Obesity and aging diminish sirtuin 1 (SIRT1)-mediated deacetylation of SIRT3, leading to hyperacetylation and decreased activity and stability of SIRT3

Received for publication, January 27, 2017, and in revised form, August 9, 2017 Published, Papers in Press, August 14, 2017, DOI 10.1074/jbc.M117.778720

Sanghoon Kwon‡1, Sunmi Seok‡1, Peter Yau§, Xiaoling Li§, Byron Kemper¶, and Jongsook Kim Kemper‡2

From the ‡Department of Molecular and Integrative Physiology and the §Proteomics Center, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 and ¶Laboratory of Signal Transduction, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Edited by John M. Denu

Sirtuin 3 (SIRT3) deacetylates and regulates many mitochondrial proteins to maintain health, but its functions are depressed in aging and obesity. The best-studied sirtuin, SIRT1, counteracts aging- and obesity-related diseases by deacetylating many proteins, but whether SIRT1 has a role in deacetylating and altering the function of SIRT3 is unknown. Here we show that SIRT3 is reversibly acetylated in the mitochondria and unexpectedly is a target of SIRT1 deacetylation. SIRT3 is hyperacetylated in aged and obese mice, in which SIRT1 activity is low, and SIRT3 acetylation at Lys57 inhibits its deacetylase activity and promotes protein degradation. Adenovirus-mediated expression of SIRT3 or an acetylation-defective SIRT3-K57R mutant in diet-induced obese mice decreased acetylation of mitochondrial long-chain acyl-CoA dehydrogenase, a known SIRT3 deacetylation target; improved fatty acid β-oxidation; and ameliorated liver steatosis and glucose intolerance. These SIRT3-mediated beneficial effects were not observed with an acetylation-mimic SIRT3-K57Q mutant. Our findings reveal an unexpected mechanism for SIRT3 regulation via SIRT1-mediated deacetylation. Improving mitochondrial SIRT3 functions by inhibiting SIRT3 acetylation may offer a new therapeutic approach for obesity- and aging-related diseases associated with mitochondrial dysfunction.

The sirtuins are NAD+-dependent protein deacetylases that play critical roles in metabolism, stress responses, and aging processes (1–5). Among the seven mammalian sirtuins, SIRT1 is best-studied and mediates transcriptional responses to fasting, exercise, or caloric restriction by deacetylating histones and non-histone gene regulatory proteins, including the key metabolic regulators, PGC-1α and SREBP-1 (6–8). Expression and enzymatic activity of SIRT1 are aberrantly low in both aging and obesity, and activation of SIRT1 delays and improves many diseases related with these conditions (1–5, 8–11). SIRT1 has received great attention as a drug target because the activity of SIRT1 can be enhanced by natural or synthetic SIRT1 activators (12) or dietary supplements that increase cellular levels of a key enzymatic co-factor, NAD+ (13, 14). Although SIRT1 deacetylates many cellular proteins, whether SIRT1 can deacetylate and alter the function of other sirtuin members has not been described.

Mitochondrial SIRT3 deacetylates and regulates the activity of proteins involved in mitochondrial functions, including intermediary metabolism, fatty acid oxidation, the urea cycle, and the oxidative stress response (15–19). Mice lacking SIRT3, but not the other mitochondrial sirtuins, SIRT4 or SIRT5, exhibited global mitochondrial protein hyperacetylation (20), indicating that SIRT3 is the major mitochondrial deacetylase. Indeed, the global mitochondrial acetyl-proteome was markedly reprogrammed in SIRT3-KO mice (21). Although SIRT3-KO mice fed normal chow did not show a metabolically remarkable phenotype (20), development of metabolic syndromes, such as adiposity, insulin resistance, and liver steatosis, were accelerated by chronically feeding these mice a high-fat diet (HFD) (22). Intriguingly, a genetic polymorphism that increases SIRT3 function has been associated with extreme longevity in humans (23), and conversely, a mutation of SIRT3 that reduces its activity is associated with increased risk for metabolic syndrome (22). The expression and enzymatic activity of SIRT3 are reduced in obesity and aging (15, 22, 24), but the underlying mechanisms are not clearly understood.

In this study, we show that the acetylation state of SIRT3 is regulated post-translationally by SIRT1 deacetylation. SIRT3 is acetylated at Lys57 and is hyperacetylated in both aged and obese mice, conditions in which SIRT1 deacetylation function is low. Utilizing acetylation-mimic and -defective Lys57-SIRT3 mutants, we provide evidence that acetylation of SIRT3 leads to decreased SIRT3 protein stability and deacetylation activity and increased levels of acetylated mitochondrial long-chain Acyl-CoA dehydrogenase (LCAD), a known target of SIRT3 deacetylation (16), which contributes to impaired fatty acid oxidation,
liver steatosis, and glucose intolerance in HFD-induced obese mice.

Results

**SIRT3 interacts with SIRT1 in the liver mitochondrial fraction**

Analysis of proteins that co-immunoprecipitated with mouse SIRT3-FLAG in the mitochondrial fraction of mouse liver extracts by LC-MS identified a SIRT1 peptide (not shown). Although identification of SIRT1 by this method did not quite reach statistical significance, we examined this possible interaction further because SIRT1 had not been reported as a SIRT3-interacting protein, and it was unexpected because SIRT1 is predominantly a nuclear protein.

To examine whether a minor fraction of SIRT1 is present in the mitochondria, endogenous SIRT1 and SIRT3 protein levels in subcellular fractions of mouse liver extracts were determined. SIRT3 was predominantly mitochondrial, whereas SIRT1 was mainly nuclear, but a small fraction of SIRT1 was detected in the mitochondrial fraction (Fig. 1A). The interaction between SIRT3 and SIRT1 in the mitochondrial fraction was confirmed by CoIP (Fig. 1B and supplemental Fig. S1). Remarkably, their interaction was substantially increased in fasted mice (Fig. 1B). In immunofluorescence studies in Hepa1c1c7 cells, whereas endogenous SIRT3 is localized in the mitochondria as expected, endogenous SIRT1 is broadly distributed in the cell, and co-localization of SIRT1 with SIRT3 and of SIRT1 with the mitochondrial marker was detected (Fig. 1C). In GST-pulldown experiments, SIRT3 interacted with SIRT1 through its N-terminal region (Fig. 1D), and both the catalytic and C-terminal domains of SIRT1 interacted with SIRT3 (Fig. 1E). These results provide evidence that a small fraction of SIRT1 interacts with SIRT3 in the liver mitochondria.

