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PRINCIPAL INVESTIGATOR: Pere Puigserver, Ph.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute, Inc.
450 Brookline Avenue
Boston, MA 02215-5450

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pere_puigserver@dfci.harvard.edu

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14. ABSTRACT

We have tested the hypothesis that acetylation of PGC-1α by GCN5 and associated proteins control hepatic glucose production. Here, a summary of the accomplished tasks is provided. Task 1, a detailed analysis of the role of Pc3 and WDR18 on GCN5-mediated transcription on gluconeogenic genes has been performed. Task 2, we have confirmed that Pc3 and WDR18 are part of the PGC-1α/GCN5 complex but are not required for its assembly. Task 3, we identified the major acetylation sites on PGC-1α that account for the pro-gluconeogenic effects. Task 4, we have identified the mechanisms whereby GCN5 suppress PGC-1α activity, in part through Pc3 and WDR18. Task 5, we have analyzed the suppressive effects of GCN5 on hepatic glucose metabolism. Task 6, we have identified that additional GCN5 interacting proteins including the Sirtuin 6 activate GCN5 and suppress hepatic glucose output. The final conclusions are that PGC-1α acetylation is a key chemical switch that in response to fed/fasting controls liver metabolism.

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Abstract (160 words)
We have tested the hypothesis that acetylation of PGC-1α by GCN5 and associated proteins control hepatic glucose production. Here, a summary of the accomplished tasks is provided. Task 1, a detailed analysis of the role of Pc3 and WDR18 on GCN5-mediated transcription on gluconeogenic genes has been performed. Task 2, we have confirmed that Pc3 and WDR18 are part of the PGC-1α/GCN5 complex but are not required for its assembly. Task 3, we identified the major acetylation sites on PGC-1α that account for the pro-gluconeogenic effects. Task 4, we have identified the mechanisms whereby GCN5 suppress PGC-1α activity, in part through Pc3 and WDR18. Task 5, we have analyzed the suppressive effects of GCN5 on hepatic glucose metabolism. Task 6, we have identified that additional GCN5 interacting proteins including the Sirtuin 6 activate GCN5 and suppress hepatic glucose output. The final conclusions are that PGC-1α acetylation is a key chemical switch that in response to fed/fasting controls liver metabolism.

Introduction
Homeostatic mechanisms in mammals, including humans, function to maintain blood glucose levels within a narrow range in response to hormones and nutrient fluctuations. For example, high stress and intense exercise conditions combined with food deprivation make soldiers very vulnerable to changes in blood glucose levels. Glucose homeostasis is dysregulated in metabolic diseases such as obesity and diabetes with high increased incidence in the US population. We study a biochemical process that controls blood glucose levels through control of hepatic glucose synthesis. This regulatory control is achieved by a chemical modification –acetylation- of the PGC-1α metabolic transcriptional coactivator (Rodgers et al. 2005) (Lerin et al. 2006) (Rodgers and Puigserver, 2007). The overall purpose and scope of this Research Proposal is to decipher how several proteins, Pc3, WDR18 and Sirt6, that control the enzymatic activity of the PGC-1α GCN5 Acetyl Transferase regulate PGC-1α acetylation and its effects on glucose metabolism. We are using biochemical and physiological approaches, both in cell culture and mouse models, to precisely identify the key acetylation sites on PGC-1α that are required and sufficient to modulate blood glucose levels. These findings have strong implications for the basic pathways of energy homeostasis, diabetes and metabolic diseases and will certainly benefit performance of personnel in the army that work in conditions of high stress. It is important to highlight that PGC-1α chemical acetylation is directly controlled by two enzymes: GCN5 and SIRT1; this strengths the possibility to use small molecules to target the catalytic activity of these proteins to manipulate PGC-1α acetylation and normalize high glucose levels in diabetic patients. The fact that PGC-1α does not contain an enzymatic activity makes it difficult to screen for drugs that affect its function, however because GCN5 and SIRT1 contain acetyl transferase and deacetylase activity, respectively, they could be potential targets for therapeutic interventions. In addition, in the last period of funding

Body

In this body section of this Final Report, we will summarize all the experimental data obtained during the funding period. Since most of the data has been previously reported in the annual reports, we will provide a general description of these experiments. In addition, we will report more detail information about the research progress in the last year. Briefly, we have succesfully accomplished the goals that were originally proposed in this application.

Task 1. Analysis of two novel proteins in the GCN5 complex (Pc3 and WDR18) that strongly repress PGC-1α glucose production function. Importantly, WDR18 is regulated by nutritional status in diabetic models. (Months 1-24).

• *Genetic Approaches using siRNAs. Design and test specific siRNAs for Pc3 and WDR-18 proteins of the GCN5 complex. (Months 1-12).* 1. Generation of specific siRNAs for Pc3 (Pc3 according to the new nomenclature has been re-named CCDC101). 2. Generation of specific siRNAs for WDR-18.

• *Test of different siRNAs (for CCDC101 and WDR-18) on PGC-1α function. Transcriptional analysis using luciferase reporter systems. (Months 12-24).* 1. Effects of CCDC101 on PGC-1α transcriptional activity. 2. Effects of WDR-18 on PGC-1α transcriptional activity.

Task 2. Identification of the mechanisms of PGC-1α's repression by acetylation and interactions with GCN5-associated proteins Pc3 and WDR18 (Months 12-36).

• *Analysis of physical interaction between PGC-1α, transcriptional regulatory proteins that are known to control gluconeogenic genes and two novel proteins in the GCN5 protein complex (Pc3 and WDR18). (Months 12-36).*

• *Chromatin Immunoprecipitation Analysis. Determination of PGC-1α chromatin occupancy on gluconeogenic genes depending on GCN5 activation modulated by association with Pc3 and WDR-18 (Months 18-36).*

Task 3. Identification of the important functional acetylated lysines involved in PGC-1α repression. (Months 1-24).

• *Generation of PGC-1α lysine mutants. Site-directed mutagenesis methodology. (Months 1-6).*

• *Transcriptional analysis of PGC-1α acetylated mutants and regulation by GCN5 associated proteins CCDC101 and WDR-18. Analysis using luciferase reporter systems (Months 6-24).*

Task 4. Identification of the mechanisms of PGC-1α's repression by acetylation and interaction with GCN5-associated proteins Pc3 and WDR-18. (Months 12-36).

• *Gene expression analysis by Northern Blot of gluconeogenic and glycolytic genes in cultured hepatocytes. Requirements of Pc3 and WDR18 by using specific siRNAs (Months 24-27).*

• *Glucose production analysis. Glucose measurement determinations from hepatocyte's – infected with adenoviruses encoding PGC-1α acetylation mutants- culture medium. Requirement of Pc3 and WDR18 by using specific siRNAs (Months 24-30).*

acetylation mutant will be more active on secreting glucose, and Pc3 –positively- and Wdr18 – negatively- will affect PGC-1a wild type but not mutant.

Task 5. Analysis of PGC-1α acetylation mutants and requirement of GCN5 to control gluconeogenic/glycolytic fatty acid oxidation and mitochondrial gene expression in the liver of obese/diabetic mice. (Months 30-48).

• *Analysis of GCN5 requirement on the hepatic gluconeogenic/glycolytic, fatty acid oxidation and mitochondrial gene expression in the liver of obese/diabetic mice. Use of mice injected with adenoviruses encoding GCN5 siRNA. (Months 30-48).*
• *Analysis of GCN5 effects on hepatic gluconeogenic/glycolytic, fatty acid oxidation and mitochondrial gene expression depending on PGC-1α in the liver of obese/diabetic mice. Use of mice injected with adenoviruses encoding GCN5 siRNA and PGC-1α. (Months 30-48).*
• *Analysis of PGC-1α acetylation to control hepatic gluconeogenic/glycolytic gene expression, fatty acid oxidation and mitochondrial gene expression. Use of mice injected with adenoviruses encoding PGC-1α and PGC-1α acetylation mutants. (Months 30-48).*

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Task 6. Physiological and metabolic analysis of GCN5 requirement and PGC-1α acetylation to control glucose production in liver of obese/diabetic mice. (Months 30-48).

• *Analysis of GCN5 and PGC-1α acetylation on blood glucose and insulin levels. Measurement of blood glucose and insulin levels in obese/diabetic mice infected with adenoviruses encoding GCN5 siRNA, PGC-1α siRNA, PGC-1α and GCN5. (Months 30-48).*
• *Analysis of GCN5 and PGC-1a acetylation on hepatic glucose production in obese/diabetic mice. Pyruvate tolerance tests in mice infected with adenoviruses encoding GCN5 siRNA, PGC-1α siRNA, PGC-1α and GCN5. (Months 30-48).*
• *Analysis of GCN5 and PGC-1α acetylation on insulin signaling in obese/diabetic mice. Glucose and insulin tolerance tests in mice infected with adenoviruses encoding GCN5 siRNA, PGC-1α siRNA, PGC-1α and GCN5. (Months 30-48).*

As described in the previous report, we have had problems in generating the GCN5 siRNA and caused problems to keep the time lines. However, we are now in a position that we are going to test these siRNAs in the livers of control and diabetic models. Unfortunately, these experiments are going to be performed in the near future. If these results form part of a publication we will include the DOD as part of the funding.

While the generation of GCN5 siRNAs have been difficult, in the frame of this task we have performed experiments that were proposed in the last scientific technical report regarding the role of SIRT6 as part of the GCN5 complex and associated proteins. SIRT6 strongly interacts with GCN5 protein and increases its acetyl transferase activity in different substrates including PGC-1α and histones. Importantly, we have performed additional experiments in primary hepatocytes as well as in mice showing that Sirt6 controls hepatic glucose production through effects on gluconeogenic genes. Thus, in primary hepatocytes both gain- and loss-of-function of Sirt6 results in changes in gluconeogenic genes and this translates into different rates of glucose production. In mice, we have performed experiments using Sirt6 overexpression using tail vein injections of adenoviruses encoding for this protein –as a control we used a Sirt6 that is catalytically inactive and does not affects GCN5-induced PGC-1α acetylation. As predicted based on the effects of Sirt6 on GCN5, hepatic expression of Sirt6 causes suppression of gluconeogenic genes and mice became hypoglycemic, however these metabolic effects were not observed with Sirt6 mutant. Together, these experiments support the main hypothesis of this grant revealing that PGC-1a acetylation in liver is a major regulator of glucose metabolism.

Key Research Accomplishments

Task 1.- • We have accomplished a detailed analysis of the role of Pc3 and WDR18 on GCN5-mediated transcription on gluconeogenic genes. These two proteins and the new addition of Sirt6, form a group of regulators of GCN5 that through acetylation of PGC-1a control genes involved in the metabolic pathway of glucose production.

Task 2.- • We have confirmed that Pc3, WDR18 and SIRT6 are part of the PGC-1α/GCN5 complex that control expression of metabolic genes and glucose production.

Task 3.- • We have identified the major acetylation sites on PGC-1α that account for the pro-gluconeogenic effects. It turns out that 13 lysines are acetylated by GCN5; how precisely all these sites control acetylation is unclear and will be the subject of investigation in the future.

Task 4.- • We have identified the mechanisms whereby GCN5 suppress PGC-1α activity, in part through Pc3, WDR18 and Sirt6.

Task 5.- • We have assess the suppressive effects of GCN5 and interacting proteins including Sirt6 on hepatic glucose metabolism in cell culture systems using hepatoma and primary cultured cells.

Task 6.- • We have shown that hepatic expression of GCN5 and Sirt6 are sufficient to decrease blood glucose levels in the fasted state. Moreover, both proteins also sufficient to decrease hepatic glucose production in life mice.

Reportable Outcomes

Part of the results reported here have been used or discussed in the following publications.

1. C. Lerin, J.T. Rodgers, D. Kalume, S.H. Kim, A. Pandey and **P. Puigserver.** GCN5 Acetyl Transferase Complex Controls Glucose Metabolism through Transcriptional Repression of PGC-1a. *Cell Metab.* 2006 Jun;3(**6**):429-38.

2. M. Lagouge, Argmann, C., Gerhart-Hines, Z., Meziane, H., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., Lerin, C., Geny, B., Laakso, M., **Puigserver, P**., Auwerx, J. Resveratrol promotes mitochondrial biogenesis and protects against metabolic disease through activating SIRT1 and PGC-1a activity. *Cell.* 2006 127(6):1109-22.

3. Z. Gerhart-Hines, J.T. Rodgers, O. Bare, C. Lerin, S.H. Kim, R. Mostoslavsky, F.W. Alt, Z. Wu and **P. Puigserver.** Metabolic Control of Mitochondrial Function and Fatty Acid Oxidation Through PGC-1a/SIRT1. *EMBO J.* 2007 26, 1913-1923.

4. J.T. Rodgers and **P. Puigserver**. Fasting-Dependent Glucose and Lipid Metabolic Response through Hepatic SIRT1. *Proc. Natl. Acad. Sci. USA.* 2007 104(31) 12861-66.

5. A. Coste , J.F. Louet , M. Lagouge , C. Lerin , M. Antal , H. Meziane , K. Schoonjans , **P. Puigserver**, B. O'Malley and J. Auwerx. The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1alpha. *Proc. Natl. Acad. Sci. USA.* 2008 105(44):17187-92.

