solution containing 35 mM Tris-HCl, pH 8.0, 270 mM glycine, 14 mM MgSO₄, 8 mM ATP, and 0.3% [wt/vol] Pb(NO₄)₂ with slow agitation. All steps were conducted at room temperature, and the reactions were stopped after the color was developed by fixing the gel for 30 min in a solution containing 50% methanol (vol/vol) and 10% acetic acid (vol/vol).

MS parameter settings. The MS run time was 96 min, MS1 scan range was 300.00–1,500.00, and the top 20 MS/MS has a mass accuracy of ±50 ppm. Isolation width was 2.0, normalized collision energy was 35.0, activation Q was 0.250, activation time was 20.0, and lock mass was 371.101237. Charge state rejection parameter was enabled, and a charge state of +1 was rejected. Dynamic exclusion was enabled, the repeat count was 1, repeat duration was 35.0, exclusion list size was 500, and exclusion duration was 40.0. Exclusion mass width was relative to mass, and exclusion mass width was 10 ppm. Informatics. MS/MS spectra were evaluated using SEQUEST 3G and the SORCERER 2 platform obtained from Sage-N Research (v4.0; Lundgren et al., 2009). Searches were performed against the most recent update of the NCBI Drosophila database with a mass accuracy of ±50 ppm for precursor ions and 1 D for product ions. Results were filtered with a mass accuracy of ±5 ppm on precursor ions and the presence of the intended motif.

Bioinformatics

Enriched GO analysis and pathway analysis were performed using the ChIPseekAnno package from Bioconductor (Zhu et al., 2010). GO terms and pathways were annotated with at least five genes in the genome, and Benjamini and Hochberg-adjusted P < 0.01 was considered significantly enriched (Benjamini and Hochberg, 1995). Amino acid sequences were obtained using the biomart package obtained from Bioconductor (Durinck, et al., 2005).
et al., 2005). Consensus amino acid patterns surrounding acetyl-Lys sites (±6 amino acids) were identified (P < 0.05) and visualized using iceLogo with nonacetylated lysines of all acetylated mitochondria proteins as the background model (Colaet, et al., 2009).

Cell culture and transfection experiments
Transfection was performed using the nucleofection device (Amago Nucleofector, Lonza) and reagents according to the manufacturer’s standard protocol. In brief, HEK293T cells were cultured in DMEM (10% FBS + 1% penicillin-streptomycin) 3 d before the experiment. 5 x 10^5 cells were used for each nucleofection. The cell pellet was resuspended in 100 µl nucleofection solution and then added to the total plasmid DNA (3 µg). The cell DNA mixture in a 1-cm cuvette is nucleoporated according to a predefined program (A-023). After electroporation, cells were incubated in media with 5 mM nicotinamide and 500 nM trichostatin A unless otherwise mentioned. Cells are harvested after 24 h for immunoprecipitation. DDK-tagged (similar to FLAG tag) ATP synthase β (RC20163B) and DDK-tagged human SIRT3 (RC200190), SIRT4 (RC212226), SIRT5 (RC200189), and SIRT1 (RC218134) plasmids were obtained from OriGene. In deacetylation experiments involving SIRT3 overexpression, DDK-tagged human SIRT3 was cotransfected with DDK-tagged ATP synthase β, and cells were incubated in media without nicotinamide and trichostatin A. For siRNA experiments, cells were transfected with each siRNA (1 µM) or the scrambled version, and cells were harvested after 72 h. The Trizol siRNAs used to reduce SIRT3 (SR308253), SIRT4 (SR308254), SIRT5 (SR308253), and SIRT1 (SR308256), and the scrambled siRNAs were obtained from OriGene. The siRNA sequences used to reduce endogenous ATP synthase β were 5′-CUCCAUUAUAGGCGGCAUU-3′ and 5′-AAUCACAAAUGUCGCCAATA-3′ (Thermo Fisher Scientific).

Immunoprecipitation and immunoblotting
After transfection, cells were lysed in radioimmunoprecipitation assay buffer with protease inhibitor cocktail (Roche). DDK-tagged proteins were immunoprecipitated using a DDK antibody (mouse), 4C5, coupled to protein G–agarose beads (OriGene). The immunoprecipitate was washed in radioimmunoprecipitation assay buffer and dissolved in SDS sample buffer. For immunoprecipitation of endogenous ATP synthase β, either HEK293T or human breast cancer cells were lysed in NP1 buffer (PBS with 0.5% Nonidet P-40) and protease inhibitor cocktail. The extract is incubated for 8–10 h at 4°C with an antibody to ATP synthase β (MitoSciences) or IgG (mock) followed by addition of immobilized protein G (Thermo Fisher Scientific) and incubated further for 12 h at 4°C. The beads were centrifuged at 5,000 rpm for 5 min and washed three times in NP1 buffer. The beads were then incubated with 2x SDS sample buffer without β-mercaptoethanol for 10 min at room temperature. The beads were centrifuged, and the supernatant was separated by SDS-PAGE after addition of β-mercaptoethanol. Blocking mouse anti-DDK was used at 1:2,000, mouse anti-ATP synthase β was used at 1:4,000 (MitoSciences), and rabbit anti–human SIRT3 antibody (Cell Signaling Technology) was used at 1:1,000. HRP-conjugated rabbit or mouse secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) were used at 1:5,000 dilution. For Western blot analysis, the rabbit anti-acetyl-Lys antibody (Cell Signaling Technology) was used at 1:500, and the HRP-conjugated rabbit secondary antibody was used at 1:3,000 dilution. The blotting was performed following a published protocol (Guan et al., 2010).

