SIRT3 Regulates Mitochondrial Protein Acetylation and Intermediary Metabolism

M.D. HIRSCHHEY¹, T. SHIMAZU¹, J.-Y. HUANG¹, B. SCHWER¹, AND E. VERDIN^{1,2}

¹*Gladstone Institute of Virology and Immunology, San Francisco, California 94158*

²*Department of Medicine, University of California, San Francisco, California 94143*

Correspondence: everdin@gladstone.ucsf.edu

The sirtuins are a family of nicotinamide adenine dinucleotide (NAD⁺)-dependent protein deacetylases that regulate cell survival, metabolism, and longevity. Humans have seven sirtuins (SIRT1–SIRT7) with distinct subcellular locations and functions. SIRT3 is localized to the mitochondrial matrix and its expression is selectively activated during fasting and calorie restriction. Activated SIRT3 deacetylates several key metabolic enzymes—acetyl-coenzyme A synthetase, long-chain acyl-coenzyme A (acyl-CoA) dehydrogenase (LCAD), and 3-hydroxy-3-methylglutaryl CoA synthase 2—and enhances their enzymatic activity. Disruption of SIRT3 activity in mice, either by genetic ablation or during high-fat feeding, is associated with accelerated development of metabolic abnormalities similar to the metabolic syndrome in humans. SIRT3 is therefore emerging as a metabolic sensor that responds to change in the energy status of the cell and modulates the activity of key metabolic enzymes via protein deacetylation.

Proper mitochondrial function requires careful regulation of the activity of multiple metabolic enzymes and is in turn required for metabolic homeostasis. Changes in mitochondrial number and activity are implicated in aging, cancer, and other diseases (Wallace 2005). Mitochondrial dysfunction appears to play a particularly important role in the pathogenesis of the metabolic syndrome—a group of metabolic abnormalities characterized by central obesity, dyslipidemia, high blood pressure, and increased fasting glucose. A number of abnormalities in mitochondria have been identified in patients and animal models with the metabolic syndrome, including reduced mitochondrial mass (Kelley et al. 2002), altered mitochondrial morphology (Civitarese et al. 2010), reduced fatty-acid oxidation (Zhang et al. 2007), lower oxidative phosphorylation (Petersen et al. 2005; Befroy et al. 2007), and increased reactive oxygen species (ROS) (Patti et al. 2003; Petersen et al. 2004; Civitarese et al. 2006; Ukropcova et al. 2007). Various post-translational modifications fine-tune the activities of metabolic enzymes, and acetylation is increasingly recognized as an important posttranslational modification for a number of key metabolic pathways. A large number of metabolic enzymes are acetylated in a variety of organisms (Wang et al. 2010; Zhao et al. 2010). This chapter focuses on the role of the major mitochondrial protein deacetylase, SIRT3, its regulation during fasting, calorie restriction and high-fat feeding, the identification and characterization of its targets, and its role in the pathogenesis of the metabolic syndrome.

ACETYLATION IS A PREVALENT MITOCHONDRIAL PROTEIN POSTTRANSLATIONAL MODIFICATION

Lysine acetylation is a reversible and highly regulated posttranslational modification. It was initially discovered on histones, but several nonhistone proteins have since been identified to be lysine acetylated (Glozak et al. 2005). Acetylation takes place on the ϵ -amino group of lysine residues and regulates diverse protein properties, including DNA–protein interactions, subcellular localization, transcriptional activity, protein stability, protein–protein interactions, and last, but not least, enzymatic activity. Lysine acetylation is under the control of competing enzymes, commonly called histone acetyltransferases (HATs) and histone deacetylases, although several of these enzymes mainly target nonhistone proteins.

Although acetylation was originally thought to affect only histones, an extensive proteomic survey of cellular proteins revealed that a large number of mitochondrial proteins are subject to reversible lysine acetylation (Kim et al. 2006). In this study, mouse liver mitochondria were purified, subjected to proteolytic digestion, and the resulting lysate subjected to immuno-affinity purification of lysine-acetylated peptides. Nano–high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) analysis of the acetylated peptides identified 277 lysine acetylation sites in 133 mitochondrial proteins, thereby conclusively establishing that lysine acetylation is an abundant posttranslational modification in the

mitochondrion. Interestingly, mitochondrial protein acetylation appeared to be linked to metabolic status because 62% of acetylated proteins were identified in mitochondrial fractions isolated from both fed and fasted animals, 14% were specific to fed mice, and 24% were specific to fasted mice. Most lysine-acetylated proteins from mitochondrial fractions were metabolic enzymes (91 proteins). Interestingly, ATP synthase F_o subunit 8, one of 15 proteins encoded by mitochondrial DNA, was also acetylated, implying that the acetylation reaction can occur *de novo* within mitochondria.