6. G. Suchankova, L. Nelson, Z. Gerhart-Hines, M. Kelly, M.S. Gauthier, A.K Saha, Y. Ido, **P. Puigserver** and N.B. Ruderman. Concurrent regulation of AMP-activated protein kinase and SIRT1 in mammalian cells. *Biochem. Biophys. Res. Commun*. 2009 378:836-41.

7. C. Canto, Z. Gerhart-Hines, J.N. Feige, M. Lagouge, J.C. Milne, L. Noriega, P.J. Elliott, **P. Puigserver** and J. Auwerx. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature*. 2009 458(7241):1056-60.

8. T.J. Kelly, C. Lerin, W. Haas, S.P. Gygi, and **P. Puigserver**. GCN5-Mediated Transcriptional Control of the Metabolic Coactivator PGC-1b through Lysine Acetylation. *J. Biol. Chem.* 2009 284(30):19945-52.

9. J.T. Rodgers, W. Haas, S.P. Gygi, and **P. Puigserver**. Cdc2-like Kinase 2 is an insulin regulated suppressor of Hepatic Gluconeogenesis. *Cell Metabolism*, 2010 11(1), 23-34.

10. J. T. Rodgers, C. Lerin, Z. Gerhart-Hines and **P. Puigserver.** Metabolic Adaptations Through PGC-1α and SIRT1 Pathways. *FEBS Letters*. 582: 46-53. 2008.

11. J. Dominy, Y. Lee, Z . Gerhart-Hines and **P. Puigserver**. Nutrient-dependent regulation of PGC-1alpha's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5. *Biochim Biophys Acta*. 1804(8):1676-83, 2010.

Additionally, these results have been partially presented in the following conferences or seminars as invited speaker.

2006.
- Beth Israel Hospital/Harvard Medical School. Division of Endocrinology. Boston.
- Gordon Conferences on Biology of Aging. Ventura, CA.
- Dana-Farber Cancer Institute/Harvard Medical School. Boston.
- The Molecular biology of Obesity and Bone Remodeling. Cantoblanco Workshops on Biology. Madrid, Spain.
- 8th Annual Conference. Frontiers in Diabetic Research. Molecular Mediators of Metabolic Homeostasis. Columbia University, College of Physicians and Surgeons. New York.
- 6th Nestle Nutrition Conference. Diet, genes and transcription factors linked to the Metabolic Syndrome. Mexico.
- University of Texas Health Science Center at San Antonio. Dept. of Physiology. San Antonio, TX.

2007.
- Keystone Symposia. Obesity: Peripheral and Central Pathways Regulating Energy Homeostasis. Keystone, Colorado.
- University of Utah. Department of Biochemistry. Salt Lake City, UT.
- NIH/NIDDK. Bethesda, MD.
- The Paul F. Glenn Symposium on Aging. Harvard Medical School.
- American Aging Association- 36th Annual Meeting. San Antonio, TX.

- FASEB Summer Research Conferences. Glucose Transporter Biology. Snowmass Village, CO.
-17th Annual Irwin M. Arias, MD Symposium. American Liver Foundation. Boston, MA.
- Nuclear Receptors in Liver and Digestive Diseases: A Research Workshop. Rockville, MD.
- University of Cincinnati. Genomic Research Institute. Cincinnati, OH.

2008.

- Keystone Symposia. Diabetes Mellitus, Insulin Action and Resistance. Breckenridge, Colorado.
- Keystone Symposia. Metabolic Pathways of Longevity. Copper Mountain, Colorado.
- Keystone Symposia. Nuclear Receptors: Orphan Brothers, Whistler, Canada.
- American Society of Biochemistry and Molecular Biology. San Diego.
- Molecular Mechanisms Modulating Skeletal Mass and Function. The Banbury Center, Cold Spring Harbor, New York.
- 8th Symposium Molecular and Physiological Aspects of Type 2 Diabetes and Obesity. Nobel Forum, Wallenbergsalen. Karolinska Institutet and Stockholm University. Stockholm, Sweden.
- Baylor College of Medicine. Department of Molecular and Cellular Biology. Houston, TX.
- Case Western Reserve University. Department of Genetics, Cleveland, OH.
- University of Michigan Medical School. Department of Molecular and Integrative Physiology. Ann Harbor, MI.
- University of California, Los Angeles. Department of Molecular Medicine and Pharmacology. Los Angeles, CA.
- University of Southern California. Department of Pharmacology and Pharmaceutical Sciences. Los Angeles, CA.
- Colloquium on the Biology of Aging. Marine Biological Laboratory, Woods Hole, MA.
- Wyeth Symposium on Metabolic Dysregulation. Boston University, Department of Pharmacology and Experimental Therapeutics. Boston, MA.
- The Hamner Institutes for Health Sciences. Research Triangle Park, NC.
- University of Geneva. Faculty of Medicine. Department of Cell Physiology and Metabolism. Geneva, Switzerland.

2009.

- Symposium on Biological Complexity: Processes of Aging. Salk Institute for Biological Sciences, Fondation IPSEN Nature and Nature Reviews Molecular Cell Biology. San Diego, CA.
- Gordon Research Conference. Biology of Aging. Ventura, CA.
- Deuel Conference on Lipids Program. Borrego Springs, CA.
- Third Annual Scientific Meeting of Cardio-Diabetes Study Group. Tokyo, Japan.
- Biomedicum Center. University of Helsinki. Finland.
- Columbia University College of Physicians & Surgeons, Naomi Berrie Diabetes Center, Department of Medicine. New York.
- 14th International Biochemistry of Exercise. Conference-Muscle as Molecular and Metabolic Machines. University of Guelph. Canada.
- Minisymposium "Models of Human Diseases IV". ETH, Zurich, Switzerland.
- Ohio State University. Dept. of Molecular Virology, Immunology and Medical Genetics. Columbus, OH.
- Symposium on Aging&Healthy Lifespan Conference. Harvard Medical School, Boston.
- ICC (Idibell Cancer Conferences on Sirtuins. Barcelona, Spain.
- CNIO Cancer Conference: The Energy of Cancer. Madrid, Spain.

- EPFL (Ecole Polytechnique Federale de Lausanne) and Merck Research Laboratories. 1st Metabolic Disease Symposium on Insulin resistance. Saas Fee, Switzerland.

2010.

- Harvard Medical School, Dept. of Pathology.
- Yale School of Medicine. Dept. of Comparative Medicine and Physiology.
- Whitaker Cardiovascular Institute. Boston University Medical Center
- American Diabetes Association. Annual Conference.
- IRB (Biomedical Research Institute). University of Barcelona, Spain
- LabLinks: Cellular Metabolism and Cancer. Broad Institute, Cambridge, MA
- University of Illinois at Urbana/Champaigne. Department of Molecular and Integrative Physiology.
- University of Madison, Wisconsin. Department of Biomolecular Chemistry.

Personnel Supported by this Grant

Joseph Rodgers, Ph.D.	Yoonjin Lee
Sharon Blättler, Ph.D.	Zachary Gerhart-Hines
John Dominy, Ph.D.	Thomas Cunningham
Christine Chin	Pere Puigserver, Ph.D.

Conclusion

We have summarized in this Final Report the key conclusions and results that have been obtained during this Award. We have accomplished all the original tasks that were proposed, except for the experiments using siRNA GCN5 that will be performed in a near future. However, we have complement these experiments with a detailed analysis of Sirt6, as a potent interactor and activator of GCN5, on gluconeogenesis both in primary hepatocytes as well as in mice. Taken together, the results obtained during this Award are entirely consistent with our overall hypothesis that PGC-1α acetylation is a key chemical modification that control glucose and lipid metabolism. As part of our laboratory goals we will continue our studies to further show the functionality of this pathway in different metabolic conditions in vivo and with the identification of novel components. Our long-term aim is to provide the basis for therapeutical targets that can be use to prevent glucose and lipid dysregulation that occur in conditions of stress or overnutrition that have a significant impact in the personnel army.

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Lerin, C., Rodgers, J.T., Kalume, D.E., Kim, S.H., Pandey, A., and Puigserver, P. 2006. GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. *Cell Metab* **3**(6): 429-438.

P. Puigserver, J. Rhee, J. Donovan, C.J. Walkey, J.C. Yoon, F. Oriente, Y. Kitamura, J. Altomonte, H. Dong, D. Accili and B.M. Spiegelman. Insulin-Regulated Hepatic Gluconeogenesis Through FOXO1/PGC-1a Interacction. *Nature* **423**, 550-5, 2003.

Rodgers, J.T. and Puigserver, P. 2007. Fasting-dependent glucose and lipid metabolic response through hepatic Sirt1. Proc. Natl. Acad. Sci. **104**: (12861-6).

Rodgers, J.T., Lerin, C., Haas, W., Gygi, S.P., Spiegelman, B.M., and Puigserver, P. 2005. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* **434**(7029): 113-118.

Appendices
Attached.

GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1α

Carles Lerin,[1] Joseph T. Rodgers,[1] Dario E. Kalume,[2] Seung-hee Kim,[1] Akhilesh Pandey,[2] and Pere Puigserver[1,*]

[1] Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205
[2] McKusick-Nathans Institute for Genetic Medicine and the Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205
*Correspondence: ppuigse1@bs.jhmi.edu

Summary

Hormonal and nutrient regulation of hepatic gluconeogenesis mainly occurs through modulation of the transcriptional coactivator PGC-1α. The identity of endogenous proteins and their enzymatic activities that regulate the functions and form part of PGC-1α complex are unknown. Here, we show that PGC-1α is in a multiprotein complex containing the acetyltransferase GCN5. PGC-1α is directly acetylated by GCN5 resulting in a transcriptionally inactive protein that relocalizes from promoter regions to nuclear foci. Adenoviral-mediated expression of GCN5 in cultured hepatocytes and in mouse liver largely represses activation of gluconeogenic enzymes and decreases hepatic glucose production. Thus, we have identified the endogenous PGC-1α protein complex and provided the molecular mechanism by which PGC-1α acetylation by GCN5 turns off the transcriptional and biological function of this metabolic coactivator. GCN5 might be a pharmacological target to regulate the activity of PGC-1α, providing a potential treatment for metabolic disorders in which hepatic glucose output is dysregulated.

Introduction

Maintenance of glucose homeostasis in mammals is accomplished through a tight regulation of glucose uptake by peripheral tissues and by production of glucose mainly in the liver. These metabolic processes constantly fluctuate in the normal physiology of feeding/fasting. After a meal, circulating blood insulin levels increase thereby blocking glucose production by the liver and inducing glucose uptake in tissues such as skeletal muscle and adipose. In contrast, in the fasted state glucagon signals to increase hepatic glucose output to maintain blood glucose levels within a narrow range, as neuronal and red blood cells utilize glucose as a main energetic fuel. In pathological states such as diabetes, insulin signaling is impaired, resulting in dysregulation of both glucose uptake in peripheral tissues and hepatic glucose output (Flier, 2004; Saltiel, 2001; Shulman, 2000). Therefore, efforts to gain insight into molecular mechanisms that control these metabolic processes are crucial to develop new therapeutic strategies.

A major contributor of fasting hyperglycemia in diabetes is an increased production of glucose by the liver. This metabolic process is largely controlled at the transcriptional level through hormonal and nutrient signals that activate key enzymes in the gluconeogenic pathway such as G6Pase and PEPCK. Several transcriptional components have been identified that control gene expression of these enzymes including transcription factors CREB, FOXO1, HNF4α, GR and C/EBPs (Herzig et al., 2001; Imai et al., 1990; Nakae et al., 2001; Rhee et al., 2003; Yoon et al., 2001). In this transcriptional regulatory network, the metabolic coactivator PGC-1α has been shown to modulate the gluconeogenic pathway in fasted and diabetic states through interaction with several of these transcription factors (Yoon et al., 2001). The coactivator PGC-1α also regulates genetic programs associated with oxidative metabolism and the nutritional food deprivation response (Knutti and Kralli, 2001; Lin et al., 2005). Notably, PGC-1α function is dysregulated in diabetic human muscle (Mootha et al., 2003) as well as in the liver of diabetic mice (Yoon et al., 2001). Specific knock-down of PGC-1α in the liver of diabetic mice is sufficient to normalize blood glucose levels (Koo et al., 2004). Furthermore, liver specific null mice present abnormal hepatic production of glucose and decreased blood glucose levels (Handschin et al., 2005).

Although in recent years signaling pathways and transcription factors that dictate the biological function of PGC-1α have been identified, the biochemical machinery that controls PGC-1α transcriptional function and how it is regulated remains largely unknown. Initial studies found that histone acetyltransferases p300 and SRC-1 bind to the N-terminal activation domain increasing the transcriptional activity after docking to transcription factors (Puigserver et al., 1999). Roeder's group have identified the TRAP/Mediator complex that binds to the C terminus of PGC-1α through direct interaction with the TRAP220 subunit and cooperates with p300 to augment transcriptional activity (Wallberg et al., 2003). However, the endogenous PGC-1α complex and the associated enzymatic activities that are being recruited to PGC-1α to modulate gene expression have remained elusive.