SIRT3 is hyperacetylated in SIRT1-LKO mice and in aged or obese mice

To determine the functional significance of the interaction of SIRT3 with SIRT1, we first tested whether the acetylation status of SIRT3 is regulated by SIRT1. Ectopic expression of SIRT1 in Cos-1 cells dramatically decreased the level of acetylated SIRT3-FLAG, and conversely, down-regulation of SIRT1 increased the acetylated SIRT3 level by more than 2-fold (Fig. 2A). These results suggest that SIRT3 is reversibly acetylated, and the SIRT3 acetylation level is regulated by SIRT1.

To test directly whether SIRT3 is a substrate for SIRT1 deacetylation, in vitro assays were performed using SIRT1-WT and two SIRT1 mutants having decreased activity, H363Y (25) and S164D (10) that were expressed in and immunoprecipitated from Cos-1 cells. The addition of SIRT1-WT, together with NAD⁺, led to a substantial decrease in acetylated SIRT3 levels (Fig. 2B, lanes 3 and 4), but these SIRT1-mediated effects were not observed with either the H363Y or S164D SIRT1 mutant (Fig. 2B, lanes 5 and 6). Similar results for SIRT1-WT were observed using commercially available purified SIRT1 (supplemental Fig. S2). Acetylation levels of SIRT3-WT or the catalytically inactive H248Y-SIRT3 mutant (16, 20) were not changed by incubation of these proteins with NAD⁺ (Fig. 2B, lanes 1, 2, 7, and 8), indicating that SIRT3 does not undergo autodeacetylation. These results suggest that SIRT3 is a direct target of SIRT1 deacetylation in vitro.

We next determined whether the acetylation level of endogenous SIRT3 in mouse liver is regulated by SIRT1 in vivo utilizing SIRT1-LKO mice. Subcellular fractions of livers from control littermates or SIRT1-LKO mice were prepared (Fig. 2C, left panel), and acetylation of SIRT3 in the mitochondrial fraction was determined by IP/IB. Levels of acetylated endogenous
mitochondrial SIRT3 were higher in the SIRT1-LKO mice than in the control mice, even though SIRT3 protein levels were markedly decreased in the SIRT1-LKO mice (Fig. 2C, center and right panels). These results, together with the cell and in vitro studies above, provide evidence that the SIRT3 acetylation status is regulated by SIRT1.

Because SIRT1 function is depressed in both obesity and aging and levels of its enzymatic co-factor, NAD$^+$, are decreased in these conditions (26, 27), we examined acetylated SIRT3 levels in aged mice and in HFD-induced obese mice.

Intriguingly, in liver mitochondrial fractions, acetylation levels of endogenous SIRT3 are elevated in both aged and obese mice compared with young lean mice (Fig. 2D, left panel), even though SIRT3 protein levels were markedly decreased (Fig. 2D, center panel; quantitation in right panel). These results show that SIRT3 acetylation levels in the liver are increased in SIRT1-LKO mice and in aged or obese mice. Because SIRT1 function is aberrantly low in aged or obese animals, these results are consistent with the conclusions that SIRT1 regulates the acetylation level of SIRT3 and that
the low SIRT1 function contributes to hyperacetylation of SIRT3 in aged and obese mice.

**Lys**57 **is the acetylation site in SIRT3**

The site of acetylation in SIRT3 was identified by tandem mass spectrometry analysis. Mouse SIRT3-FLAG was adenovirally expressed in livers of mice chronically fed a HFD, and SIRT3-FLAG in the mitochondrial fraction was purified under stringent conditions with SDS in the buffers (Fig. 3A). Lys57 was the only acetylated site identified in SIRT3 in HFD obese mice by this analysis (Fig. 3B). To confirm Lys57 as the acetylation site in SIRT3, Lys57 was mutated to Arg, and acetylation of SIRT3-FLAG was assessed by an in-cell acetylation assay. Acetylation of exogenously expressed WT SIRT3-FLAG was detected in Cos-1 cells, and mutation of Lys57 to Arg as a negative control did not (Fig. 3C). We also tested whether SIRT3 can deacetylate itself utilizing a catalytically inactive SIRT3 mutant, H248Y-SIRT3, or K223R-SIRT3 as a negative control, and Ac-SIRT3-FLAG levels were determined by IP/IB (left panel). Input SIRT3-FLAG protein levels are shown (right panel). In C, the SIRT3 band intensities were quantified, Ac-SIRT3 levels relative to total SIRT3 levels were calculated, and mean values ± S.E. are presented (n = 3 independent assays) (right panel). In D, consistent results were observed in two independent assays. E, amino acid sequences adjacent to Lys57 in SIRT3 in the indicated species.

These results indicate that SIRT3 is a target of post-translational acetylation at Lys57 and are consistent with the direct interaction of SIRT3 and SIRT1 through the N-terminal domain of SIRT3 and catalytic and C-terminal domain of SIRT1 (Fig. 1, G and H). Remarkably, Lys57 in mouse SIRT3 (Lys122 in humans) is highly conserved among species from human to yeast (Fig. 3E), suggesting the functional importance of this residue.

Expression of SIRT1 or acetylation-defective K57R-SIRT3 increases the stability of SIRT3

Reversible protein acetylation profoundly modulates multiple protein functions, including subcellular localization, protein-protein and protein-DNA interaction, enzymatic activity, and protein stability (16, 28–30). Protein levels of endogenous SIRT3 in mitochondrial fractions were decreased, and conversely, its acetylation levels were increased in aged, HFD obese, and SIRT1-LKO mice (Fig. 2, C and D), which suggests that acetylation of SIRT3 may influence protein stability. To test this idea, SIRT3-FLAG was expressed in Cos-1 cells, and the levels of acetylated H248Y-SIRT3 and SIRT3-WT were similar (Fig. 3D), suggesting that SIRT3 does not autodeacetylate.
Surprisingly, SIRT3-FLAG was rapidly degraded after CHX treatment with a half-life $\approx 45$ min (Fig. 4, A–D). Exogenous expression of SIRT1 reduced the degradation rate of SIRT3, whereas down-regulation of SIRT1 increased it (Fig. 4, A and B).