Plate assay for measuring complex V activity
An siRNA-resistant ATP synthase β was synthesized by making the following changes to 5′-TTGTAAATAAAGITGCGA-3′ (corresponding to amino acid sequence LINWNA) and 5′-AAGCTCTTCCTGGCAAGG-3′ (corresponding to amino acid sequence SALG). Either the nondegradable wild-type construct or each of the nondegradable site-specific lys substitutions was transfected along with the siRNAs. Cells were harvested after 75 h, and mitochondrial-enriched fractions were prepared. The two-step complex V assay was performed using the ATP synthase-specific activity microplate assay kit according to the manufacturer’s instructions (MS543; MitoSciences). For this assay, the FO1-ATPase holoenzyme is immunocaptured within the wells of a 96-well microplate that is coated with an antibody that recognizes all subunits of the complex. The enzymatic hydrolysis of ATP to ADP is coupled to the oxidation of NADH to NAD+, which results in a decrease in absorbance at 340 nm. Subsequently, in the same wells, the quantity of ATP synthase β is determined by adding an ATP synthase β antibody conjugated with alkaline phosphatase. An increase in absorbance at 405 nm is measured, and this is proportional to the amount of ATP synthase β captured in the wells. The ratio of activity to quantity represents the relative specific activity of ATP synthase β. The mitochondrial extract was solubilized with digitonin, and 40–60 µg was used per well. The plate was read using a microplate reader (Infinite M200 Pro; Tecan). Specific activity was taken as the ratio of complex V activity to quantity of ATP synthase β in each well.

Structural observations of ATP synthase β
The structure of the F1–stator complex was generated with PyMOL (Delano Scientific LLC) using the bovine F1–stator complex structure.

Preparation of soluble and nuclear extracts
Soluble extracts were prepared from w1118 and dcker1 flies by washing them with buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 2 mM mercaptoethanol, 50 mM KCl, 10 mM nicotinamide, and 500 nM trichostatin A) followed by homogenization in the same buffer. The homogenate was clarified by a 15-min centrifugation at 12,000 g, and then, the supernatant was centrifuged at 150,000 g for 1 h at 4°C (Malcovati et al., 1973). For preparation of nuclear extracts, flies are grounded in 10 mM Tris-Cl buffer, pH 8.0, containing 300 mM sucrose, 2 mM magnesium acetate, 3 mM CoCl2, 0.1% Triton X-100, 0.5 mM DTT, 10 mM nicotinamide, and 500 nM trichostatin A. The homogenate is filtered through two sheets of 100-µm nylon mesh to remove large debris. Frillates are transferred to a Teflon glass homogenizer and stroked 40 times on ice. Homogenates are filtered through two sheets of 35-µm nylon mesh twice and then mixed with 10 mM Tris-Cl buffer, pH 8.0, containing 1.75 M sucrose, 5 mM magnesium acetate, 0.5 mM DTT, 10 mM nicotinamide, and 500 nM trichostatin A. The mixture is then divided into two equal portions and is layered over a sucrose gradient consisting of 8 ml of 10 mM Tris-Cl buffer, pH 8.0, containing 1.9 M sucrose, 5 mM magnesium acetate, and 0.5 mM DTT over 4 ml of 10 mM Tris-Cl buffer, pH 8.0, containing 25% glycerol, 5 mM magnesium acetate, 5 mM DTT, 0.1 mM EDTA, 10 mM nicotinamide, and 500 nM trichostatin A in tubes of a rotor (SW 28; Beckman Coulter). The sample is then spun at 14,000 rpm for 15 min at 4°C. The supernatant is discarded, and the pellet is removed with a Kimwipe. Each pellet is resuspended in 500 µl of 10 mM Tris-Cl buffer, pH 8.0, containing 25% glycerol, 5 mM magnesium acetate, 5 mM DTT, 0.1 mM EDTA, 10 mM nicotinamide, and 500 nM trichostatin A, and the suspension is spun for 1 min at maximum speed. Nuclei are recovered as a pellet (Hirayoshi and Lis, 1999).