Hormonal signaling through glucagon and glucocorticoids increases PGC-1α gene expression via glucocorticoid receptor and the CREB/TORC pathway (Herzig et al., 2001; Koo et al., 2005; Yoon et al., 2001). In contrast, insulin decreases PGC-1α function through AKT-mediated phosphorylation of FOXO1 (Puigserver et al., 2003). In parallel to this hormonal regulation, we have recently found that a nutrient pathway also modulates the gluconeogenic activity of PGC-1α in the fasted state through the NAD^+-dependent deacetylase SIRT1 (Rodgers et al., 2005).

SIRT1 deacetylates PGC-1α in the fasted liver to control secretion of glucose. This might provide a metabolic adaptation in caloric restriction and how it could influence life span-dependent biological processes. A key question remaining from these studies was what is the endogenous acetyltransferase that, by functioning in opposite direction of SIRT1 deacetylase, would negatively impact PGC-1α function.

Taken together, our studies have identified the endogenous PGC-1α complex that contains at least two different protein complexes, GCN5 and TIP60 acetyltransferases. Moreover, we have found that GCN5 is the specific acetyltransferase for PGC-1α. Consistent with the activation effect of SIRT1 on PGC-1α through deacetylation, we show here that acetylation of PGC-1α by GCN5 is critical to block its activity through localization to nuclear inactive transcriptional domains. Finally, GCN5 represses PGC-1α ability to induce gluconeogenic gene expression and hepatic glucose secretion in cultured hepatocytes and in mice.

Results

Identification of GCN5 and TIP60 acetyltransferase complexes as part of the PGC-1α protein complex

We have previously reported that SIRT1 is a specific deacetylase of PGC-1α and is part of a nutrient signaling pathway in the food deprivation response (Rodgers et al., 2005). In order to identify the endogenous PGC-1α acetyltransferase, we purified PGC-1α protein holo-complexes from cultured hepatocytes as described in Experimental Procedures. Basically, nuclear extracts from Fao hepatocytes expressing a dually tagged Flag-HA-PGC-1α (FH-PGC-1α) were fractionated by FPLC through a gel filtration column. Fractions containing FH-PGC-1α were subjected to immunoaffinity chromatography. Polypeptides that copurified with PGC-1α were analyzed and sequenced by LC-MS/MS. A complete list of proteins that associated specifically with PGC-1α is provided in Figure S1 available in the Supplemental Data available with this article online. We purified two proteins with histone acetyltransferase (HAT) activity, GCN5 and TIP60. Both proteins have been shown to form large protein complexes (Ikura et al., 2000; Martinez et al., 2001), and most of their respective subunits were also present in the PGC-1α purified material (Figure 1A). We confirmed the specificity of some of these interactions by coimmunoprecipitation and Western blot analysis of different proteins from the GCN5 complex (GCN5, SAP130, $TAF_{II}31$, and PAF65β) and the TIP60 complex (RuvBL1) as well as KAP-1 (Figure 1B). To test for the presence of distinct PGC-1α complexes, we performed glycerol gradient ultracentrifugation using the purified PGC-1α fraction after immunoaffinity chromatography. GCN5 and $TAF_{II}31$ (GCN5 complex) separated in different fractions as compared to RuvBL1 (TIP60 complex) (Figure 1C), suggesting that PGC-1α is present in at least two different protein complexes associated with either GCN5 or TIP60 complexes. We next determined whether the interaction between GCN5 and TIP60 with PGC-1α was direct by performing in vitro binding assays with fragments of PGC-1α fused to GST and GCN5 or TIP60. GCN5 and TIP60 strongly interacted with the N-terminal activation domain of PGC-1α, a region that has been shown to interact with other acetyltransferases such as CBP/p300 and SRC-1 (Puigserver et al., 1999) (Figure 1D, top and middle panels). In the case of GCN5, the main interaction domain with PGC-1α was the bromodomain (Figure 1D, bottom panel), a region known to mediate interactions with proteins involved in regulation of transcription (Hassan et al., 2002). As expected, purified PGC-1α complexes contained strong HAT activity, mainly toward histone 3 and to a lesser extent histone 4 (Figure 1E), consistent with the presence of GCN5 and TIP60 in the PGC-1α complexes. Taken together, these results indicate that PGC-1α is part of large endogenous protein complexes in hepatic cells containing the GCN5 and TIP60 acetyltransferases subcomplexes.

GCN5 is the specific acetyltransferase for PGC-1α

We have previously shown that PGC-1α is highly acetylated at multiple lysines after treatment with the SIRT1 inhibitor nicotinamide (Rodgers et al., 2005), indicating that there must exist a protein acetyltransferase that directly acetylates PGC-1α. As both GCN5 and TIP60 were the only protein acetyltransferases identified in the complex, either could potentially acetylate PGC-1α. As shown in Figure 2A, expression of GCN5 (and its close homolog PCAF) induced acetylation of PGC-1α in HEK293 cells. In contrast, other acetyltransferases such as p300, SRC1—both previously shown to interact with PGC-1α (Puigserver et al., 1999; Wallberg et al., 2003)—as well as TIP60 did not induce acetylation of PGC-1α, while p300 did acetylate p53 (Figure 2A, bottom panel). Adenoviral-mediated expression of GCN5 in hepatic cells also acetylated PGC-1α (Figure S2). To determine whether PGC-1α acetylation was dependent on GCN5 acetyltransferase activity, we used a previously described catalytically inactive GCN5 mutant (Y621A/P622A) (Liu et al., 2003). As shown in Figure 2B, this GCN5 mutant (GCN5m) was unable to acetylate PGC-1α. To determine whether endogenous GCN5 was required for PGC-1α acetylation, we used a GCN5 RNAi construct to reduce GCN5 protein levels. As shown in Figure 2C, cells expressing GCN5 RNAi showed a decreased PGC-1α acetylation in response to nicotinamide. Furthermore, in vitro acetylation experiments using recombinant GCN5 and a fragment of PGC-1α (1–400 aa) showed that GCN5 directly acetylates PGC-1α (Figure 2D). Together, these data indicate that GCN5 is an endogenous acetyltransferase of PGC-1α.

GCN5 acetyltransferase is a repressor of PGC-1α transcriptional activity

To analyze the functional consequences of the interaction and acetylation of PGC-1α by GCN5, we performed transcriptional luciferase reporter assays using GAL4-DBD- PGC-1α. As shown in Figure 3A, GCN5 repressed the transcriptional activity of GAL4-DBD- PGC-1α by 35-fold. Conversely, p300, which did not acetylate PGC-1α (see Figure 2A), strongly activated GAL4-DBD- PGC-1α (Figure 3A and Puigserver et al., 1999). As a negative control, we showed that GCN5 did not repress but slightly activated GAL4-DBD-VP16 (Figure 3B). These results indicate that GCN5 repressed PGC-1α intrinsic transcriptional activity. To further determine whether GCN5 also repressed coactivation function on a transcription factor context, we used the hormone nuclear receptor HNF4α. As shown previously (Yoon et al., 2001), PGC-1α strongly activated HNF4α -dependent transcriptional activity. However, expression of GCN5 repressed this activity by 6-fold (Figure 3C). Similar results were also obtained using PEPCK promoter (Figure 3D), a previously described endogenous target of PGC-1α (Puigserver et al., 2003). Importantly, this GCN5 repression was dependent on its acetyltransferase

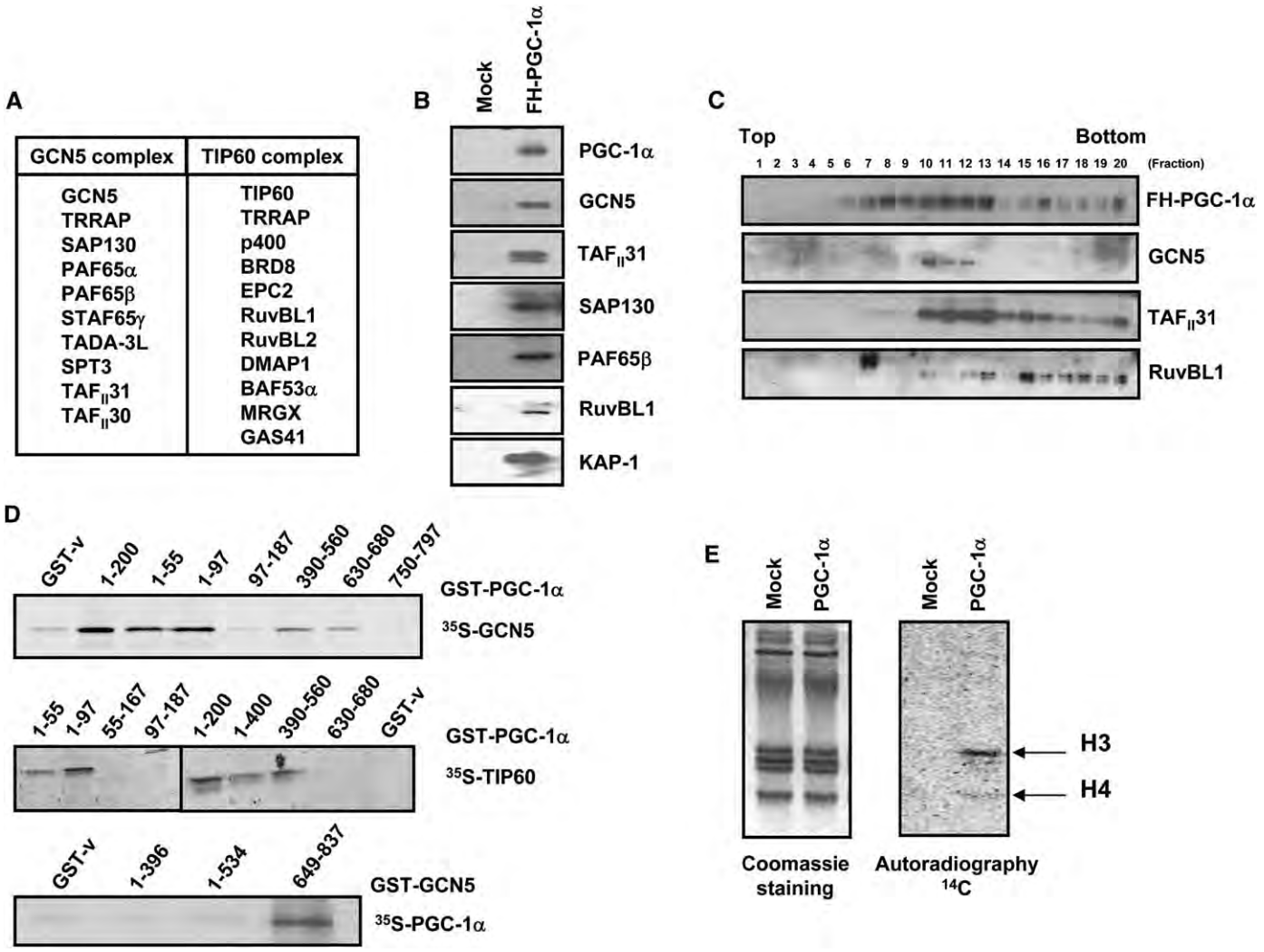

Figure 1. Physical association of PGC-1α with GCN5 and TIP60 acetyltransferase complexes

A) GCN5 and TIP60 complex subunits are associated with PGC-1α. Proteins identified by LC-MS/MS in the purified PGC-1α complexes belonging to either GCN5 or TIP60 complexes are listed.

B) Coimmunoprecipitation of PGC-1α with proteins identified in PGC-1α complexes. PGC-1α complexes were subjected to Western blot using the indicated antibodies.

C) Glycerol gradient analysis of PGC-1α complexes. PGC-1α complexes were separated on a glycerol gradient by ultracentrifugation and fractions were analyzed by Western blot using the indicated antibodies.

D) PGC-1α physically interacts with GCN5 and TIP60. GST- PGC-1α fusion proteins bound to sepharose beads were incubated with ^{35}S-methionine in vitro translated GCN5 or TIP60 proteins. After the binding reaction, precipitates were separated by SDS-PAGE and analyzed by autoradiography.

E) PGC-1α complexes contain histone acetyltransferase activity. Purified PGC-1α complexes were used in an in vitro histone acetylation reaction. Histones were separated by SDS-PAGE and analyzed by Coomassie staining and autoradiography.

activity, as the catalytically inactive GCN5 mutant did not significantly affect PGC-1α activity in luciferase assays (Figures 3C and 3D). Moreover, consistent with the inhibitory effect of GCN5 overexpression on PGC-1α transcriptional activity, decreasing GCN5 levels using GCN5 RNAi increased HNF4α activation by PGC-1α (Figure 3E). Taken together, these results indicate that GCN5 functions as a transcriptional repressor of PGC-1α and that this repression depends on its acetyltransferase activity.