We further determined the effects of mutation of Lys$^{57}$ on the stability of SIRT3. The degradation rate of the acetylation-defective K57R-SIRT3 mutant was markedly reduced compared with SIRT3-WT, whereas that of the acetylation-mimic K57Q-SIRT3 mutant was modestly, although significantly, increased (Fig. 4, C and D). The reason for the modest effect of the K57Q mutation, compared with the K57R mutation, on protein degradation is not clear but may be due to substantial basal levels of acetylated WT-SIRT3 in these cells, as detected by pan-acetyl-lysine antibody (Fig. 2A). These results indicate that acetylation of SIRT3 at Lys$^{57}$ promotes protein degradation.

To further test whether SIRT1-mediated deacetylation of SIRT3 at Lys$^{57}$ affects SIRT3 stability, we examined the effect of co-expression of SIRT1 or a catalytically inactive H363Y-SIRT1 mutant (25) with SIRT3-WT or K57R-SIRT3 on the stability of SIRT3. Because hepatic factors may affect the degradation rate of SIRT3, these experiments were done in primary mouse hepatocytes. In hepatocytes, the degradation rate of SIRT3-FLAG was very rapid, with a half-life shorter than 30 min (Fig. 4E). The degradation rate of SIRT3-WT was decreased by addition of SIRT1-WT but not by addition of catalytically inactive SIRT1 mutants as expected (Fig. 4E). Compared with SIRT3-WT, the degradation rate of the K57R-SIRT3 mutant was relatively insensitive to the addition of SIRT1-WT or H363Y mutant. These results indicate that acetylation of SIRT3 at Lys$^{57}$, regulated by SIRT1, promotes SIRT3 protein degradation.
Acetylation-mimic mutation of SIRT3 at Lys$^{57}$ results in decreased SIRT3 activity and increased acetylation of LCAD, which is associated with increased long-chain acylcarnitine levels.

**Acetylation-mimic mutation of SIRT3 at Lys$^{57}$ results in decreased SIRT3 activity**

In addition to effects on protein stability, acetylation of SIRT3 may also affect its activity. We thus determined the deacetylase activities of acetylation-defective K57R-SIRT3 and acetylation-mimic K57Q-SIRT3. In assays utilizing SIRT3 substrate peptides with a C-terminally attached fluorophore (31), deacetylase activity of the acetylation-mimic K57Q mutant was substantially decreased compared with SIRT3-WT or the K57R mutant (Fig. 5A), consistent with previous results (22). In vitro deacetylase assays of FLAG-LCAD proteins bound to M2 agarose were incubated with SIRT3-WT or the K57Q mutant in the presence of NAD$^+$ for 1 h, and Ac-LCAD levels in the input samples were detected by IB (Fig. 5B). Consistent results were observed from two independent assays. Effects of SIRT3 Lys$^{57}$ mutations on LCAD acetylation in liver extracts in vivo were observed. C–G, effects of SIRT3 Lys$^{57}$ mutations on LCAD acetylation levels and liver acylcarnitine levels in vivo. C, experimental outline (five mice/group). D, SIRT3 and control GFP protein levels detected by IB in liver extracts from mice fed a ND or HFD and infected with control Ad-GFP or Ad-GFP-SIRT3-WT, Ad-GFP-K57R-SIRT3, or Ad-GFP-K57Q-SIRT3. E, acetylated LCAD levels were determined by IP/IB and input protein levels were detected by IB. In D and E, liver samples from three mice from the total of five mice in each group were randomly selected and used for IB. F and G, liver acylcarnitine levels (F) and serum β-hydroxybutyrate levels (G) in HFD mice were determined by metabolomic analysis. Statistical significance between the K57R and K57Q groups was determined by the Student’s t test (S.E., n = 5). *, p < 0.05; **, p < 0.01.

**Acetylation-mimic K57Q mutation of SIRT3 at Lys$^{57}$ results in increased acetylation of LCAD in vivo and impaired β-oxidation**

To further determine whether acetylation of SIRT3 at Lys$^{57}$ influences its activity on LCAD deacetylation in mice in vivo, SIRT3-WT, an acetylation-defective K57R mutant or an acetylation-mimic K57Q mutant were adenovirally expressed in liver extracts.
ers of dietary obese mice for 3 weeks (Fig. 5, C and D), and levels of acetylated LCAD were determined. Similar levels of SIRT3-WT and the mutants were detected in liver extracts, even though the stability is affected by the mutants in cells (Fig. 4). This may be due to overexpression of the proteins in vivo, which diminishes the differences in stability. Protein levels of endogenous SIRT3 in the liver extracts were markedly decreased in HFD mice compared with lean ND mice injected with control Ad-GFP (Fig. 5D) as shown in previous studies (22).

Acetylated levels of LCAD were readily detected in HFD obese mice as previously reported (22) but were substantially reduced below detection levels by exogenous expression of SIRT3-WT or the acetylation-defective K57R mutant (Fig. 5E). In contrast, levels of acetylated LCAD were substantially higher for the acetylation-mimic K57Q-SIRT3 mutant compared with SIRT3-WT or K57R-SIRT3 (Fig. 5E). These results, together with in vitro deacetylation studies above (Fig. 5A and B), indicate that acetylation of SIRT3 inhibits its deacetylase activity.

Because increased acetylation of LCAD in HFD mice is associated with its decreased enzymatic activity in the fatty acid oxidation pathway (16, 22), we further tested whether SIRT3 acetylation affects liver acylcarnitine levels. Impaired β-oxidation results in accumulation of acylcarnitine, particularly long-chain species (34). Long-chain acylcarnitine levels in liver homogenates were increased by expression of K57Q-SIRT3 compared with expression of SIRT3-WT or K57R-SIRT3 (Fig. 5F). Consistent with these results, the serum level of a ketone body, β-hydroxybutyrate, was decreased in mice expressing K57Q-SIRT3, compared with mice expressing SIRT3-WT or K57R-SIRT3 (Fig. 5G). These results, together with in vitro studies above (Fig. 5B), suggest that acetylation of SIRT3 at Lys57 is associated with increased LCAD acetylation and increased long-chain acylcarnitine levels in HFD obese mice, indicative of impaired fatty acid oxidation.