These early results suggested that as much as 20% of all mitochondrial proteins are lysine acetylated (Kim et al. 2006). However, more recent proteomic analyses of lysine-acetylated mitochondrial proteins showed that as much as 50% of all mitochondrial proteins are acetylated, and that proteins involved in energy metabolism are overrepresented. This includes proteins in the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, β -oxidation of lipids, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and the urea cycle (Wang et al. 2010; Zhao et al. 2010). Transporter and channel proteins are also lysine acetylated. Interestingly, 44% of mitochondrial dehydrogenases are acetylated. Among them, 14 use NAD^+ as the electron acceptor to catalyze biochemical reactions in oxidative, catabolic routes.

SIRTUINS ARE NAD^+ -DEPENDENT PROTEIN DEACETYLASES

SIRT3 belongs to a larger family, the sirtuins, that have important roles in controlling metabolism in a variety of organisms (for review, see Schwer and Verdin 2008). Named after the yeast silent information regulator 2 (Sir2), sirtuins regulate important biological pathways in eubacteria, archaea, and eukaryotes. Yeast Sir2 and some sirtuins are protein deacetylases (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). Sirtuins mediate a deacetylation reaction that couples lysine deacetylation to NAD^+ hydrolysis. This hydrolysis yields *O*-acetyl-ADP-ribose, the deacetylated substrate, and nicotinamide (for review, see Denu 2005; Sauve et al. 2006).

Bacteria and archaea encode one or two sirtuins, but mice and humans possess seven sirtuins (SIRT1–7). Mouse and human sirtuins occupy different subcellular compartments, such as the nucleus (SIRT1, -2, -3, -6, and -7), cytoplasm (SIRT1 and -2), and mitochondria (SIRT3, -4, and -5) (Blander and Guarente 2004; North and Verdin 2004; Michishita et al. 2005; Chen et al. 2006; Haigis et al. 2006; Tanno et al. 2006). The sirtuins are assigned to five subclasses (I–IV and U) based on the phylogenetic conservation of a core domain of approximately 250 amino acids (Frye 1999, 2000). Among mammalian sirtuins, SIRT1, -2, and -3 are closely related class I sirtuins, show high homology to the yeast sirtuins Sir2, Hst1, and Hst2, and exhibit robust deacetylase activity. Class II sirtuins (SIRT4) have no detectable deacetylase activity and instead show weak ADP-ribosyltransferase activity (Haigis et al. 2006; Ahuja et al. 2007). Class

III sirtuins (SIRT5) have only weak deacetylase activity on histone substrates (Verdin et al. 2004; Nakagawa et al. 2009); class IV sirtuins have ADP ribosyltransferase and deacetylase activity (SIRT6) or unknown activity (SIRT7; Kawahara et al. 2009; Haigis and Sinclair 2010). Class U sirtuins are intermediate between classes I and IV and have only been observed in bacteria.

The dependence of sirtuins on NAD^+ suggests that their enzymatic activity is directly linked to the energy status of the cell via the cellular NAD^+ :NADH ratio; the absolute levels of NAD^+ , NADH, or nicotinamide; or a combination of these variables (Lin et al. 2000, 2002, 2004; Bitterman et al. 2002; Anderson et al. 2003).

SIRT3 IS A MITOCHONDRIAL DEACETYLASE

Three sirtuins, SIRT3, SIRT4, and SIRT5, are located in mitochondria (Onyango et al. 2002; Schwer et al. 2002; Michishita et al. 2005; Haigis et al. 2006), and endogenous SIRT3 is a soluble protein in the mitochondrial matrix (Schwer et al. 2002, 2006). Interestingly, SIRT3 is translated in the cytoplasm as a longer, enzymatically inactive precursor that is imported into the mitochondrion. Following import, the first 100 amino acids of SIRT3 are proteolytically cleaved, leading to a final enzymatically active SIRT3 with a molecular mass of 28 kDa (Fig. 1).