GCN5 acetyltransferase induces translocation of PGC-1α to subnuclear domains

It has been previously shown that PGC-1α activates gluconeogenic gene expression by recruitment to the regulatory promoter regions through interaction with transcription factors (Puigserver et al., 2003). To determine the mechanism of GCN5 transcriptional repression, we performed ChIP experiments to analyze whether GCN5 could relocalize PGC-1α from endogenous gluconeogenic PEPCK and G6Pase promoters. As shown in Figure 4A, PGC-1α occupies these promoters and expression of GCN5 decreased its binding. This suggests that repression of gene transcription by GCN5-mediated acetylation of PGC-1α is through diminished binding to the active promoter sequence. We then examined whether PGC-1α nuclear localization was affected by performing immunofluorescence microscopy analysis in hepatocytes. As shown in Figure 4B, PGC-1α localized uniformly in the nucleus. However, when GCN5 was expressed both PGC-1α and GCN5 colocalized to

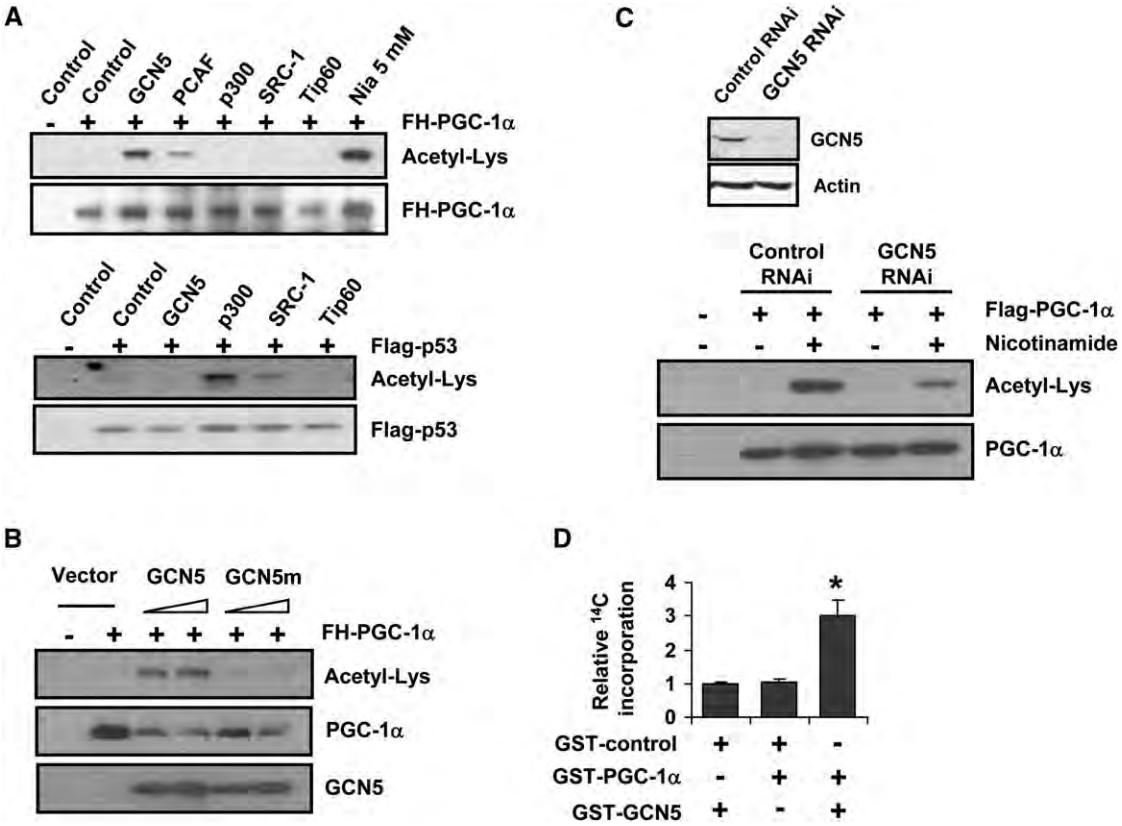

Figure 2. Acetylation of PGC-1α through GCN5 acetyltransferase activity

A) GCN5 induces PGC-1α acetylation. HEK293 cells were transfected with the indicated plasmids. FH- PGC-1α (top panel) or Flag-p53 (midle panel) were immunoprecipitated and acetylation levels were analyzed by Western blot.

B) A catalytically inactive GCN5 mutant does not acetylate PGC-1α. HEK293 cells were transfected with the indicated plasmids and PGC-1α acetylation levels were analyzed by Western blot.

C) GCN5 RNAi decreases PGC-1α acetylation. HEK293 cells were transfected with the indicated plasmids and treated with 10 mM nicotinamide for 16 hr. PGC-1α acetylation levels were analyzed by Western blot.

D) In vitro acetylation of PGC-1α by GCN5. Recombinant GST- PGC-1α (1–400 aa) and GST-GCN5 (352–837 aa) proteins were used in an in vitro acetylation assay. Values represent means ± SEM of three independent experiments performed in duplicate; *p < 0.0001, versus PGC-1α.

distinctive nuclear punctuate pattern (Figure 4B, middle panel). This new nuclear foci distribution of PGC-1α depended on GCN5 acetyltransferase activity as GCN5m did not promote this nuclear distribution (Figure 4B, bottom panel). Moreover, PGC-1α localization to these nuclear foci required the C-terminal domain of PGC-1α, as GCN5 did not relocalize a PGC-1α deletion (1–570 aa) (Figure 4C). Interestingly, it was previously reported that in certain cell types PGC-1α is localized to nuclear speckles with splicing factors (Monsalve et al., 2000). However, after extensive microscopic analysis we found that PGC-1α -GCN5 containing nuclear punctuate structures did not colocalize with nuclear speckles containing splicing factors or snRNPs (SC35, Sm, U1snRNP70K, and U2-B″) (Lamond and Spector, 2003), PML bodies (SP100) (Hodges et al., 1998), nucleolar organizing regions (fibrillarin) (Bubulya et al., 2004), polycomb bodies (Pc2) (Kagey et al., 2003), or matrix-associated deacetylase bodies (SMRT) (Downes et al., 2000) (Figure S3). Instead, we found that PGC-1α largely colocalized with a nuclear corepressor for hormone nuclear receptors, RIP140 (Christian et al., 2004; Zilliacus et al., 2001) (Figure 4D), suggesting that these nuclear structures represent spatial sites to which regulators of gene transcription are recruited. An interesting possibility is that these nuclear foci could represent structures that contain repressive chromatin similar to the *Drosophila* insulators (Capelson and Corces, 2004).

GCN5 acetyltransferase blocks PGC-1α-induced gluconeogenic gene expression and hepatic glucose secretion

We have recently shown that deacetylation of PGC-1α through SIRT1 deacetylase is part of a nutrient pathway in response to food deprivation and is required to activate hepatic gluconeogenic PGC-1α target genes such as G6Pase and PEPCK (Rodgers et al., 2005). The fact that GCN5 acetylates and represses PGC-1α prompted us to investigate the effects of GCN5 on PGC-1α -dependent gluconeogenic gene expression. Hepatocytes were infected with adenoviruses expressing PGC-1α and GCN5 and mRNA levels for G6Pase and PEPCK were analyzed. Consistent with both the effects of SIRT1 on PGC-1α (Rodgers et al., 2005) as well as the transcriptional assays (see Figure 3), expression of GCN5 largely decreased PGC-1α -induction of gluconeogenic genes (Figure 5A). Interestingly, the repression of PGC-1α by GCN5 also affected the mitochondrial genes cytochrome c and β-ATP-synthase (Figure 5A). These results indicate that GCN5 acts as a general repressor of PGC-1α. Notably, these effects were mediated to a

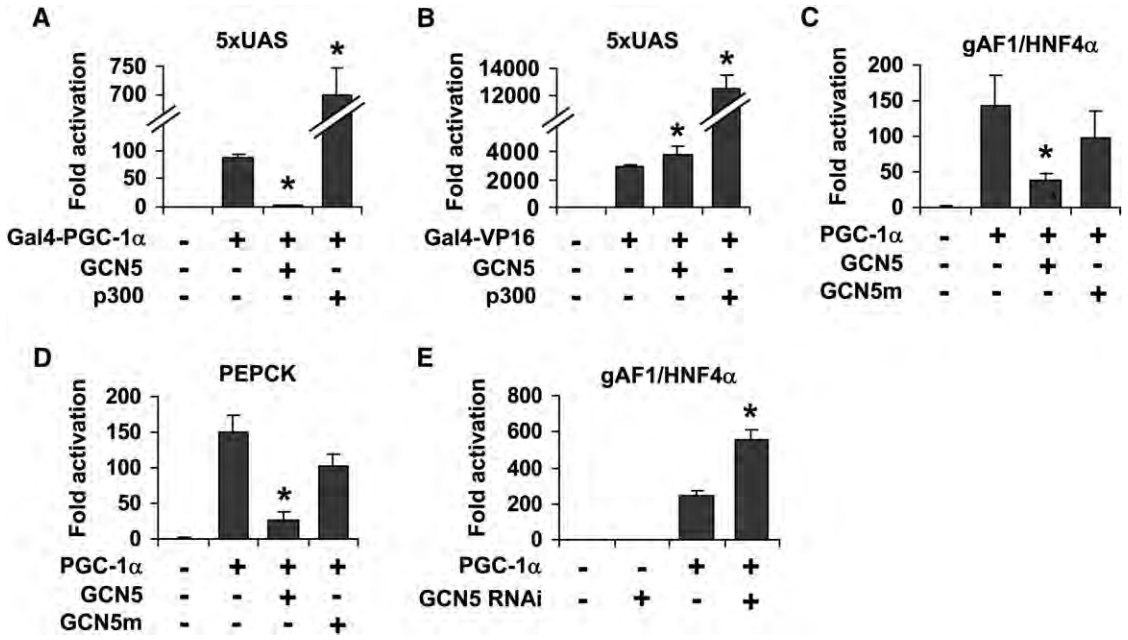

Figure 3. Repression of PGC-1α transcriptional activity through GCN5 acetyltransferase activity

HEK293 cells were transfected with the indicated plasmids together with a 5xUAS reporter luciferase construct (**A** and **B**), HNF4α and gAF1 reporter luciferase construct (**C** and **E**), or HNF4α and PEPCK reporter luciferase construct (**D**). Luciferase activity was measured 36 hr after transfection. Values represent means ± SEM of at least three independent experiments performed in quadruplicate; *$p < 0.005$, versus GAL4-PGC-1α (**A**), GAL4-VP16 (**B**), or PGC-1α (**C**, **D**, and **E**).

large extent through GCN5 acetyltransferase activity as GCN5m only slightly repressed PGC-1α -target genes. We next determined whether changes in gluconeogenic gene expression reflected increases in hepatic glucose production. Overexpression of PGC-1α increased glucose secretion by 78%, compared to control. Expression of GCN5 greatly reduced the ability of PGC-1α to induce glucose secretion (11% of induction) compared to GCN5m (40% of induction) (Figure 5B). We next tested whether decreasing endogenous GCN5 levels could affect the expression of gluconeogenic genes. Adenoviral-mediated expression of GCN5 RNAi in hepatic cells decreased both mRNA and protein levels (Figure S4). As expected, knock down of GCN5 increased PGC-1α's ability to induce gluconeogenic genes PEPCK and G6Pase (Figure 5C), correlating with an elevated induction of glucose production by PGC-1α (Figure 5D). Taken together, these results indicate that GCN5, through its acetyltransferase activity, is sufficient to repress PGC-1α function on endogenous genes and to abolish glucose secretion in hepatic cultured cells.

GCN5 acetyltransferase controls blood glucose levels and gluconeogenic gene expression in the mouse liver during fasting

Finally, to determine whether the PGC-1α/GCN5 pathway was also operative in live animals, we delivered adenovirus encoding GCN5 via tail injection to the mouse liver. As shown in Figure S5A, hepatic GCN5 mRNA levels were moderately elevated compared to endogenous GCN5 mRNA levels. As expected from previous experiments (Rodgers et al., 2005), ectopic expression of GCN5 led to an increase in endogenous PGC-1α acetylation levels (Figure S5B).To determine if GCN5 expression affected gluconeogenic gene expression, we analyzed mRNA levels of G6Pase and PEPCK (Figure 6A). Although in the fed state, expression of both genes was not significantly changed, GCN5 partially blocked the induction of PEPCK and G6Pase expression after a 16 hr fasting period. Correlative with these effects on gluconeogenic gene expression, fasted blood glucose levels were reduced in mice expressing GCN5 (Figure 6B). We next analyze whether this decrease in blood glucose levels was due to a lower gluconeogenic activity using a pyruvate tolerance test. As seen in Figure 6C, blood glucose levels were consistently lower in GCN5 expressing mice at different time points following pyruvate administration, indicating that hepatic conversion of pyruvate into glucose was partially inhibited by GCN5 expression. Taken together, these results show that changes of GCN5 levels in hepatocytes are sufficient to control glucose homeostasis in mice.

Discussion

A precise regulation of the genes that control glucose production is required to maintain blood glucose levels in food deprivation states. This control is markedly dysregulated in both type I and II diabetes and leads to persistent hyperglycemia. In this complex hormonal and nutrient regulation, PGC-1α is controlled and recruited to regulate gene expression of key hepatic gluconeogenic enzymes (Puigserver et al., 2003; Rhee et al., 2003). In this molecular context and using a biochemical approach based on the purification of PGC-1α endogenous protein complexes, we have identified GCN5 acetyltransferase as a component of the PGC-1α transcriptional pathway that negatively regulates expression of gluconeogenic genes through direct acetylation and nuclear localization of PGC-1α.