Expression of K57R-SIRT3 reverses fatty liver symptoms in HFD obese mice

SIRT3-KO mice exhibit accelerated metabolic symptoms in response to chronic HFD feeding, which include adiposity, hyperlipidemia, insulin resistance, and liver steatosis (22). Impaired fatty acid oxidation results in abnormal accumulation of triglyceride (TG) and pathological symptoms of fatty liver (35). We therefore further investigated the effects of adenovirus-mediated exogenous expression of SIRT3-WT and its Lys57 acetylation mutants on fatty liver symptoms in HFD obese mice.

In mice fed a HFD, body weight (supplemental Fig. S3A) and liver size (Fig. 6A), liver and adipose weights (supplemental Fig. S3, B and C), liver weight/body weight (Fig. 6B), and neutral lipid content (Fig. 6C) and triglyceride levels in the liver (Fig.
6D) all increased as expected compared with lean mice fed a normal diet. Strikingly, expression of SIRT3-WT or the acetylation-defective K57R-SIRT3 largely reversed all the increases in HFD mice, whereas expression of the acetylation-mimic K57Q-SIRT3 mutant had little effect (Fig. 6, A–D). Food intake was not markedly changed among groups (supplemental Fig. S3D). Impaired glucose regulation frequently occurs in liver steatosis (36). Expression of SIRT3-WT or K57R-SIRT3 in HFD mice led to decreased fasting blood glucose levels (Fig. 6E) and improved glucose tolerance (Fig. 6F), but expression of K57Q-SIRT3 had little effect. These results, taken together, suggest that hyperacetylation of SIRT3 in mice chronically fed a HFD contributes to symptoms of fatty liver.

Changes in mitochondrial function can cause altered gene activity in the nucleus (37) so that hepatic gene expression associated with fatty liver might be affected by acetylation of mitochondrial SIRT3. Indeed, expression of SIRT3-WT or K57R-SIRT3 in HFD mice significantly increased mRNA levels of fatty acid oxidation enzymes Cpt1 and Mccd; key gene activators of mitochondrial fatty acid oxidation, Pgc-1α and Ppara, a key hormone promoting fatty acid oxidation, Fgf21; and ketogenic genes, including the rate-limiting Hmgcs1/2, whereas mRNA levels of lipogenic and pro-inflammatory genes were decreased (Fig. 6G). In contrast, expression of the acetylation-mimic K57Q-SIRT3 had little effect on gene expression (Fig. 6G). These effects on gene expression are consistent with the effects on fatty liver symptoms of the LysS7 mutants of SIRT3.

Overall, adenovirus-mediated expression of SIRT3-WT or the acetylation-defective K57R mutant in livers of HFD obese mice led to remarkable metabolic effects with decreased adiposity and liver TG levels and improved glucose tolerance. Conversely, these SIRT3-mediated beneficial effects were not detected with the acetylation-mimic K57Q-SIRT3 mutant, leading to little improvement of the impaired fatty acid oxidation and fatty liver symptoms in HFD mice.

Discussion

In this study, we present evidence that SIRT3 is reversibly acetylated in the mitochondria and is a target of SIRT1 deacetylation. The acetylation level of SIRT3 is highly elevated in obesity and aging, conditions where SIRT1 function is low. SIRT3 is acetylated at LysS7, which leads to decreased SIRT3 deacetylation activity and protein stability. From metabolic studies utilizing adenovirus-mediated expression of SIRT3-WT or acetylation-defective or -mimic SIRT3-LysS7 mutants in HFD obese mice, we further show that hyperacetylation of SIRT3 contributes to impaired fatty acid β-oxidation, liver steatosis, and glucose intolerance.

An unexpected finding of this study was that levels of mitochondrial acetylated SIRT3 are regulated by SIRT1, because SIRT1 is mostly a nuclear-cytoplasmic shuttling protein (38) that mediates fasting transcriptional responses by deacetylating histones and gene regulatory proteins in the nucleus (2, 3) and is not considered to be a mitochondrial protein. Detection of SIRT1 in mitochondria, however, has been previously reported (39, 40). Consistent with these earlier studies, the present study provides proteomic, imaging, and biochemical evidence that that a minor fraction of SIRT1 is present in liver mitochondria and further directly interacts with SIRT3 under fasting conditions. SIRT3 acetylation levels were regulated by SIRT1 in vitro and in cells and increased in SIRT1-LKO mice in vivo. Because SIRT3 is a deacetylase, it is possible that the SIRT1 affects acetylation of SIRT3 indirectly by affecting SIRT3 levels or activity. However, results from in vitro and in-cell deacetylation studies indicate that this is not the case. Our experimental data support the conclusion that SIRT3 acetylation status is regulated by SIRT1 and is the first example of a sirtuin being a target of another sirtuin.

A significant functional consequence of acetylation of SIRT3 is decreased deacetylase activity. A single amino acid residue, LysS7, in the N-terminal domain of SIRT3, was identified as the acetylation site in dietary obese mice, and mutation of acetylation-mimic K57Q led to decreased SIRT3 activity. The catalytic domain of SIRT3 is located in the central and C-terminal regions, so acetylation at LysS7 may alter the conformation of SIRT3 and inhibit the deacetylase activity. Allosteric modulation of SIRT1 activity has been reported, which is mediated through its N-terminal domain that binds STACs and SIRT1-interacting proteins (12, 41). Further, it was shown that obesity-linked phosphorylation of SIRT1 by CK2 at Ser164 in the N-terminal domain inhibits its activity (10). In the present study, expression of the acetylation-mimic K57Q-SIRT3 is associated with increased LCAD acetylation and long-chain acylcarnitine levels, indicative of impaired β-oxidation, which contributes to liver steatosis and glucose intolerance. A single-nucleotide polymorphism in SIRT3, V208I, in fatty liver disease patients, reduces SIRT3 activity, thereby playing a pathological role in liver steatosis and insulin resistance (22). The basis for the decrease in activity of the Val208 mutant is not known, but it could affect the conformation of SIRT3 as we suggest for acetylation at LysS7.