Initial controversies regarding the subcellular localization of SIRT3 have been resolved. For example, a small fraction of full-length, unprocessed human SIRT3 was also reported to be localized to the nucleus. Recent results from the Reinberg laboratory indicate that this nuclear form of SIRT3 interacts with specific genes (Scher et al. 2007; Reinberg 2011). Another question centered on whether SIRT3 is a primary mitochondrial protein in mice. This controversy was linked to the finding that mouse SIRT3 cDNA lacked the amino-terminal mitochondrial targeting domain identified in the human protein. However, we used an antiserum for SIRT3 to confirm that mouse endogenous SIRT3 is exclusively mitochondrial: We could not detect any nuclear SIRT3 (Lombard et al. 2007), and a recently identified mouse SIRT3 cDNA encodes a protein that is imported to the mitochondrial matrix, like human SIRT3 (Cooper et al. 2009; Jin et al. 2009).

Most importantly, the identification of a protein deacetylase within the mitochondrial matrix suggested that mitochondrial proteins were acetylated and that SIRT3 might regulate their acetylation level and their biological activities.

In support of this model, mitochondrial proteins were strikingly hyperacetylated in mice lacking SIRT3 (SIRT3KO mice) (Lombard et al. 2007). In contrast, mice lacking either SIRT4 or SIRT5 showed no obvious change in mitochondrial protein acetylation (Lombard et al. 2007). These observations supported the model that SIRT3 is the major mitochondrial deacetylase. However, despite this striking biochemical abnormality, SIRT3KO mice were healthy under normal laboratory conditions and conditions of mild stress, such as short-

Figure 1. SIRT3 is a mitochondrial NAD^+ -dependent protein deacetylase. SIRT3 is encoded in the nucleus and imported into the mitochondrial matrix by a canonical mitochondrial targeting sequence. After import, a mitochondrial protein peptidase cleaves the targeting sequence and activates the deacetylase SIRT3 into its active form. Using NAD^+ as a cofactor, SIRT3 removes acetyl groups from protein lysine residues within mitochondrial proteins and generates *O*-acetyl-ADP ribose and nicotinamide.

term food deprivation, and showed normal overall metabolism and cold resistance.

SIRT3 DEACETYLATES MULTIPLE MITOCHONDRIAL PROTEINS DURING FASTING

Because SIRT3KO mice had no overt phenotype under basal conditions, we tested various stress conditions where SIRT3 could play a possible role. In wild-type (WT) mice, SIRT3 protein and mRNA expression is up-regulated during fasting, a clue that SIRT3 might be involved in the fasting response. The fasting response in mammals is characterized by dramatic changes in metabolic fluxes. Fatty acids are released from triglycerides in white adipose tissue and transported to the liver where they become oxidized. In contrast to other tissues, hepatic fatty-acid oxidation is incomplete and does not progress much beyond acetyl-coenzyme A (acetyl-CoA). The accumulating acetyl-CoA is used as a substrate for the generation of ketone bodies and acetate. Both acetate and ketone bodies diffuse within the organism and are used by peripheral tissues as energy sources.

Metabolomic analysis of livers from SIRT3KO mice revealed multiple abnormalities in lipid metabolism, including increased triglycerides and accumulation of acylcarnitines, which are intermediate products of mitochondrial fatty-acid oxidation. Histological examination of liver tissue from SIRT3KO mice revealed hepatic steatosis. These data suggested that SIRT3KO mice have a defect in fatty-acid oxidation. Measurement of palmitate oxidation in liver extracts from WT and SIRT3KO mice revealed a primary defect in β -oxidation in the absence of SIRT3.

To identify SIRT3 targets, we purified mitochondria from SIRT3KO mice, digested mitochondrial matrix lysates with trypsin, immunoprecipitated the acetylated

peptides with an antiacetyllysine antibody, and processed the immunoprecipitated peptide for mass spectrometry analysis. This analysis identified more than 1000 acetylated peptides (Fig. 2) (DB Lombard, unpubl.). Multiple enzymes in the mitochondrial β -oxidation pathway were found to be acetylated in mice lacking SIRT3, including carnitine *O*-palmitoyltransferase 1 and 2, carnitine/acylcarnitine translocase, acyl-CoA dehydrogenase (very long, long, medium, and short chain), enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. Because the metabolomic data indicated a selective accumulation of acylcarnitines with a chain length greater than 16, we focused our analysis on long-chain acyl-CoA dehydrogenase (LCAD) as a critical enzyme targeted by SIRT3. We identified a single lysine (K42) in LCAD whose acetylation was regulated by SIRT3. The acetylated enzyme was inhibited and its deacetylation by SIRT3 enhanced its activity in vitro and in vivo.