The experimental data presented here show the endogenous protein components of the PGC-1α complex. Notably, two different protein complexes have been identified with PGC-1α, the GCN5 and the TIP60 protein complexes, with their respective

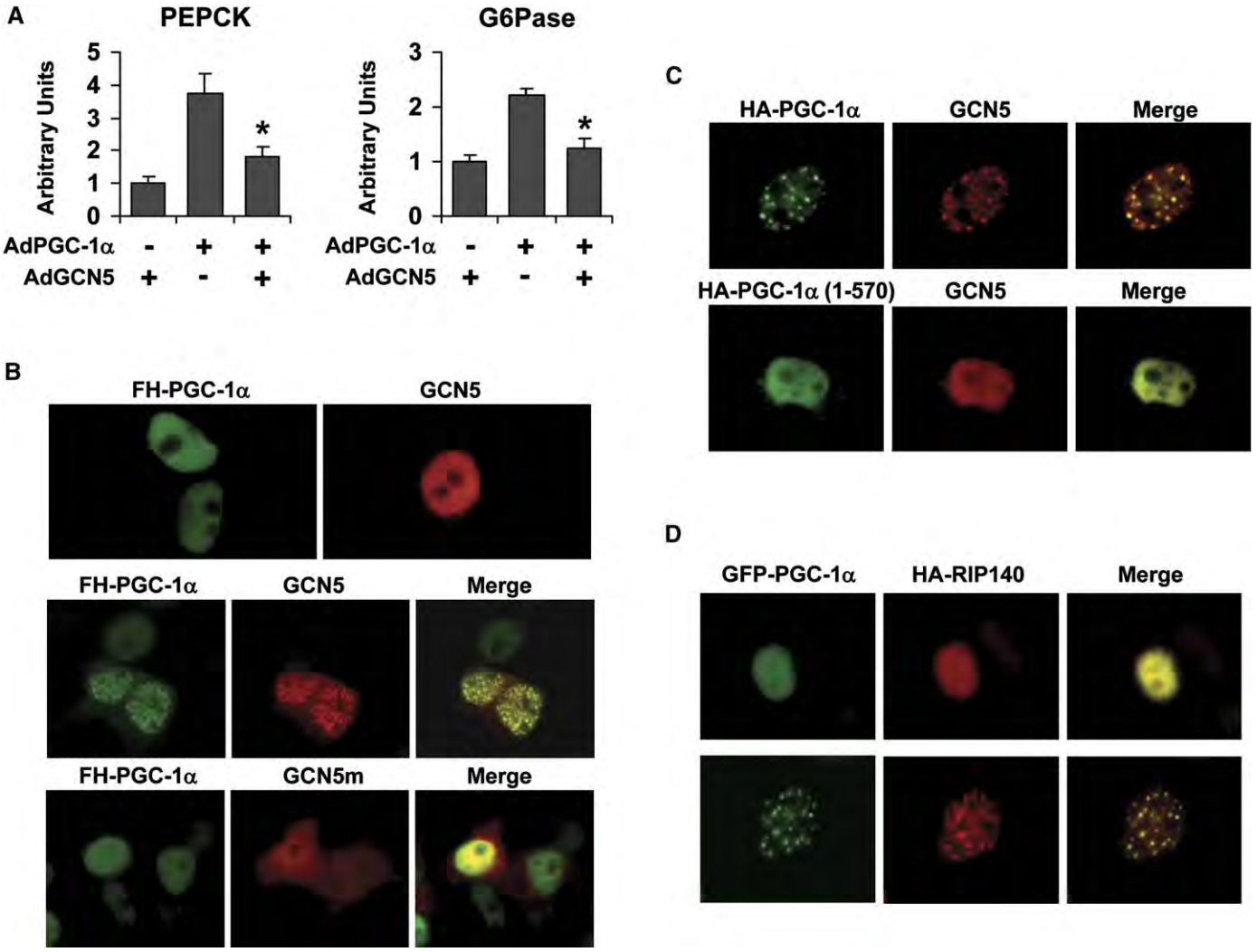

Figure 4. Nuclear redistribution of PGC-1α by GCN5 acetyltransferase activity

A) Decreased PGC-1α PEPCK and G6Pase promoters occupancy by GCN5. Fao cells were infected with the indicated adenovirus and chromatin immunoprecipitation was performed. Immunoprecipitates were analyzed by the presence of the PEPCK and G6Pase promoter by RT-PCR. Values represent means ± SEM of two independent experiments performed in duplicate; *$p < 0.05$, versus PGC-1α.

B) Localization of PGC-1α to nuclear foci by GCN5. Fao cells were infected with adenoviruses expressing FH- PGC-1α or GCN5 (top panel), FH- PGC-1α and GCN5 (middle panel), or FH- PGC-1α and GCN5m (bottom panel). Immunofluorescence was performed using a mouse anti-HA antibody (shown in green) and a rabbit anti-GCN5 antibody (shown in red).

C) PGC-1α C-terminal domain is necessary for its nuclear foci localization. HEK293 cells were transfected with HA-PGC-1α and GCN5 (top panel) or HA-PGC-1α (1-570 aa) and GCN5 (bottom panel). Immunofluorescence was performed as in (**B**).

D) Colocalization of PGC-1α and RIP140 in nuclear foci. HEK293 cells were transfected with GFP-PGC-1α and HA-RIP140 with (bottom panel) or without (top panel) GCN5. Immunofluorescence was performed using an anti-HA antibody (shown in red).

subunits. Of importance, the GCN5 acetyltransferase complex associated with PGC-1α provides a mechanistic basis by which acetylation of PGC-1α represses expression of gluconeogenic genes. Specifically, it clearly delineates a mechanism by which metabolic gene expression is regulated through intranuclear localization of a particular transcriptional coactivator. It is conceivable that control of gene expression by localizing key metabolic regulators such as PGC-1α to repressive spatial domains is a mechanism to efficiently modulate gene expression under different hormonal and nutrient signals. Although the biochemical identity of these nuclear foci is unknown, it is remarkable that we have shown that it colocalizes with a transcriptional repressor of hormone nuclear receptors, RIP140. Interestingly, RIP140-deficient mice have altered energy metabolism with increased energy expenditure (Leonardsson et al., 2004). To what extent repression of PGC-1α by GCN5 requires colocalization and/or interaction with RIP140 is unknown. However, it is clear from our studies that the gluconeogenic promoters are not present in these repressive structures, suggesting that the main mission of this new localization and spatial distribution is to recruit transcriptional activators away from the chromatin.

We have found that most subunits of the TIP60 protein complex are also associated with the PGC-1α complex. Moreover, we show here that both proteins directly interact, but the role of TIP60 complex on PGC-1α function is currently under investigation. The TIP60 complex has been linked to the DNA damage

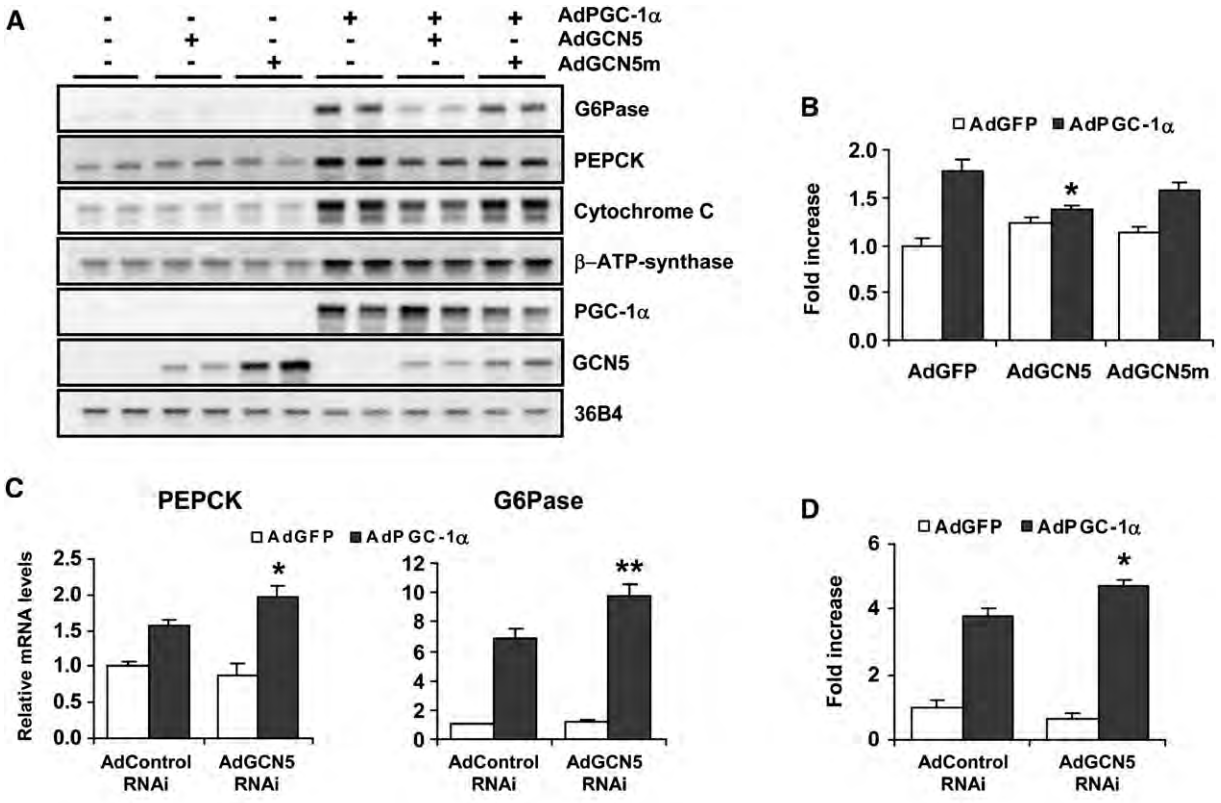

Figure 5. Inhibition of gluconeogenic PGC-1α function through GCN5 acetyltransferase activity

A) Reduction of gluconeogenic and mitochondrial PGC-1α target genes by GCN5. Fao cells were infected with the indicated adenoviruses and total RNA was analyzed by Northern blot 2 days after infection.

B) Inhibition of hepatic glucose secretion by GCN5. Fao cells were infected as in (**A**) and incubated with a gluconeogenic medium before measuring glucose secretion. Values represent means ± SEM of three independent experiments performed in duplicate; *p < 0.001 versus AdGFP.

C) Increased PGC-1α gluconeogenic function by GCN5 RNAi. Fao cells were infected with the indicated adenovirus and total RNA was analyzed by RT-PCR 3 days after infection. Values represent means ± SEM of three independent experiments performed in duplicate; *p < 0.05 and **p < 0.01 versus AdControl RNAi.

D) Increased PGC-1α-induced hepatic glucose secretion by GCN5 RNAi. Fao cells were infected as in (**C**) and incubated with a gluconeogenic medium before measuring glucose secretion. Values represent means ± SEM of two independent experiments performed in triplicate; *p < 0.01 versus AdControl RNAi.

and repair response (Ikura et al., 2000; Kusch et al., 2004). Interestingly, PGC-1α has been associated with an increased in ROS detoxification enzymes (St-Pierre et al., 2003). It could be conceivable that in certain promoters of genes involved in oxidative stress and DNA repair the TIP60 complex could recruit PGC-1α to regulate expression of these genes. Another important mechanistic question is how the GCN5 and TIP60 complexes complement the function of two other classes of proteins shown to interact with PGC-1α, the HATs p300 and SRC-1 (Puigserver et al., 1999) as well as the TRAP/mediator complex (Wallberg et al., 2003). As it relates to GCN5 as a negative regulator of PGC-1α, a possibility is a different spatial localization that precludes activation with these other set of proteins. Interestingly, TIP60 complex contains RuvBL1 and RuvBL2, two proteins with DNA helicase enzymatic activity that could cooperate with p300 and TRAP complex to remodel chromatin and modulate gene expression. Further studies will address the interplay between these different set of proteins to regulate PGC-1α transcriptional activity.

In a food deprivation physiological response, SIRT1 deacetylase regulates the function of PGC-1α on gluconeogenic genes and hepatic glucose production (Rodgers et al., 2005). The results presented here indicate that there exists an opposite mechanism through GCN5 acetylation of PGC-1α. This provides strong evidence that the acetylation status of PGC-1α is critical to the transcriptional function of this coactivator. We show here that modulating the activity of GCN5 in hepatic cells and in the mouse liver results in altered expression of gluconeogenic genes, glucose production and blood glucose levels, indicating that this pathway is operative in mice. Whether GCN5 is directly regulated in response to hormonal and/or nutrient signals to control the biological function of PGC-1α in key metabolic tissues is currently under investigation. Alternatively, it is conceivable that GCN5 might be constitutively active and the ''true'' metabolic regulatory sensor modulating acetylation of PGC-1α is SIRT1. As SIRT1 has been implicated in biological processes affecting life span in different organisms (Bitterman et al., 2003; Bordone and Guarente, 2005; Rogina and Helfand, 2004), it could be possible that certain activities of SIRT1 are antagonized by GCN5. At least, what we show here is that the metabolic gluconeogenic pathway that is activated by caloric restriction and SIRT1 (Rodgers et al., 2005) is oppositely regulated by GCN5, whether the same antagonism is applied to other SIRT1 pathways such as resistance to oxidative stress is an attractive hypothesis.