Ceramide estimation
Sphingolipid-enriched fractions were prepared from mitochondria isolated from w1118 or dcker1 flies. Mitochondria were homogenized in 2.0 ml methanol/chloroform [2:1] using a Teflon homogenizer in a glass tube followed by 500 µl of water and vortexed. The homogenate was sonicated in a water bath–type sonicator for 20 min and incubated for 2 h at 37°C. To the extract, 1 ml of water and 500 µl chloroform were added, vortexed, and centrifuged at 1,000 rpm for 10 min at room temperature. The organic phase was collected and dried under nitrogen. Extracts were redissolved in 2 ml of synthetic upper (methanol/water/chloroform of 94:9:6:6) and applied to a pretreated column for solid-phase extraction (Sep-Pak C18, Waters Corporation). The column was washed with 4 ml of water, and lipids were extracted in 4 ml methanol followed by 4 ml methanol/ chloroform. The samples were dried under nitrogen and redissolved in the requisite amount of chloroform/methanol [1:1]. The d14 sphingoid base containing cerebrosides was estimated by ultra-HPLC/MS (Dasgupta et al., 2009, Yanamine et al., 2011).

Measurement of citrate synthase activity
Citrate synthase activity was measured by following the decrease in absorbance at 412 nm because of the reduction of DTNB [5, 5′-dithiobis[2-nitrobenzoic acid]]. The reaction mixture containing 0.1 M Tris-HCl, pH 8.0, 0.3 mM acetyl-CoA, 0.1 mM DTNB, and 10 µg mitochondrial protein was incubated for 10 min. The reaction was initiated by adding 0.5 mM oxaloacetate, and the change in absorbance was monitored for 3 min. Citrate synthase activity was calculated by using an extinction coefficient of 13.6 mM cm^-1.

Online supplemental material
Fig. S1 shows that the NAD+ level is decreased in the dcerk1 mutant. Fig. S2 shows separation of OXPHOS complexes by BN-PAGE. Fig. S3 depicts that dsirt2 and dcker1 mutants show increased ROS levels. Fig. S4 shows a strategy for identification of Drosophila mitochondrial acetyltransferase and dsirt2-regulated acetylome. Table S1 shows details of acetyl-Lys peptides in the mitochondrial acetylome identified by MS. Table S2 shows...
details of acetyl-lys peptides that increase in dSir2 mutant mitochondrial acetyome identified by MS. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201404118/DC1.

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Supplemental material

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Figure S1. **MS measurements of NAD$^+$ and metabolites involved in the salvage pathway and de novo synthesis of NAD$^+$ from tryptophan in cdase$^{	ext{1}}$ mutants**. NAD$^+$ level is decreased in cdase$^{	ext{1}}$ mutants. Extracts were prepared from 100 flies, and three replicates were performed. Error bars represent SDs, and statistical significance was determined using Student's $t$-test; *, $P \leq 0.05$–0.01; **, $P \leq 0.01$–0.001; ***, $P \leq 0.001$–0.0001.

Figure S2. **Separation of OXPHOS complexes by BN-PAGE**. (A) Separation of mitochondrial OXPHOS complexes from $w^{1118}$ and dcerk$^{	ext{1}}$ by BN-PAGE and their activity staining. (B) Mass spectrometric measurements of NAD$^+$ and nicotinamide in $w^{1118}$ flies fed nicotinamide. Error bars represent SDs, and statistical significance was determined using Student's $t$-test; **, $P \leq 0.01$–0.001.
Table S1 shows details of acetyl-Lys peptides in the mitochondrial acetylome identified by MS and is provided in an Excel file.

Table S2 shows details of acetyl-Lys peptides that increase in dsirt2 mutant mitochondrial acetylome identified by MS and is provided in an Excel file.

Figure S3. **dsirt2 mutant flies show increased ROS levels.** Mitochondria were prepared from \( w^{1118} \), dcerk\(^1\), and dsirt2 mutants, and ROS production was measured using pyruvate + proline (left) or sn-glycerol 3-phosphate (right) as a substrate. ROS levels are increased in dcerk\(^1\) and dsirt2 compared with \( w^{1118} \) mitochondria. Mitochondria were prepared from \( \sim 1,000 \) flies for each batch. \( n = 3 \); error bars represent SDs. Statistical significance was determined using Student’s \( t \) test: **, \( P \leq 0.01–0.001 \); ***, \( P \leq 0.001–0.0001 \).

Figure S4. **Strategy for identification of Drosophila mitochondrial acetylome and dSirt2-regulated acetylome.** Mitochondria were isolated from 3,000 flies (\( w^{1118} \) or dsirt2) per batch. The mitochondrial extracts were digested with trypsin and desalted. Acetyl-Lys peptides were immunoprecipitated using an acetyl-Lys antibody. The enriched peptides were separated by LC-MS/MS. The MS/MS spectra were evaluated using SEQUEST 3G and SORCERER 2 platform, and label-free quantitation was performed from the acetyl-Lys peptide intensities. The experiment was performed in duplicate.