Mice lacking SIRT3 exhibited other hallmarks of fatty-acid oxidation disorders: reduced ATP levels and intolerance to cold exposure, particularly during fasting (Fig. 3) (Hirschey et al. 2010).

In a parallel study, we found that another key enzyme of the fasting response, 3-hydroxy,3-methylglutaryl-CoA synthase (HMGCS2), is also regulated by SIRT3. HMGCS2 catalyzes the rate-limiting step in ketone body synthesis (Fig. 3). During fasting, SIRT3 deacetylates three lysine residues on HMGCS2, inducing an increase in its enzymatic activity and ketone body production. Using molecular dynamics simulation modeling,

Figure 2. Identification of acetylated mitochondrial proteins from mouse tissue. Mitochondria are purified from mouse tissue lacking SIRT3, lysed, and subjected to trypsin protein digestion. Acetylated peptides are enriched using an antiacetyllysine affinity matrix, eluted with dilute acid, and analyzed by liquid chromatography/tandem mass spectrometry (LC/MS-MS).

Figure 3. SIRT3 regulates metabolism during fasting. During metabolic stress, such as fasting, lipids are liberated from storage in adipose tissues, transported through the blood bound to albumin, and imported into the liver for oxidation and ATP production. SIRT3 is up-regulated in response to fasting in the liver and deacetylates several mitochondrial proteins, including long-chain acyl-CoA dehydrogenase (LCAD) and 3-hydroxy,3-methyl-glutaryl-CoA synthase 2 (HMGCS2), increasing their enzymatic activity. By-products of lipid oxidation such as acetate and the ketone body β -hydroxybutyrate are exported from the liver and used for energy production in extrahepatic tissues. SIRT3 also deacetylates acetyl-CoA synthetase 2 (AceCS2) in extrahepatic tissues to generate acetyl-CoA from acetate, which can be consumed in the TCA cycle.

we found that deacetylation of these three lysine residues changed the conformation of the catalytic pocket and positioned key catalytic residues nearby the substrate acetyl-CoA. Finally, mice lacking SIRT3 showed decreased ketone body levels during fasting, highlighting another role of SIRT3 in the fasting response (Fig. 3) (Shimazu et al. 2010).

Under ketogenic conditions, such as fasting, the liver of mammals releases substantial amounts of acetate into the bloodstream (Seufert et al. 1974; Buckley and Williamson 1977; Yamashita et al. 2001), at least in part via the activation of an acetyl-CoA hydrolase (Matsunaga et al. 1985). The released acetate freely diffuses to peripheral tissues but must be activated before it can be utilized for metabolism (Fig. 3). The cytoplasmic (AceCS1) and mitochondrial (AceCS2) acetyl-CoA synthases activate acetate and are differentially regulated: Fasting induces mitochondrial AceCS2 expression (Fujino et al. 2001) and decreases cytoplasmic AceCS1 expression in the liver and other tissues (Fujino et al. 2001; Sone et al. 2002). These observations point to an interesting model. Under fasting and ketogenic conditions, acetate could be released from the liver and utilized by AceCS2 to generate acetyl-CoA in extrahepatic tissues (Fujino et al. 2001).

AceCS2 was identified by our group and John Denu's group as the first acetylated target of SIRT3 (Hallows et al. 2006; Schwer et al. 2006). In the prokaryote *Salmonella enterica*, a sirtuin called CobB deacetylates acetyl-CoA synthetase, activates its enzymatic activity, and allows the bacteria to grow on acetate as a carbon source (Starai et al. 2002). Remarkably, the site of