SIRT3 acetylation also has significant effects on protein stability. Overexpression of SIRT1 or the acetylation-defective K57R-SIRT3 mutant increased stability, whereas down-regulation of SIRT1 or overexpression of the acetylation-mimic K57Q mutant had the opposite effects. In biochemical studies, we observed that inhibition of proteasomal degradation with MG132 substantially increased SIRT3 levels (supplemental Fig. S4). Further, expression of SIRT1 decreased, whereas down-regulation of SIRT1 increased, ubiquitinated SIRT3 levels (supplemental Fig. S5), suggesting that the ubiquitin-proteasomal degradation pathway might be involved and regulated by SIRT1-mediated deacetylation of SIRT3. It is therefore possible that acetylated SIRT3 is targeted for ubiquitination and retro-transported from the mitochondria to the cytoplasm for proteasomal degradation, as has been observed for other mitochondrial proteins (42), but further experiments will be necessary to fully elucidate the mechanisms by which SIRT3 acetylation promotes protein degradation.

SIRT1 deacetylates and increases expression of Pgc-1α and Ppara, both of which increase Sirt3 gene expression (22, 43, 44), so that SIRT1 likely increases transcription of SIRT3. Consistent with this, we observed that mRNA levels of hepatic Sirt3 and Pgc-1α were decreased in SIRT1-LKO mice (supplemental Fig. S6). Further, SIRT1 occupancy at the PPARα-bound Sirt3 promoter was detected in fasted mice (supplemental Fig. S7).
suggesting a direct role of SIRT1 in transcriptional induction of Sirt3 during fasting. Thus, SIRT1 appears to increase SIRT3 levels by at least two mechanisms: increasing transcription of Sirt3 and increasing SIRT3 stability via deacetylation. The relative importance of these two mechanisms remains to be determined, but because SIRT3 has a short half-life of ~30–45 min, changes in SIRT3 protein levels via reversible acetylation could provide a rapid mechanism to respond to environmental cues.

Although deacetylation by SIRT1 regulates the acetylation status of SIRT3, the acetylation of Sirt3 could also play a role in determining the levels of acetylated SIRT3. Little is known about acetylation of mitochondrial proteins, even though global analysis has shown that nearly every major mitochondrial metabolic enzyme is reversibly acetylated (21, 45). Because non-enzymatic acetylation of histones with acetyl-CoA occurs under physiological conditions (46), SIRT3 could be acetylated by increased acetyl-CoA levels. Mitochondrial acetyltransferases in vivo have not been identified, but Scott et al. (47) reported that GCN5L1 acetylase counteracts SIRT3 activities in regulation of mitochondrial function by promoting acetylation of SIRT3 targets, suggesting GCN5L1 is a mitochondrial acetyltransferase. Thus, it remains possible that acetylation of SIRT3 may be aberrantly up-regulated in aging and obesity and contribute, with decreased SIRT1 deacetylation, to the hyperacetylation of SIRT3 and consequently to elevated acetylation levels of mitochondrial proteins observed in these conditions (22).

This study identifies SIRT3 as a target of post-translational acetylation, which is regulated by SIRT1. Hyperacetylation of SIRT3 in obesity and aging contributes to decreased SIRT3 activity, leading to increased LCAD acetylation, defective β-oxidation, and metabolic symptoms, including liver steatosis and glucose intolerance. Reversible mitochondrial protein acetylation is now recognized as a key metabolic regulatory mechanism (15, 16, 18, 32). By targeting the hyperacetylation of SIRT3 in obesity and aging, depressed SIRT3 activity might be restored, and consequently, dysregulated mitochondrial function be improved. SIRT3 acetylation may thus provide a new therapeutic target for obesity- and other aging-related diseases associated with mitochondrial dysfunction.

**Experimental procedures**

**Reagents and materials**

Antibodies for SIRT3 (5490S), cytochrome c (11940S), and acetyl-Lys (9441S) were purchased from Cell Signaling; antibodies for SIRT1 (sc-47765), lamin (sc-20680), tubulin (sc-5274), GAPDH (sc-47724), GFP (sc-8334), and LCAD (sc-82466) were from Santa Cruz Biotechnology; and antibodies for SIRT1 (sc-47765), lamin (sc-20680), tubulin (sc-5274), and β-actin (sc-1040) were from Sigma. The siRNAs for SIRT1 (s96764 and s174220) were purchased from Applied Biosystems. Expression plasmids for SIRT3-WT and its H248Y mutant and SIRT1 H363Y mutant were obtained from Addgene. Purified recombinant SIRT1 was obtained from Sigma (cs1040).

**Animal experiments**

To develop diet-induced obesity, 8–12-week-old C57BL/6J male mice were fed a HFD (42% fat; Harlan Teklad) for 16 weeks. For adenoviral experiments, adenovirus expressing SIRT3-WT-FLAG or Lys57 mutants (2.5–5.0 × 10⁸ active viral particles in 100 μl of saline) was injected via the tail vein, and 3 weeks later, the mice were sacrificed at approximately 9:00 a.m., and the livers were collected. Infection of mice with these viral doses does not cause marked inflammation (28, 48). Ad-SIRT3-FLAG was constructed using mouse SIRT3-FLAG cDNA (49) inserted into the XhoI/HindIII site in the Ad-Track-CMV vector. For fasting/feeding experiments, the mice were fasted for 12 h and then fasted or refed a normal chow for 6 h. For the glucose tolerance test, the mice were fasted for 16 h and injected i.p. with 2 g/kg glucose (Sigma), and blood glucose levels were measured using an Accu-check Aviva glucometer (Roche). Liver acylcarnitine and serum β-hydroxyl butyrate levels were measured as described (10) by metabolomics analysis in the University of Illinois at Urbana-Champaign Metabolomics Facility. All animal use and adenoviral protocols were approved by the Institutional Animal Care and Use and Institutional Biosafety Committees.

**Tandem mass spectrometry**

SIRT3-FLAG was expressed in mice fed a normal chow diet or a HFD by tail vein injection of Ad-SIRT3-FLAG. Five days after injection, the mice were sacrificed at 9:00 a.m., the mitochondrial fraction was prepared, and SIRT3-FLAG protein in the fraction was purified by binding to M2 agarose and then subjected to LC-MS or LC-MS/MS mass spectrometry-based proteomic analysis as described previously (7, 28, 29, 48). LC/MS or MS/MS spectra were screened against the SIRT3 sequence using SEQUEST (Thermo Finnigan, San Jose, CA), and the identified acetylated peptides were confirmed by manual inspection of the MS2 and MS3 spectra.