Finally, it is important to note that GCN5 acetyltransferase enzymatic activity may provide a target for therapeutic drugs to

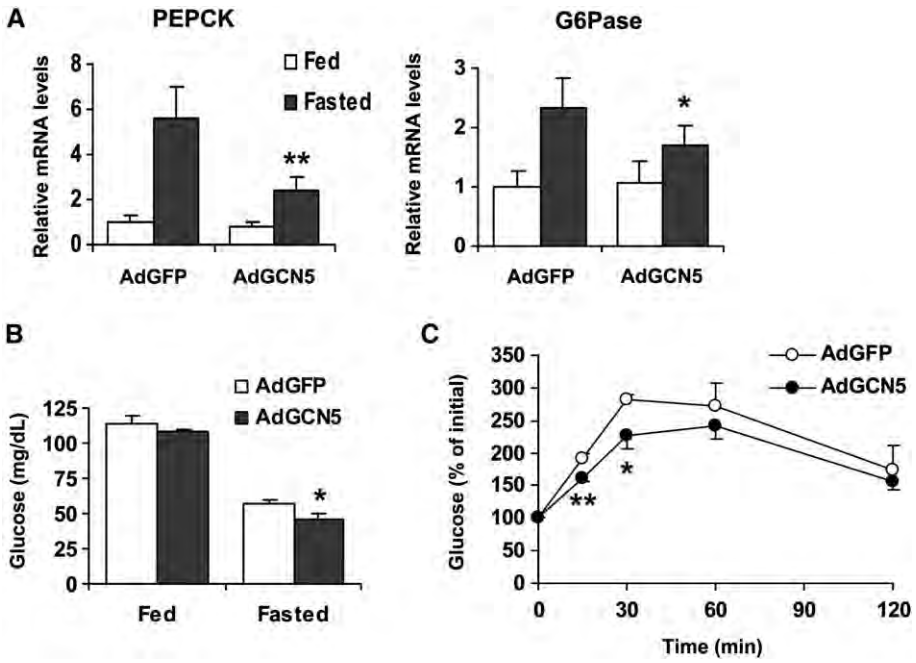

Figure 6. Inhibition of gluconeogenic gene expression by GCN5 acetyltransferase activity in the mouse liver

A) Inhibition of gluconeogenic gene induction during fasting by GCN5. Total RNA was isolated from mice injected with either GFP or GCN5 adenoviruses. mRNA levels for PEPCK and G6Pase were analyzed by RT-PCR in fed and fasted states. Values represent means ± SEM, with n ≥ 6 from two independent experiments; *p < 0.01 and **p < 0.005, versus AdGFP.

B) Decreased blood glucose levels during fasting by GCN5. Blood glucose levels were measured in the fed or fasted states from mice injected with either GFP or GCN5 adenovirus. Values represent means ± SEM with n ≥ 13 from three independent experiments; *p < 0.05, versus AdGFP.

C) GCN5 decreases glucose production from pyruvate. Pyruvate was injected intraperitoneally at a dose of 2 g/Kg into fasted mice expressing either GFP or GCN5. Blood glucose levels were monitored after pyruvate injection. Values represent means ± SEM with n = 7 from two independent experiments; *p < 0.01 and **p < 0.005 versus AdGFP.

treat glucose metabolic disorders in diabetes or aging, by potentially modulating the function of PGC-1α in tissues such as the liver to control glucose production.

Experimental Procedures

Constructs

The catalytically inactive acetyltransferase mutant GCN5 (Y621A/P622A) (GCN5m) was generated by site-directed mutagenesis and verified by DNA sequencing. Target sequence of the RNAi directed against GCN5 was tgttcgagctctcaaagat. Wild-type and GCN5m, Flag-HA-PGC-1α (FH-PGC-1α) and GCN5 RNAi adenovirus were constructed using the pAd-Easy system. Basically, inserts were cloned into the pAdTrack shuttle vector and adenovirus constructs were created by recombination of the shuttle vector and pAdEasy vector by electroporation into BJ5183-AD-1 bacteria (Stratagene). Plasmids maps and sequences of constructs used in this study are available upon request.

Cell culture and treatments

HEK293 cells were maintained in DMEM with 10% fetal bovine serum (FBS). Fao rat hepatocytes were cultured in Ham's F-12 Coon's modified media (Biosources) with 5% FBS. Fao cells were infected with adenoviruses for 3 hr and incubated in the same media for an additional 24 hr. Cells were then washed with PBS and incubated with RPMI with 0.5% BSA for 16 hr before harvesting.

Protein complex purification

PGC-1α complexes were purified from approximately 40 mg of nuclear extracts prepared from Fao cells infected with adenoviruses expressing PGC-1α protein fused with N-terminal Flag- and HA-epitope tags (FH-PGC-1α). As a control, we performed mock purification from Fao cells infected with adenoviruses expressing GFP. Nuclear extracts were obtained as previously described (Dignam et al., 1983) and dialyzed against a buffer containing 20 mM Tris-HCl (pH 8), 100 mM KCl, 0.2 mM EDTA, 0.1% Tween-20, 10% glycerol, 20 mM β-mercaptoethanol, and 0.1 mM PMSF. Protein complex purification was performed as described in (Nakatani and Ogryzko, 2003) with several modifications. Basically, nuclear extracts were separated through a Sephacryl S-300 size exclusion column (Amersham Biosciences) and fractions containing FH-PGC-1α were collected. Protein complexes were further purified by immunoaffinity chromatography with Flag M2 antibody linked to agarose beads (Sigma). Protein complexes were then eluted by incubating with Flag peptide (0.2 mg/ml) and the eluted material was separated in a 4%–20% acrylamide gradient gel. Protein bands were excised from SDS-PAGE gels, digested with trypsin, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (Ibarrola et al., 2003). The mass spectra were acquired on a Micromass-Waters Q-TOF API-US mass spectrometer (Manchester, UK). In order to identify the proteins, mass spectrometric data were searched against NCBI non-redundant database by using Mascot version 1.9. For density gradient sedimentation, 0.2 ml of purified material was loaded onto a 4 ml glycerol gradient (15%–40%) and centrifuged at 55.000 rpm in a Beckman SW55Ti rotor for 2.5 hr. Fractions were collected from the top and proteins were detected by Western blot using the indicated antibodies.

Protein interaction analysis

Protein-protein interaction analysis were performed by either coimmunoprecipitation or in vitro binding assays. For coimmunoprecipitation experiments, Fao cells were infected with adenoviruses expressing FH-PGC-1α or GFP and nuclear extracts were obtained as described in (Dignam et al., 1983). Immunoprecipitation was performed by incubating with M2 Flag antibody and eluting with Flag peptide. The eluted material was then analyzed by Western blot using the indicated antibodies. Antibodies used in this study are anti-GCN5 (Biolegends), anti-$TAF_{II}31$ (provided by Robert G. Roeder), anti-SAP130 (Santa Cruz), anti-PAF65β (provided by Yoshihiro Nakatani), anti-RuvBL1 (provided by Anyndia Dutta), and anti-KAP-1 (provided by Frank J. Rauscher). For in vitro binding assays, GST–PGC-1α and GST–GCN5 fragments were expressed in bacteria (BL21) by isopropyl thiogalactoside induction for 3 hr at 30°C and purified on sepharose beads containing glutathione. [^{35}S]-labeled proteins were made with a TNT reticulocyte lysate kit (Promega). Equal amounts of GST fusion proteins (1 μg) were mixed with 5 μl of the in vitro translated proteins in a binding buffer containing 20 mM HEPES buffer (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP40, 2 mM dithiothreitol, and 10% glycerol. After 1 hr of in vitro binding reaction, agarose beads were washed three times with the binding buffer. Bound proteins were separated by SDS-PAGE and analyzed by autoradiography.

Analysis of protein acetylation

Flag-tagged proteins were expressed in 293 cells using PolyFect (Qiagen) or in Fao cells by adenoviral infection. Whole-cell extracts were obtained 36 hr after transfection or infection and subjected to immunoprecipitation with anti-Flag M2 antibody linked to agarose beads. For in vivo acetylation analysis, PGC-1α was immunoprecipitated with anti-PGC-1α antibody (H-300, Santa Cruz) from nuclear extracts obtained from fasted livers of mice injected

with either GFP or GCN5 adenovirus. The immunoprecipitates were then separated by SDS-PAGE and immunoblotted using the acetyl-Lysine antibody (Cell Signaling and Technology) and the M2 Flag antibody (Sigma) or anti-PGC-1α H300 antibody to detect lysine acetylation and total protein levels, respectively.

In vitro protein acetyltransferase assay

Histone acetyltransferase assays were performed using 5 μg of core histones (Upstate) and 5 μl of the PGC-1α-purified or the mock-purified material in acetylation buffer (50 mM Tris-HCl [pH 8], 10% glycerol, 40 mM sodium butyrate, 20 mM nicotinamide, 4 mM DTT, and 1 mM PMSF) with 1 nmol [^{14}C]-acetyl-CoA (55 mCi/mmol). After incubation at 30ºC for 10 min, histones were separated in 15% SDS-PAGE and analyzed by autoradiography. Same protocol was used to analyze PGC-1α acetylation but using 1 μg of recombinant GST-PGC-1α (1–400 aa) and 1 μg of recombinant GST-GCN5 (352–837 aa) and incubating at 30ºC for 1 hr. After extensive washing, incorporated radioactivity was measured by liquid scintillation counting.

Transcriptional activation assays

HEK293 cells were transiently transfected in 24-well dishes using PolyFect (Quiagen) with the indicated plasmids. The ratio DNA:PolyFect was 1:2. Cells were lysed 36 hr after transfection and luciferase assays were performed.

Gene expression analysis

Gene expression was analyzed either by Northern blot or real-time PCR. Total RNA prepared from Fao cells or mouse livers was extracted with Trizol (Invitrogen). RNA messages were analyzed by Northern blot using specific ^{32}P-labeled probes. Alternatively, complementary DNA generated by Superscript II enzyme (Invitrogen) was analyzed by quantitative reverse-transcriptase-mediated PCR (Q-RT−PCR) using an iQ SYBR Green Supermix (Bio-Rad). All data were normalized to tubulin expression. The oligonucleotide primers used are provided as Supplemental Data (Figure S6).

Hepatic glucose output

Fao cells were grown in 6 well dishes and infected with the indicated adenovirus. Two or three days after infection, culture medium was replaced with 1 ml of glucose-free RPMI supplemented with 0.5% BSA, 20 mM sodium lactate and 2 mM sodium pyruvate. After 4 hr incubation, glucose concentration in the culture medium was measured using the glucose-6-phosphate dehydrogenase method (Williamson et al., 1967). Glucose values were normalized to the total protein content and expressed as fold increase versus control (AdGFP or AdControl RNAi) cells.

Chromatin immunoprecipitation analysis

Fao cells were infected with the indicated adenovirus. Cells were cross-linked with 1% formaldehyde at 37ºC for 10 min and sonicated in lysis buffer containing 50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS and protease inhibitors. Supernatants were incubated with the anti-HA antibody (clone 3F10) linked to agarose beads (Roche) at 4ºC for 16 hr. After extensive washing immunoprecipitants were eluted with 2% SDS in 0.1 M NaH_2CO_3. Cross-linking was reversed by heating at 65ºC for 4 hr and eluates were treated with proteinase K (Roche) at 45ºC for 1 hr. DNA was analyzed by Real-Time PCR using an iQ SYBR Green Supermix (Bio-Rad). Forward and reverse primers used were 5′ tggcctggcttcgaggaccagg 3′ and 5′ aacctagccctgatctttggactc 3′, for G6Pase promoter, and 5′ gtgggagtgacacctcacagc 3′ and 5′ aggacagg gctggccgggacg 3′ for PEPCK promoter. Values were normalized to the amount of G6Pase and PEPCK promoters in the input.

Immunofluorescence microscopy

Immunofluorescence was performed 36 hr after transfection or infection with the indicated antibodies. Cells were fixed by incubating in 4% paraformaldehyde for 10 min at room temperature and permeabilized by incubating with 0.2% Triton X-100 in PBS for 10 min. Cells were then blocked by incubating with 3% BSA in PBS for 30 min and treated with the indicated primary antibody for 1 hr. Primary antibodies used in this study are mouse HA.11 (Babco), rabbit anti-GCN5 (Biolegends), mouse anti-Sm (provided by Michael J. Matunis), mouse anti-SC35, mouse anti-U2-B′′ (provided by David L. Spector) and human anti-U1snRNP70K (provided by Antony Rosen). After four washes with 0.1% NP-40 in PBS, cells were incubated with anti-human TR-conjugated, anti-rabbit Cy3-conjugated or anti-mouse Cy5-conjugated secondary antibodies (Jackson Immunoresearch) for 1 hr. After being washed with PBS 0.1% NP-40 four times, cells were examined using a fluorescence microscope (Carl Zeiss Axiovert 135) and IPLab software (Scanalytics) was used to collect digital images.