acetylation in *S. enterica* acetyl-CoA synthase is highly conserved throughout evolution, including the lysine that becomes acetylated. Similar to what is observed in *S. enterica*, SIRT3 deacetylates AceCS2 and activates the enzyme (Schwer et al. 2006). Denu and colleagues made the same observation and further showed that the cytoplasmic acetyl-CoA synthase, AceCS1, which is involved in lipid synthesis, is regulated in a similar manner, but the deacetylase in this case is SIRT1 (Hallows et al. 2006). Recent experiments indicate that activation of acetate by AceCS2 has a specific and unique role in thermogenesis during fasting. Mice lacking AceCS2 (AceCS2KO) show 50% decreased muscle ATP levels during fasting in comparison to WT (Sakakibara et al. 2009). Fasted AceCS2KO mice become significantly hypothermic and exhibit reduced exercise capacity. These findings demonstrate that activation of acetate by AceCS2 is pivotal in thermogenesis, especially under low-glucose or ketogenic conditions, and is crucially required for survival. Interestingly, the phenotypes of mice lacking SIRT3 or AceCS2 overlap significantly because mice lacking SIRT3 also show defective thermogenesis and significant mortality when fasted in the cold (Hirschey et al. 2010).

Because a large number of mitochondrial proteins are subject to reversible lysine acetylation (Kim et al. 2006), several other SIRT3 substrates likely exist. For example, mice lacking SIRT3 have reduced ATP production (>50%), several components of complex I of the electron transport chain are hyperacetylated, and complex I activity is inhibited (Ahn et al. 2008). Furthermore, glutamate dehydrogenase and isocitrate dehydrogenase 2 were also identified as targets of SIRT3 (Schlicker et al. 2008).

These studies showed that SIRT3 regulates energy homeostasis during nutrient deprivation. It controls fatty-acid catabolism (Hirschey et al. 2010), ketone body synthesis (Shimazu et al. 2010), and acetate metabolism (Hallows et al. 2006; Schwer et al. 2006), crucial metabolic pathways that are activated during fasting.

SIRT3 ACTIVITY IS INDUCED DURING CALORIE RESTRICTION

Calorie restriction (CR) is a low-calorie dietary regimen without malnutrition. It extends the life span of yeast, worms, flies, and mammals and decreases the incidence of age-associated disorders, such as cardiovascular disease, diabetes, and cancer in animal models (Bordone and Guarente 2005; Masoro 2005). In rodents, a 20%–40% reduction of calorie intake extends life span by up to 50% (McCay et al. 1935). Whereas the positive effects of CR in mammals are well studied, the molecular mechanism of CR is not fully understood (Koubova and Guarente 2003).

Mitochondrial protein acetylation levels change in a tissue-specific manner during calorie restriction in mice. The acetylation level of more proteins increases in the liver, whereas the opposite is observed in brown adipose

tissue (Schwer et al. 2009). These observations suggest that changes in mitochondrial protein acetylation could represent an important signal during CR. SIRT3 is implicated in metabolic regulation during CR because both fatty-acid oxidation and ketone body production increase during CR (Shi et al. 2005). Importantly, fasting and CR are not equivalent and induce only partially overlapping physiological responses. For example, acute starvation increases the $\text{NAD}^+:\text{NADH}$ ratio in liver, but CR decreases this ratio (Hagopian et al. 2003a,b).

Interestingly, SIRT3 expression is activated in brown adipose tissue by CR and by exposure to cold (Shi et al. 2005). SIRT3 is also essential for CR-mediated reduction in oxidative stress. In the absence of SIRT3, the reduction in oxidative stress normally observed during CR is lost (Qiu et al. 2010; Someya et al. 2010). Two distinct enzymes that control oxidative stress, superoxide dismutase 2 (SOD2) and isocitrate dehydrogenase 2 (IDH2), are acetylated enzymes and their deacetylation by SIRT3 enhances their enzymatic activities (Fig. 4) (Qiu et al. 2010; Tao et al. 2010).

In addition to its effect on life span, CR also slows the progression of age-related hearing loss, a common age-related disorder associated with oxidative stress. Importantly, mice lacking SIRT3 show no protective effect of CR on age-related hearing loss (Someya et al. 2010). This observation suggests that SIRT3 might be an important mediator of other beneficial aspects of CR, including increased life span. Thus, SIRT3 plays an essential role in mediating at least some of the beneficial effects of CR.