**Mitochondrial fractionation**

Mouse liver mitochondrial fractions were prepared using a mitochondrial fractionation kit (Active Motif, Inc.) according to the manufacturer’s instruction with modifications. Mouse livers were collected in 10 ml of ice-cold PBS and minced with a razor blade. The minced tissue was washed with ice-cold PBS and resuspended in 3 ml of 1× cytosolic buffer. The sample was incubated on ice for 15 min then transferred to a prechilled Dounce homogenizer. The tissue was homogenized by 20 strokes and centrifuged at 800 × g for 20 min at 4 °C. The resulting pellet was the nuclear fraction. The supernatant was centrifuged three times at 800 × g for 10 min at 4 °C to remove any residual nuclei. The supernatant was then centrifuged at 10,000 × g for 20 min at 4 °C to pellet the mitochondria. The resulting supernatant was centrifuged three times at 16,000 × g for 20 min at 4 °C to produce the cytosol fraction. The mitochondrial pellet was washed five times by resuspension in 300 μl of cytosolic buffer and centrifugation at 10,000 × g for 10 min at 4 °C. The final mitochondrial fraction pellet was lysed by adding 100 μl of mitochondrial buffer, incubated on ice for 15 min, and then vortexed for 10 s. All the buffers and solutions were supplemented with protease and phosphatase inhibitors,
1 mM DTT, 1 μM TSA, and 20 mM NAM. For IB analysis, 10 μl of each cellular fraction of a total of 500 μl, 1.5 ml, and 100 μl for the nuclear, cytosolic, and mitochondrial fractions, respectively, were analyzed.

In-cell and in vivo acetylation assay

For in-cell SIRT3 acetylation studies, Cos-1 cells were transfected with plasmids as indicated and/or siRNA, and 36–48 h later, the cells were treated with 1 μM TSA and 20 mM NAM, in the presence or absence of 1 μM MG132 for 3 h and harvested. Cos-1 cell extracts or mouse liver extracts were prepared by brief sonication of cell pellets or liver tissue in SDS-containing post-translational modification buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS, 5% glycerol) supplemented with protease and phosphatase inhibitors, 1 mM DTT, 1 μM TSA, and 20 mM NAM. After centrifugation, supernatants were incubated with 1–2 μg of antibodies for SIRT3 or pan acetyl-Lys for 2 h and isolated by binding to protein G-agarose. Bound acetylated proteins or SIRT3 were detected by IB using antibodies for pan acetyl-Lys or SIRT3, respectively.

In vitro fluorometric SIRT3 deacetylase activity assay

SIRT3-WT-FLAG (49) and FLAG-SIRT3 mutants (K57R or K57Q) were expressed in Cos-1 cells and immunoprecipitated with M2 agarose from mitochondria fractions, and the level of SIRT3 was determined by IB. The immunoprecipitated SIRT3-FLAG proteins were incubated with fluoro-substrate peptide (Abcam, ab156067) and increasing concentrations of NAD⁺. SIRT3 activity was determined by measuring fluorescent emission at 460 nm, following excitation at 360 nm according to the manufacturer’s instruction.

In vitro deacetylation assays

SIRT3-WT-FLAG (49), SIRT3-FLAG mutants (K57R or K57Q), FLAG-SIRT1, FLAG-SIRT1 mutants (S164D or H363Y), and FLAG-LCAD were expressed in Cos-1 cells and purified by binding to M2 agarose. Proteins were incubated with 50 μl of acetylation buffer (Tris-HCl, pH 8.8, 5% glycerol, 50 mM NaCl, 4 mM MgCl₂, 1 mM DTT) in the presence of 50 μM NAD⁺ at 37 °C for 1 h, and levels of acetylated SIRT3 or LCAD or input levels were measured by IB. Consistent results were observed from two independent experiments.

Histological microscopy

Frozen liver sections were stained with Oil Red O, and paraffin-embedded liver sections were stained with hematoxylin and eosin as described previously (28, 48). Stained slides were imaged with a NanoZoomer Scanner.

ColP and GST pulldown

Mitochondrial fractions were prepared from fasted or fed mice and briefly sonicated in ColP buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 5% glycerol) supplemented with protease and phosphatase inhibitors, 1 mM DTT, 1 μM TSA, and 20 mM NAM. After centrifugation, the supernatant was incubated with 1–2 μg of antibodies for 2 h, and 30 μl of a 25% protein G-agarose slurry was added. 1 h later, samples were washed with ColP buffer three times, and bound proteins were detected by IB. GST-pulldown assays were done as previously described (7, 28, 29, 48). 1–2 μg of GST-fusion proteins were incubated with in vitro synthesized proteins (Promega), and bound proteins were detected by IB. For construction of GST-SIRT3 plasmids, DNA fragments that contained different regions of SIRT3 sequences were amplified by PCR from the SIRT3 expression plasmid and cloned into the EcoRI and XhoI site of the pGEX-4T1 vector.

Construction of K57R and K57Q SIRT3 mutants

The acetylation-defective K57R-SIRT3 mutant and acetylation-mimic K57Q-SIRT3 mutants were constructed by site-directed mutagenesis (Stratagene) and confirmed by DNA sequencing. For construction of adenoviral SIRT3, SIRT3 DNA fragments were amplified by PCR from the SIRT3 WT or Lys⁵⁷ mutant expression plasmid and cloned into the XhoI and HindIII sites of the AdTrack-CMV vector.

CHX study

Protein stability studies using CHX were done as previously described (7, 50). Briefly, Cos-1 cells or primary mouse hepatocytes were transfected with expression plasmids for SIRT3-FLAG WT (49) or Lys⁵⁷ mutants, along with either SIRT1 WT or deacetylase activity-defective mutants, H363Y or S164D, or siRNA for SIRT1 (mixture of s96764 and s174220; Applied Biosystems) or control RNA, and then, 36 h later, cells were treated with CHX (10 μg/ml) for times indicated in the figure legends. The cell extracts were prepared and SIRT3-FLAG protein levels were detected by IB.