Animal experiments

Male, 8-week-old BALB/c mice were purchased from Harlan Laboratories. Recombinant adenovirus (5×10^9 pfu) was delivered by tail-vein injection to mice. Fed and 16 hr fasting blood glucose levels were measured from tail-vein blood 4 days after injection, using the Ascensia ELITE® XL Blood Glucose Monitoring System (Bayer). Animals were sacrficed and the livers were removed and snap-frozen. Where indicated, pyruvate was administered intraperitoneally to fasted mice at a dose of 2 g/Kg.

Statistical analysis

Results are given as means ± SEM Statistical analyses were performed using the unpaired two-tailed Student's t test, and the null hypothesis was rejected at the 0.05 level.

Supplemental data

Supplemental data include six figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/6/429/DC1/.

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Cell

Resveratrol Improves Mitochondrial Function and Protects against Metabolic Disease by Activating SIRT1 and PGC-1α

Marie Lagouge,[8,1] Carmen Argmann,[8,1] Zachary Gerhart-Hines,[2] Hamid Meziane,[3] Carles Lerin,[2] Frederic Daussin,[4] Nadia Messadeq,[3] Jill Milne,[5] Philip Lambert,[5] Peter Elliott,[5] Bernard Geny,[4] Markku Laakso,[6] Pere Puigserver,[2] and Johan Auwerx[1,3,7,*]

[1] Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS / INSERM / ULP, 67404 Illkirch, France
[2] Department of Cell Biology, John Hopkins University School of Medicine, Baltimore, MD 21205, USA
[3] Institut Clinique de la Souris, BP10142, 67404, Illkirch, France
[4] Department of Respiratory, Cardiocirculatory and Exercise Physiology, Hôpitaux Universitaires, 67000 Strasbourg, France
[5] Sirtris Pharmaceutical, Cambridge, MA 02139, USA
[6] Department of Medicine, University of Kuopio, 70211 Kuopio, Finland
[7] IGBMC-ICS, 67404 Illkirch, France
[8] These authors contributed equally to this work.
*Contact: auwerx@igbmc.u-strasbg.fr
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SUMMARY

Diminished mitochondrial oxidative phosphorylation and aerobic capacity are associated with reduced longevity. We tested whether resveratrol (RSV), which is known to extend lifespan, impacts mitochondrial function and metabolic homeostasis. Treatment of mice with RSV significantly increased their aerobic capacity, as evidenced by their increased running time and consumption of oxygen in muscle fibers. RSV's effects were associated with an induction of genes for oxidative phosphorylation and mitochondrial biogenesis and were largely explained by an RSV-mediated decrease in PGC-1α acetylation and an increase in PGC-1α activity. This mechanism is consistent with RSV being a known activator of the protein deacetylase, SIRT1, and by the lack of effect of RSV in SIRT1$^{-/-}$ MEFs. Importantly, RSV treatment protected mice against diet-induced-obesity and insulin resistance. These pharmacological effects of RSV combined with the association of three *Sirt1* SNPs and energy homeostasis in Finnish subjects implicates SIRT1 as a key regulator of energy and metabolic homeostasis.

INTRODUCTION

Mitochondria are the principal energy sources of the cell that convert nutrients into energy through cellular respiration (Wallace, 2005). Compromised mitochondrial function has been linked to numerous diseases, including those of the metabolic and cardiovascular systems (Petersen et al., 2003). The genetic basis of such a tight link in the rat was illustrated by the cosegregation of cardiovascular and metabolic risk factors with low aerobic capacity and reduced muscle expression of genes required for mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) (Wisloff et al., 2005). In humans, insulin resistance in the skeletal muscle has been associated with a lower ratio of oxidative type 1 to type 2 glycolytic type muscle fibers, decreased mitochondrial oxidative capacity and ATP synthesis, and, finally, decreased expression of genes that control mitochondrial activity (Mootha et al., 2003, 2004; Patti et al., 2003; Petersen et al., 2003). One gene whose decreased expression is consistently implicated in the human or animal diabetic muscle is the peroxisome proliferator-activated receptor γ coactivator, PGC-1α (Mootha et al., 2004; Patti et al., 2003; Sparks et al., 2005). PGC-1α is a coactivator with pleiotropic functions (Knutti and Kralli, 2001; Lin et al., 2005). Most importantly, PGC-1α controls mitochondrial biogenesis and function, which in the muscle can contribute to fiber-type switching (Lin et al., 2002a) and, in the brown adipose tissue (BAT), to adaptive thermogenesis (Puigserver et al., 1998).

Recently SIRT1 has been shown to function together with PGC-1α to promote adaptation to caloric restriction (CR) by regulating the genetic programs for gluconeogenesis and glycolysis in the liver (Rodgers et al., 2005). SIRT1 is one of seven mammalian homologs of Sir2 that catalyzes NAD^+-dependent protein deacetylation, yielding nicotinamide and *O*-acetyl-ADP-ribose (Blander and Guarente, 2004). Originally described as a factor regulating longevity, apoptosis and DNA repair (Blander and Guarente, 2004; Sinclair, 2005), SIRT1 also facilitates the conversion of changes in the nutritional status, which it senses via NAD^+ levels, into modulation of cellular metabolism (Brunet et al., 2004; Lin et al., 2002b; Picard et al., 2004;

Rodgers et al., 2005). SIRT1 physically interacts with and deacetylates PGC-1α at multiple lysine sites, consequently increasing PGC-1α activity leading to the induction of liver gluconeogenic gene transcription (Rodgers et al., 2005). Given the role of SIRT1 as a mediator of CR and longevity and the central role for reactive oxygen species (ROS), mainly produced as a consequence of mitochondrial functioning in promoting aging, it is plausible that PGC-1α and SIRT1 functions converge in tissues beyond the liver that have a high level of mitochondrial activity, such as the muscle and BAT. Since such a convergence could potentially impact on metabolic diseases, we addressed our hypothesis not in the context of CR but under conditions of caloric excess using the specific SIRT1 activator, resveratrol (RSV) (Borra et al., 2005; Howitz et al., 2003).

RSV is a natural polyphenolic compound mainly found in the skin of grapes and is well known for its phytoestrogenic and antioxidant properties (Baur and Sinclair, 2006). It has been shown to significantly increase SIRT1 activity through an allosteric interaction, resulting in the increase of SIRT1 affinity for both NAD^+ and the acetylated substrate (Howitz et al., 2003). These findings are consistent with the fact that in various species, RSV treatment mimics Sir2-dependent lifespan extension during CR (Howitz et al., 2003; Lin et al., 2000; Rogina and Helfand, 2004).

In this study we tested whether RSV, through increasing SIRT1 activity, could modulate PGC-1α functions in vivo and ultimately impact on the regulation of energy homeostasis. Our data reveal that RSV potently induces mitochondrial activity, through activating PGC-1α, as evidenced by the increase in oxidative type-muscle fibers, enhanced resistance to muscle fatigue, and increased tolerance to cold, all PGC-1α-dependent effects. Importantly, these effects, induced by RSV, rendered the animals resistant to diet-induced obesity and insulin resistance. In support of the importance of SIRT1 in the control of energy homeostasis, we also report a significant association between three single-nucleotide polymorphisms (SNPs) in the human *Sirt1* gene and energy homeostasis, extending the impact of our animal studies to human pathophysiology.

RESULTS

Metabolic Consequence of RSV in Diet-Induced Obesity

The metabolic effect of RSV was initially evaluated in a cohort of male C57Bl/6J mice that were given a dose of 200 or 400 mg/kg/day (mpk) of RSV administered in either a chow diet or high fat (HF) diet for 15 weeks. With this protocol, the plasma level of RSV was dose-related and ranged from 10–120 ng/ml. Under chow-fed conditions, RSV-treated mice tended to gain less weight as compared to controls (Figure 1A). However, this effect became significant when the animals were challenged with an HF diet, such that RSV-treated, HF-fed mice weighed almost the same as the chow-fed mice (Figure 1B). This decreased body mass was accounted for by a decrease in fat as illustrated by dual X-ray absorptiometry (Figure 1B) and was also reflected in the mass of the different white fat pads (Figure 1C). Morphological analysis of epididymal white adipose tissue (WAT) sections by hematoxylin and eosin (HE) staining also showed smaller adipocytes upon RSV treatement (Figure S1). These beneficial effects of RSV on body weight and fat mass were not due to decreased food intake, as the amount of kcal of food consumed per mouse over a 24 hr period was unchanged (Figure 1D). RSV, at the dose given, did not induce hepatic toxicity, since the serum levels of alanine aminotransferase and aspartate aminotransferase (data not shown) were unchanged, as was the liver histo-morphology (Figure S1). In addition, stool composition, coat maintenance, and water intake (data not shown) were unaffected, indicating that overall, RSV was well tolerated by the animals. Finally, fecal lipid kcal content was minimally affected by RSV treatment, and greater than 98% of all dietary-derived lipid was absorbed in both groups (data not shown).

The critical parameters contributing to body-weight maintenance include caloric intake and energy homeostasis (Lowell and Spiegelman, 2000). As caloric intake is unaffected by RSV (Figure 1D), we assessed the effect of this compound on energy expenditure (EE) by indirect calorimetry. Basal EE, as measured by oxygen (O_2) consumption, was significantly increased in HF-fed mice treated with RSV (Figure 1E), but their respiratory quotient (RQ) was not changed (data not shown). To assess the effect of RSV on the capacity for adaptive thermogenesis, we performed a cold test. RSV enhanced this capacity, since it maintained the body temperature higher as compared to that of nontreated animals (Figure 1F). In the mouse, the major contributor to the production of heat is the BAT, and morphometric analysis of the BAT mitochondria, by electron microscopy, revealed clearly larger mitochondrial structures attributed to an increased presence of cristae in RSV-treated mice as compared to that of HF-fed animals (Figure 2A). This amplification of the mitochondria was reflected both in the quantification of mitochondrial size (Figure 2A, right panel) and mitochondrial DNA content (mtDNA, Figure 2D). Consistent with enhanced mitochondrial activity, a marked decrease in the lipid-droplet size was also noted.

RSV Increases the Aerobic Capacity of the Muscle

In the adult human, little BAT is present, and it is mainly the skeletal muscle that possesses the mitochondrial capacity for EE. The changes in the muscle mitochondrial morphology, however, paralleled those observed in the BAT of RSV-treated mice (Figure 2B). Whereas the oxidative fibers of the gastrocnemius were unaffected, the nonoxidative fibers in RSV-treated mice had larger and denser mitochondria aggregated between adjacent myofibrils. Mitochondrial expansion was evidenced by increased mitochondrial size (Figure 2B, right panel) and mtDNA content (Figure 2D). Histological sections of muscle stained for the presence of the mitochondrial enzyme, succinate

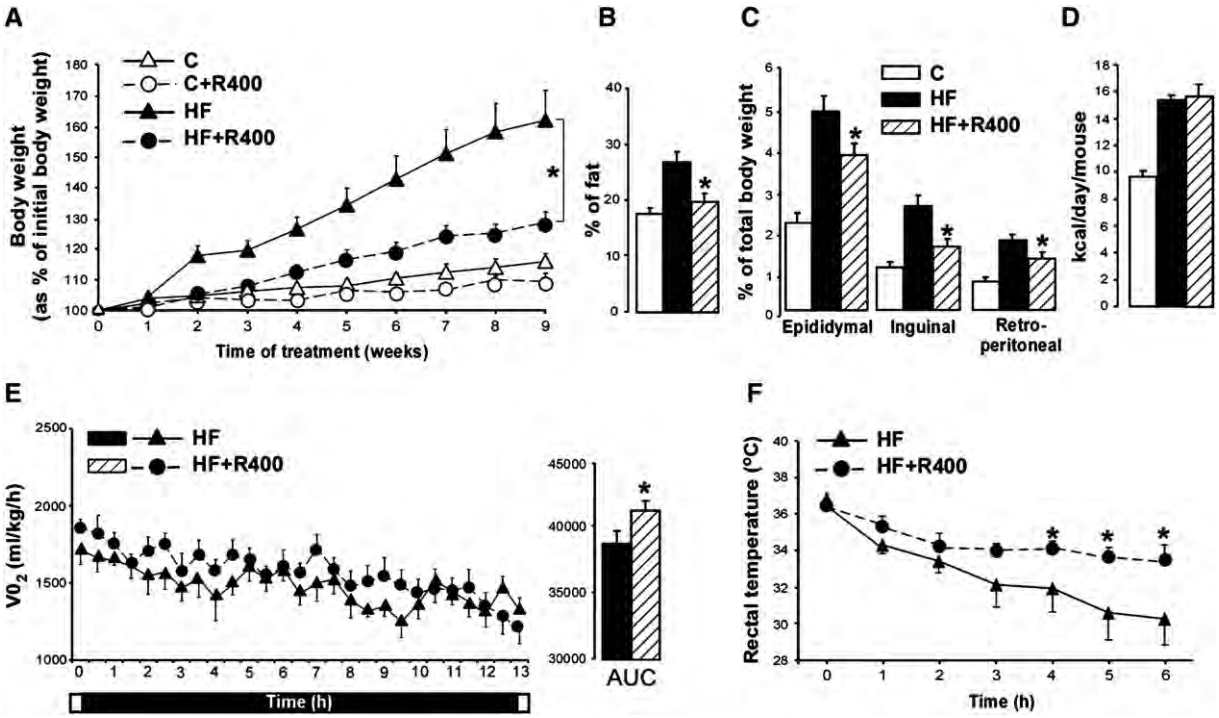


Figure 1. RSV Prevents Diet-Induced Obesity

C57Bl/6J mice were fed a chow diet (C) or high-fat diet (HF) alone or supplemented with RSV (400 mpk, R400) for 15 weeks.