SIRT3 EXPRESSION IS DOWN-REGULATED DURING HIGH-FAT FEEDING

Whereas the previous sections have focused on the role of SIRT3 under conditions characterized by restricted calorie input (fasting and CR), it is important to know that SIRT3 also plays a significant role under conditions of calorie excess. The metabolic syndrome is defined by central obesity, insulin resistance, hyperlipidemia, hyperglycemia, and hypertension (Reaven 1988). Prevalence of the metabolic syndrome is rising in the Western world and will lead to future increases in diabetes and cardiovascular disease (Ford et al. 2008). Sedentary lifestyles (Arderm et al. 2004) and high-fat “Western” diets (Feldstein and Tucker 2007) have been implicated in the increase in metabolic syndrome.

In addition to lifestyle and diet, several genes are implicated in the pathogenesis of metabolic disease, such as those encoding leptin, β -3-adrenergic receptor, hormone-sensitive lipase, lipoprotein lipase, insulin receptor substrate 1, PC-1, and skeletal muscle glycogen synthase (Zhang et al. 1994; Groop 2000; Poulsen et al. 2001; Pollex and Hegele 2006). In addition to candidate genes, multiple metabolic pathways are also implicated, including aberrant lipogenesis (Roden et al. 1996; Samuel et al. 2004), increased inflammation (Hotamisligil et al. 1993; Uysal et al. 1997), and reduced fatty-acid oxidation (Ji and Friedman 2007, 2008). Identifying the molecular mechanisms underlying the metabolic syndrome has been described as one of the most critical endeavors in modern medicine (Taubes 2009).

Figure 4. SIRT3 protects against ROS-induced damage. ROS are generated in the mitochondria from the oxidation of metabolic substrates. ROS such as superoxide (O_2^-) are converted into hydrogen peroxide (H_2O_2) by mitochondrial manganese superoxide dismutase (SOD2), which is further converted into water by glutathione peroxidase (GPX). GPX requires reduced glutathione (GSH) for its enzymatic activity, which is regulated by glutathione reductase (GSR) and NADPH. Mitochondrial isocitrate dehydrogenase 2 (IDH2) generates NADPH from NADP^+ . SIRT3 influences this process by deacetylating and activating both SOD2 and IDH2 and thereby regulates oxidative damage in cells. αKG , α -Ketoglutarate; GSSG, oxidized glutathione disulfide.

We have recently observed that lack of SIRT3 and mitochondrial protein hyperacetylation lead to accelerated development of the metabolic syndrome and its manifestations (Hirschey et al. 2011). WT mice fed a high-fat diet (HFD) develop obesity, hyperlipidemia, type 2 diabetes mellitus, insulin resistance, and nonalcoholic steatohepatitis (Surwit et al. 1995; Rossmeisl et al. 2003; Collins et al. 2004; Petro et al. 2004). We find that the development of each of these consequences of HFD feeding is significantly accelerated in mice lacking SIRT3 (Hirschey et al. 2011). In addition, we also find that mice lacking SIRT3 show dramatically enhanced levels of proinflammatory cytokines, including interferon- γ , IL-10, IL-12p70, IL-6, and TNF- α , in agreement with the discussed role of SIRT3 in the control of oxidative stress. Interestingly, LCAD deficiency is also associated with accelerated development of insulin resistance and steatohepatitis in mice (Zhang et al. 2007), primarily attributed to lipid accumulation from reduced fatty-acid oxidation (Kurtz et al. 1998). Additionally, ablation of malonyl-CoA decarboxylase (MCD), an enzyme that regulates mitochondrial fatty-acid oxidation, also leads to reduced fatty-acid oxidation and insulin resistance (Koves et al. 2008). Thus, primary lesions in fatty-acid oxidation upon ablation of SIRT3, LCAD, or MCD result in insulin resistance and support a role for mitochondrial lipid oxidation in the maintenance of insulin signaling and metabolic homeostasis (Fig. 5).