Quantitative RT-PCR assay

Total RNA was isolated from liver by TRIzol (Invitrogen) and real-time RT-PCR was performed with an iCycler iQ (Bio-Rad) using SYBR Green PCR master mix. Target gene mRNA levels were normalized to those of 36B4. Primer sequences for quantitative real-time PCR are shown in supplemental Table S1.

Statistical analysis

Statistical significance between two groups was determined by Student’s t test, and the mean ± S.E. are presented. p < 0.05 was considered as statistically significant.

Author contributions—S. K., B. K., and J. K. K. designed research; S. K. and S. S. performed experiments; P. Y. performed the mass spectrometry-based proteomic analyses; X. L. provided SIRT1-LKO mice; S. K., S. S., P. Y., B. K., and J. K. K. analyzed data; and S. K., B. K., and J. K. K. wrote the paper.

Acknowledgments—We thank to Qiang Tong at Baylor College of Medicine for providing the pcDNA-mouse SIRT3-FLAG expression plasmid and Mark Leid at the Oregon State University for GST-SIRT1 constructs. We also thank to Li Zhong in the Metabolomic Center at University of Illinois at Urbana-Champaign for measuring liver acylcarnitines and serum β-hydroxyl butyrate levels.

References

1. Guarente, L. (2011) Sirtuins, aging, and metabolism. Cold Spring Harb. Symp. Quant. Biol. 76, 81–90

Hyperacetylation of SIRT3 in obesity and aging
Hyperacetylation of SIRT3 in obesity and aging

2. Finkel, T., Deng, C. X., and Mostoslavsky, R. (2009) Recent progress in the biology and physiology of sirtuins. Nature 460, 587–591

3. Haigis, M. C., and Sinclair, D. A. (2010) Mammalian sirtuins: biological insights and disease relevance. Annu. Rev. Pathol. 5, 253–295

4. Giblin, W., Skinner, M. E., and Lombard, D. B. (2014) Sirtuins: guardians of mammalian healthspan. Trends Genet. 30, 271–286

5. Yamamoto, H., Schoonjans, K., and Auwerx, J. (2017) Sirt3 complexes with 4′-bromo-resveratrol reveal binding sites and inhibition mechanism. Cell Mol. Biol. 13, 395–402

6. He, W., Newman, J. C., Wang, M. Z., Ho, L., and Verdin, E. (2012) Mitochondrial sirtuins: regulators of protein acylation and metabolism. Trends Endocrinol. Metab. 23, 467–476

7. Hirschy, M. D., Shimazu, T., Goetzman, E., Jing, E., Schwer, B., Lombard, D. B., Grueter, C. A., Harris, C., Biddinger, S., Ilkayeva, O. R., Stevens, R. D., Li, Y., Saha, A. K., Ruderman, N. B., Bain, J., et al. (2010) Sirt3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 464, 121–125

8. Qiu, X., Brown, K., Hirschy, M. D., Verdin, E., and Chen, D. (2010) Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell Metab. 12, 662–667

9. Hallow, W. C., Lee, S., and Denu, J. M. (2006) Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. Proc. Natl. Acad. Sci. U.S.A. 103, 10230–10235

10. Hallow, W. C., Yu, W., Smith, B. C., Devries, M. K., Ellinger, J. J., Someya, S., Shortreed, M. R., Prolla, T., Markley, J. L., Smith, L. M., Zhao, S., Guan, K. L., and Denu, J. M. (2011) Sirt3 promotes the urea cycle and fatty acid oxidation during dietary restriction. Mol. Cell 41, 139–149

11. Lombard, D. B., Alt, F. W., Cheng, H. L., Bunkenborg, J., Streeper, R. S., Mostoslavsky, R., Kim, J., Yancopoulos, G., Valenzuela, D., Murphy, A., Yang, Y., Chen, Y., Hirschy, M. D., Bronson, R. T., Haigis, M., et al. (2007) Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. Mol. Cell. Biol. 27, 8807–8814

12. Hebert, A. S., Dittenhafer-Reed, K. E., Yu, W., Bailey, D. J., Selen, E. S., Boersma, M. D., Carson, J. J., Tonelli, M., Balloon, A. J., Higbee, A. J., Westphall, M. S., Pagliarini, D. J., Prolla, T. A., Assadi-Porter, F., Roy, S., et al. (2013) Calorie restriction and SIRT3 trigger global reprogramming of the mitochondrial protein acetylome. Mol. Cell 49, 186–199

13. Hirschy, M. D., Shimazu, T., Jing, E., Grueter, C. A., Collins, A. M., Aouizerat, B., Stančáková, A., Goetzman, E., Lam, M. M., Schwer, B., Stevens, R. D., Muehlbauer, M. J., Kakar, S., Bass, N. M., Kuusisto, J., et al. (2011) SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. Mol. Cell 44, 177–190

14. Alben, D., Ateri, E., Mazzuco, S., Ghilardi, A., Rodilossi, S., Biella, G., Ongaro, F., Antuono, P., Boldrini, P., Di Giorgi, E., Frigato, A., Durante, E., Cabelotto, L., Zanardo, A., Siculi, M., et al. (2014) Modulation of human longevity by SIRT3 single nucleotide polymorphisms in the prospective study “Treviso Longeva (TRELONG).” Age 36, 469–478

15. Kendrick, A. A., Choudhury, M., Rahman, S. M., McCurdy, C. E., Friederich, M., Van Hove, J. L., Watson, P. A., Birdsey, N., Bao, J., Gius, D., Sack, M. N., Jing, E., Kahn, C. R., Friedman, E. J., and Jonscher, K. R. (2011) Fatty liver is associated with reduced SIRT3 activity and mitochondrial protein hyperacetylation. Biochem. J. 433, 505–514

16. Brunet, A., Sweeney, L. B., Sturgill, J. F., Chua, K. F., Greer, P. L., Lin, Y., Tran, H., Ross, S. E., Mostoslavsky, R., Cohen, H. Y., Hu, L. S., Cheng, H. L., Jedrzychowski, M. P., Gysy, S. P., Sinclair, D. A., et al. (2004) Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 303, 2011–2015