We also found that prolonged exposure to HFD feeding in WT mice results in a reduction of hepatic SIRT3 expression. Similar observations were previously reported by Bao et al. (2010). Whereas acute HFD feeding leads to an increase in SIRT3 protein expression, chronic HFD feeding (13 wk) suppresses SIRT3 protein expression and induces global mitochondrial protein hyperacetylation, LCAD hyperacetylation, and reduced LCAD activity. The reduction in LCAD activity is phenocopied in SIRT3KO mice. The suppression of SIRT3 occurs at the transcriptional level and is primarily driven by the HFD-induced suppression of PGC-1 α (Crunkhorn et al. 2007; Li et al. 2007), a major regulator of SIRT3 expression (Kong et al. 2010; JY Huang and E Verdin, unpubl.). Overexpression of exogenous PGC-1 α was sufficient to rescue the loss of SIRT3 in HFD-fed mice. Fatty-acid oxidation is also suppressed by HFD feeding, although the molecular mechanism is incompletely understood (Ji and Friedman 2007, 2008). Our observations support the model that PGC-1 α and SIRT3 down-regulation and mitochondrial protein hyperacetylation play a critical role in this process.

Finally, we have studied the possible role of human polymorphisms in the SIRT3 gene in the development of the metabolic syndrome in humans. Because single-nucleotide polymorphisms (SNPs) in *SIRT3* have not been identified in large-scale genome-wide association studies in obesity (Lindgren et al. 2009; Heid et al. 2010; Speliotes et al. 2010), diabetes (Prokopenko et al. 2009; Dupuis et al. 2010), or cholesterol and lipid metabolism (Musunuru et al. 2010; Teslovich et al. 2010), we focused our initial analysis on a population characterized

by fatty liver disease (the NASH-CRN), reasoning that such a population should show increased frequency of the metabolic syndrome and might therefore be enriched in patients carrying predisposing SIRT3 alleles. We found that patients meeting the criteria for metabolic syndrome were more likely to carry the SIRT3 rs11246020 “A” minor allele. In a follow-up study of ~8000 Finnish men focusing specifically on rs11246020, we sought to validate this SNP association with the metabolic syndrome (Stancáková et al. 2009). We observed a significant correlation between the frequency of this allele and a metabolic syndrome diagnosis, supporting the findings in the NASH-CRN study. However, this association was relatively weak (odds ratio, 1.3) and was not observed with all definitions of the metabolic syndrome. Given the heterogeneity of the metabolic syndrome as a clinical entity, it will be important to further validate these observations in larger cohorts of patients and to further determine whether the SIRT3 polymorphism associates more strongly with unique manifestations of the metabolic syndrome rather than with the syndrome as a whole. Remarkably, the SIRT3 rs11246020 polymorphism is present with an exon of SIRT3 and induces a mutation within the catalytic domain of SIRT3 (V208I). Mutation of valine 208 into isoleucine reduces SIRT3 enzyme efficiency, both by increasing the K_M for NAD⁺ and reducing the V_{max} . These data are consistent with the model that reduction in SIRT3 enzymatic activity associated with the SIRT3 rs11246020 polymorphism and the consequent V208I mutation play a pathogenic role in humans, as in mice, and increases susceptibility to the metabolic syndrome. Together, these observations highlight the importance of using primary cellular and mouse data to direct human genetic studies and the power of integrating these data to glean insights into the relationships between human SNPs and the underlying biology.

MITOCHONDRIAL PROTEIN ACETYLATION AND METABOLIC REGULATION

In conclusion, every metabolic pathway contains acetylated proteins in both bacteria and human liver (Wang et al. 2010; Zhao et al. 2010) and acetylation has emerged as an important regulatory posttranslational modification in mitochondria (Hirschey et al. 2010). Changes in metabolic status, including CR (Schwer et al. 2009) and HFD feeding (Hirschey et al. 2011), lead to changes in mitochondrial protein acetylation. Interestingly, fatty-acid oxidation is elevated during both CR (Koubova and Guarante 2003) and HFD feeding (Kim et al. 2004). Because increased fatty-acid oxidation leads to higher intramitochondrial acetyl-CoA levels, mitochondrial protein acetylation increases via either nonenzymatic acetylation of mitochondrial proteins or the activity of a yet unidentified mitochondrial acetyltransferases (MAT) (Fig. 6). This model, however, brings a significant conundrum: How could two feeding regimens (CR and HFD) that induce such different outcomes (insulin resistance vs. insulin sensitivity; increased vs. decreased life span) be

Figure 5. Role of mitochondrial protein acetylation and SIRT3 in the pathogenesis of the metabolic syndrome. SIRT3 deacetylates several mitochondrial proteins and increases fatty-acid oxidation and ATP production. In SIRT3KO mice or mice fed a high-fat diet, specific mitochondrial proteins become hyperacetylated, resulting in less energy expenditure and lower fatty-acid oxidation levels, which both contribute to insulin resistance, obesity, and increased inflammation. Similarly, humans with a unique single-nucleotide polymorphism in the SIRT3 gene have reduced SIRT3 enzymatic efficiency and could have increased risk of developing the metabolic syndrome.

characterized by enhanced mitochondrial protein acetylation? We propose that changes in SIRT3 protein expression represent the key difference between these two conditions (Fig. 5). As discussed above, SIRT3 expression is highly sensitive to the overall metabolic status of the cell, where caloric deprivation (e.g., fasting, CR, exercise) results in increased SIRT3 expression (Shi et al. 2005; Lanza et al. 2008; Palacios et al. 2009; Hirschey et al. 2010), whereas caloric excess (e.g., HFD feeding) results in reduced SIRT3 expression (Palacios et al. 2009; Hirschey et al. 2011). Additionally, SIRT3 protein expression is sensitive to aging, where reduced protein expression is observed in aged human populations (Lanza et al. 2008) as well as in aged mice (M Hirschey and E Verdin, unpubl.). Based on our study of individual targets of SIRT3, we also note that global mitochondrial protein acetylation does not always correlate with the acetylation status of individually relevant targets. For example, the specific SIRT3 target LCAD becomes deacetylated during fasting in WT mice when global

mitochondrial protein acetylation is increased and SIRT3 expression is also high (Hirschey et al. 2010). However, LCAD becomes hyperacetylated during HFD feeding in WT mice when global mitochondrial protein acetylation is high but SIRT3 expression is low (Hirschey et al. 2011). Thus, we propose as a working model that SIRT3 plays a crucial role in determining the fate of mitochondrial protein acetylation and whether acetylation results in an overall beneficial or detrimental metabolic effect (Fig. 5).

FUTURE QUESTIONS

Further work will be required to identify how protein acetylation and deacetylation by SIRT3 are balanced in the mitochondria. Because histone acetyltransferases regulate protein acetylation in the nucleus, a MAT could acetylate proteins in the mitochondria (Fig. 6). Acetyl-CoA levels could also directly regulate protein acetylation via

Figure 6. Regulation of mitochondrial protein acetylation. Acetyl-CoA levels rise during fasting and calorie restriction. HFD feeding increases fatty-acid oxidation and could also lead to increased acetyl-CoA levels. Increased mitochondrial acetyl-CoA levels probably increase mitochondrial protein acetylation, either nonenzymatically or via the activity of a yet to be identified mitochondrial acetyltransferase (MAT). Hyperacetylation of mitochondrial proteins inhibits fatty-acid oxidation, at least in part, via inhibition of LCAD activity. This process could function as a negative-feedback inhibition loop suppressing fatty-acid oxidation when acetyl-CoA levels become elevated. Under prolonged fasting conditions or under CR, however, SIRT3 expression is activated and deacetylates LCAD and other enzymes in the fatty-acid oxidation pathway. This activity suppresses the negative-feedback loop imposed by protein hyperacetylation and allows fatty-acid oxidation to proceed in the presence of elevated acetyl-CoA levels. Aberrant SIRT3 down-regulation under high-fat feeding conditions is associated with mitochondrial protein hyperacetylation, accumulation of fatty-acid oxidation intermediary products, and the induction of insulin resistance.

nonenzymatic acetylation. Interestingly, increased fatty-acid oxidation during calorie restriction or fasting leads to increased mitochondrial acetyl-CoA concentrations and therefore increased mitochondrial protein acetylation; however, SIRT3 expression and activity are induced under the same conditions and lead to a compensatory decrease in mitochondrial protein acetylation. HFD feeding is also associated with increased fatty-acid oxidation and increased mitochondrial acetyl-CoA levels, and SIRT3 expression is induced early after initiation of high-fat feeding (Hirschey et al. 2011). However, in contrast to CR or fasting-induced SIRT3 expression, chronic HFD feeding suppresses SIRT3 expression, increases mitochondrial protein acetylation, and ultimately reduces fatty-acid oxidation.

We conclude that mitochondrial protein acetylation is a critical posttranslational modification, whose regulation by SIRT3 is necessary to maintain metabolic health in mice and humans. Future studies will examine the therapeutic potential of manipulating SIRT3 expression or activity in ameliorating manifestations of the metabolic syndrome.

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