17. Imai, S., and Kiess, W. (2009) Therapeutic potential of SIRT1 and NAMPT-mediated NAD biosynthesis in type 2 diabetes. Front. Biosci. (Landmark Ed.) 14, 2983–2995

18. Houtkooper, R. H., Pirinen, E., and Auwerx, J. (2012) Sirtuins as regulators of metabolism and healthspan. Nat. Rev. Mol. Cell Biol. 13, 225–238

19. Kim, D. H., Xiao, Z., Kwon, S., Sun, X., Ryerson, D., Tkac, D., Ma, P., Wu, S. Y., Chiang, C. M., Zhou, E., Xu, H. E., Palvimo, J. J., Chen, L. F., Kemper, B., and Kemper, J. K. (2015) A dysregulated acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity. EMBO J. 34, 184–199

20. Kemper, J. K., Xiao, Z., Pongoulti, B., Mao, J., Fang, S., Kanamalur, D., Tsang, S., Wu, S. Y., Chiang, C. M., and Veenstra, T. D. (2009) FXR acetylation is normally dynamically regulated by p300 and SIRT1 but constitutively elevated in metabolic disease states. Cell Metab. 10, 392–404

21. Flick, F., and Lüschke, B. (2012) Regulation of sirtuin function by post-translational modifications. Front. Pharmacol. 3, 29

22. Nguyen, G. T., Gertz, M., and Steegborn, C. (2013) Crystal structures of Sirt3 complexes with 4′-bromo-resveratrol reveal binding sites and inhibition mechanism. Chem. Biol. 20, 1375–1385

23. Shimazu, T., Hirschy, M. D., Hua, L., Dittenhafer-Reed, K. E., Schwer, B., Lombard, D. B., Li, Y., Bunkenborg, J., Alt, F. W., Denu, J. M., Jacobson, M. P., and Verdin, E. (2010) Sirt3 deacetylates mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 and regulates ketone body production. Cell Metab. 12, 654–661

24. Bharathi, S. S., Zhang, Y., Mohnsen, A. W., Uppala, R., Balasubramani, M., Schreiber, E., Uechi, G., Beck, M. E., Rardin, M. J., Vockley, J., Verdin, E., Gibson, B. W., Hirschy, M. D., and Goetzman, E. S. (2013) Sirtuin 3 (SIRT3) protein regulates long-chain acyl-CoA dehydrogenase by deacetylating conserved lysines near the active site. J. Biol. Chem. 288, 33837–33847

25. Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopeschuck, G. D., and Muoio, D. M. (2008) Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 7, 45–56

26. Li, H., and Friedman, M. I. (2008) Reduced hepatocytic fatty acid oxidation in outbred rats prescreened for susceptibility to diet-induced obesity. Int. J. Obes. (Lond.) 32, 1331–1334

27. Kahn, S. E., Hull, R. L., and Utzschneider, K. M. (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444, 840–846

28. Tian, Y., Garcia, G., Bian, Q., Stenzl, D., Mesdade, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopeschuck, G. D., and Muoio, D. M. (2008) Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 7, 45–56
39. Aquilano, K., Baldelli, S., Pagliei, B., and Ciriolo, M. R. (2013) Extranuclear localization of SIRT1 and PGC-1α: an insight into possible roles in diseases associated with mitochondrial dysfunction. *Curr. Mol. Med.* 13, 140–154

40. Aquilano, K., Vigilanza, P., Baldelli, S., Pagliei, B., Rotilio, G., and Ciriolo, M. R. (2010) Peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) and sirtuin 1 (SIRT1) reside in mitochondria: possible direct function in mitochondrial biogenesis. *J. Biol. Chem.* 285, 21590–21599

41. Ghisays, F., Brace, C. S., Yackly, S. M., Kwon, H. J., Mills, K. F., Kashentseva, E., Dmitriev, I. P., Curiel, D. T., Imai, S. I., and Ellenberger, T. (2015) The N-terminal domain of SIRT1 is a positive regulator of endogenous SIRT1-dependent deacetylation and transcriptional outputs. *Cell Rep.* 10, 1665–1673

42. Taylor, E. B., and Rutter, J. (2011) Mitochondrial quality control by the ubiquitin-proteasome system. *Biochem. Soc. Trans.* 39, 1509–1513

43. Kong, X., Wang, R., Xue, Y., Liu, X., Zhang, H., Chen, Y., Fang, F., and Chang, Y. (2010) Sirtuin 3, a new target of PGC-1α, plays an important role in the suppression of ROS and mitochondrial biogenesis. *PLoS One* 5, e11707

44. Giralt, A., Hondares, E., Villena, J. A., Ribas, F., Díaz-Delfín, J., Giralt, M., Iglesias, R., and Villarroya, F. (2011) Peroxisome proliferator-activated receptor-γ coactivator-1α controls transcription of the Sirt3 gene, an essential component of the thermogenic brown adipocyte phenotype. *J. Biol. Chem.* 286, 16958–16966

45. Choudhary, C., Kumar, C., Ngiad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 325, 834–840

46. Paik, W. K., Pearson, D., Lee, H. W., and Kim, S. (1970) Nonenzymatic acetylation of histones with acetyl-CoA. *Biochim. Biophys. Acta* 213, 513–522

47. Scott, I., Webster, B. R., Li, J. H., and Sack, M. N. (2012) Identification of a molecular component of the mitochondrial acetyltransferase programme: a novel role for GCN5L1. *Biochem. J.* 443, 655–661

48. Kim, D. H., Kwon, S., Byun, S., Xiao, Z., Park, S., Wu, S. Y., Chiang, C. M., Kemper, B., and Kemper, J. K. (2016) Critical role of RanBP2-mediated SUMOylation of small heterodimer partner in maintaining bile acid homeostasis. *Nat. Commun.* 7, 12179

49. Shi, T., Wang, F., Stieren, E., and Tong, Q. (2005) SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. *J. Biol. Chem.* 280, 13560–13567

50. Miao, J., Xiao, Z., Kanamaluru, D., Min, G., Yau, P. M., Veenstra, T. D., Ellis, E., Strom, S., Suino-Powell, K., Xu, H. E., and Kemper, J. K. (2009) Bile acid signaling pathways increase stability of small heterodimer partner (SHP) by inhibiting ubiquitin-proteasomal degradation. *Genes Dev.* 23, 986–996