(A) Evolution of body weight gain expressed as percentage of initial body weight.

(B) Body fat content expressed as percentage of total body mass as analyzed by DEXA.

(C) Weight of the WAT depots, expressed as percentage of total body weight.

(D) Average food intake expressed as kcal/mouse/day.

(E) EE as measured by changes in VO_2 consumption in indirect calorimetry during 13 hr (time 0 is 7:00 p.m.). The mean areas under the curves (AUC) are shown in the right side graph (n = 7).

(F) The evolution of the body temperature during a cold test (4°C for 6 hr). * = $P < 0.05$ and n = 10 animals/group unless stated otherwise. Values represent means ± SEM.

dehydrogenase (SDH, Figure 2C), and the increase in citrate synthase activity in muscle homogenates, furthermore indicates that RSV enhanced mitochondrial enzymatic activity (Figure 3A). Finally, in the isolated nonoxidative muscle fibers of RSV-treated mice, there was a significantly higher maximum VO_2 rate, indeed suggesting an increased oxidative capacity (Figure 3B). The combination of the increased mitochondria size and density, mtDNA content, SDH, and citrate synthase activities and oxidative capacity is highly suggestive that RSV increases the ratio of oxidative to nonoxidative type-muscle fibers.

Following the hypothesis that RSV induces a fiber-type switch and knowing that oxidative type 1 fibers are associated with an increased resistance to muscle fatigue (Booth et al., 2002), we evaluated the effect of RSV administration in an endurance test. In HF-fed animals treated with RSV, the distance run to exhaustion was twice that of the HF-fed controls (Figure 3C). To account for the potentially confounding significant weight difference between RSV-treated and nontreated HF-fed mice, we redid the test using RSV-treated and nontreated chow-fed mice, which did not significantly differ in body weight (Figure 1A). The RSV-treated mice, however, still outran the control chow-fed mice by nearly double the distance (Figure 3C). Thus, RSV treatment significantly increases the animal's resistance to muscle fatigue, consistent with increased mitochondrial activity and the transformation of muscle toward a slow type phenotype.

No Behavioral Defects, but Improved Motor Function in RSV-Treated Mice

Since it was reported that SIRT1 is required for increased physical activity in response to CR (Chen et al., 2005), we carefully investigated whether the RSV-mediated increase in resistance to muscle fatigue was a result of a behavioral response or was truly a metabolic consequence. We initially examined the effect of RSV on spontaneous activity in mice by assessing their circadian activity. No significant difference was observed between chow- and HF-fed mice (data not shown). However, in RSV-treated HF-fed mice, there was a significant decrease in ambulatory locomotor activity as well as a tendency to decrease the number of rears (Figure 4A). These observations indicated that the effect of RSV on EE and weight gain could not be explained

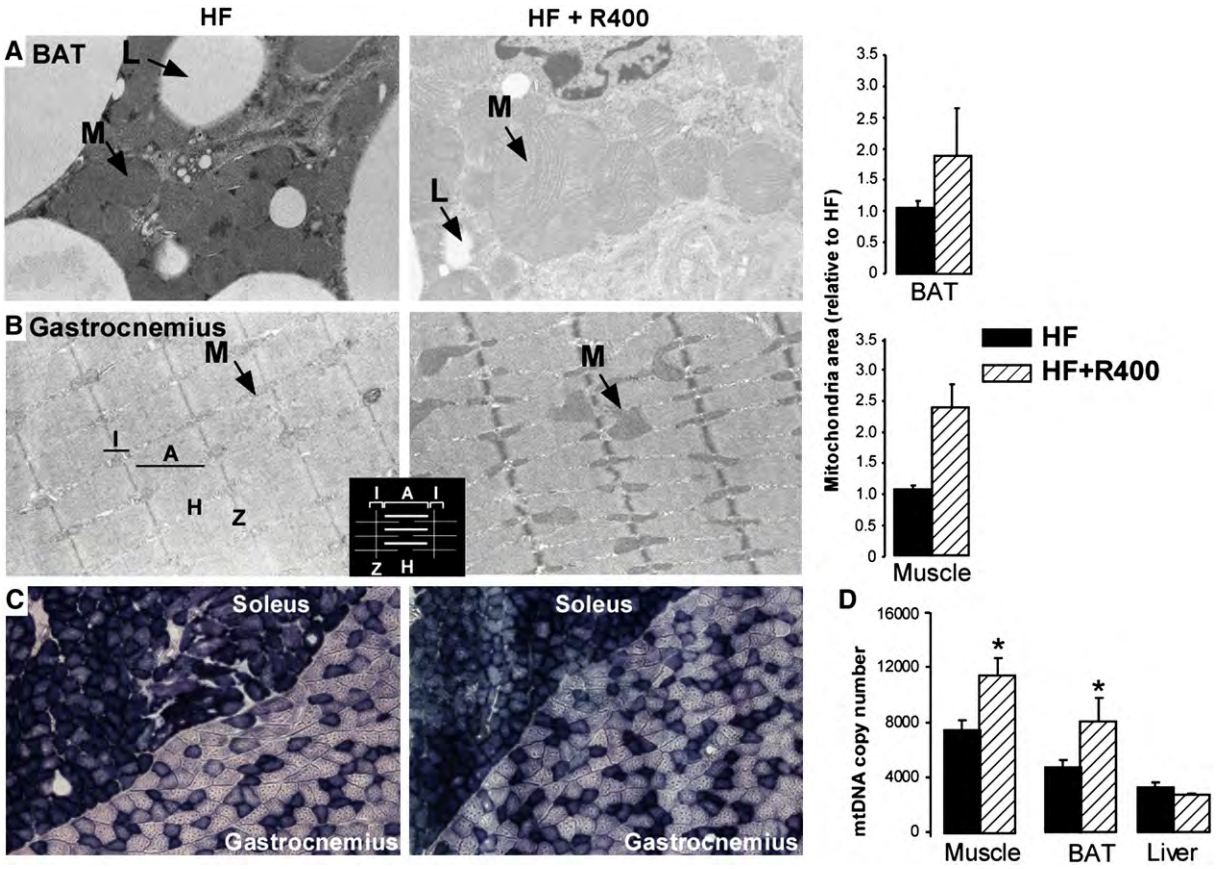


Figure 2. RSV Increases Mitochondrial Activity in the BAT and Muscle

(A and B) Transmission electronic microscopy (magnification of 20,000) image and corresponding quantification of mitochondria size in BAT (A) and nonoxidative fibers of gastrocnemius muscle (B) from RSV-treated (HF+R400) and nontreated HF-fed animals. Arrows indicate the position of mitochondria (M) and lipid droplets (L), and the inset shows the schematic organization of muscle fiber anatomy. Quantification was performed on 2 animals/group and is expressed relative to HF controls.

(C) SDH staining of gastrocnemius and soleus muscle from RSV-treated and nontreated HF-fed animals.

(D) mtDNA copy number of gastrocnemius muscle, BAT, and liver from RSV-treated and nontreated HF-fed mice (n = 4 animals/group). Values represent means ± SEM.

by an increase in spontaneous activity. In fact, the reduced level of activity in RSV-treated mice is in line with the decrease in resting heart rate (Figure 4B). Cardiodetrimental effects are, however, not suspected due to the lack of significant effect of RSV on blood pressure (Figure 4C), various echocardiography parameters (data not shown), PGC-1 activity (see below), and cardiac gene expression (Figure S3).

To discount the potential of central nervous system (CNS)-mediated behavioral effects and to determine the effect of RSV treatment on other motor abilities, we evaluated anxiety and sensorimotor function. No significant effects were observed between RSV-treated and nontreated HF-fed mice on anxiety, as evaluated by open field (Figure 4D), light/dark box (Figure 4E), and elevated-plus-maze tests (Figure 4F). The absence of a difference between RSV-treated and nontreated HF-fed mice in pain sensitivity, as measured in the hot-plate test (data not shown), also discounted the possibility that fatigue resistance might be due to altered pain sensitivity. Interestingly, as compared to nontreated HF-fed mice, the RSV-treated mice displayed increased muscle strength (Figure 4G) and markedly improved motor coordination and traction force as revealed in the rotarod (Figure 4H) and string tests (Figure 4I). These tests support the data obtained in the exercise test and suggest that RSV may improve neuromuscular function.

RSV Reprograms Muscle Gene Expression

To make the molecular connection between RSV treatment and the apparent myofiber remodeling, we profiled the expression of ±40,000 genes by microarray analysis. As the coordination of muscle plasticity is a complex event, composed of many small but cumulatively significant changes, we used a gene-set enrichment analysis (GSEA) to look for coordinate expression within treated samples of a priori-defined groups of genes (Mootha et al., 2003; Subramanian et al., 2005). Genes were ranked

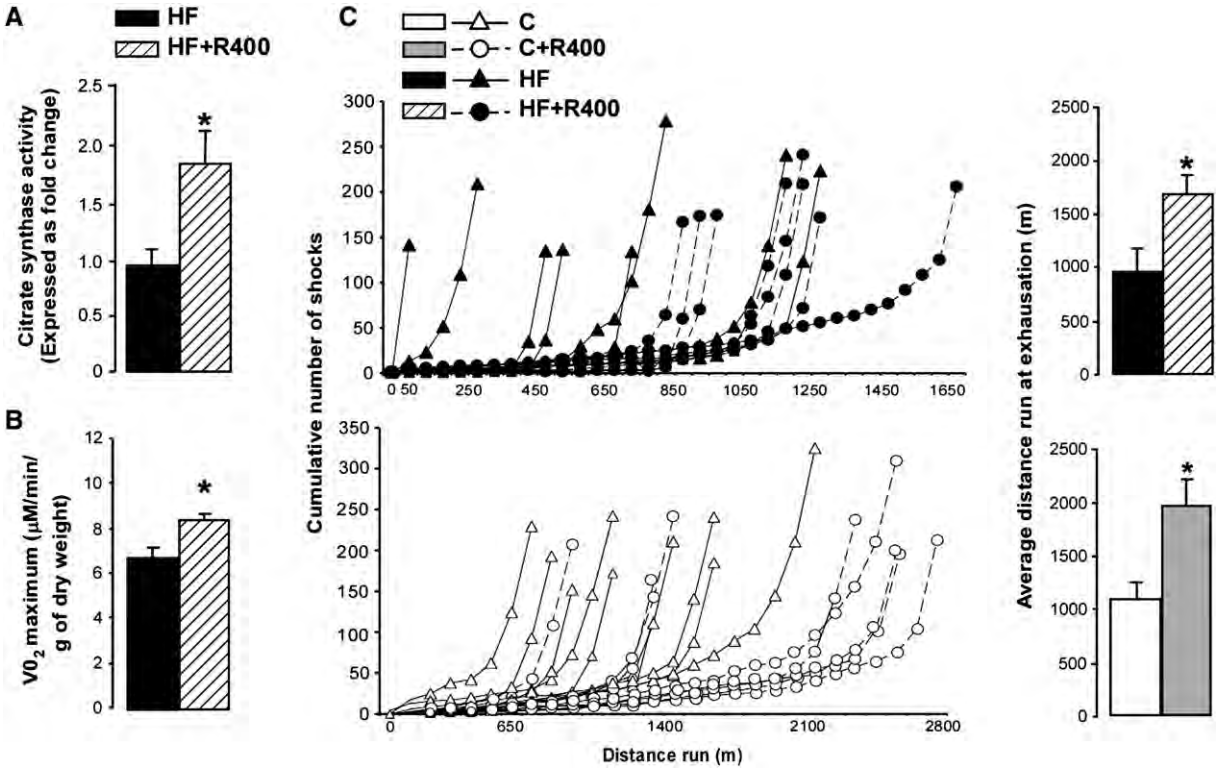


Figure 3. Enhanced Oxidative Capacity and Endurance in RSV-Treated Mice

(A) Activity of the citrate synthase, as measured in homogenates of gastrocnemius fibers isolated from RSV-treated (HF + R400) and nontreated HF-fed mice. N = 3 animals/group, and values are expressed relative to control.

(B) Maximum VO_2 consumption in isolated gastrocnemius fibers measured ex vivo. N = 5 animals/group.

(C) The effect of RSV on endurance, as measured by an exercise test. Individual animal performances (graphs on the left) as well as the average distance run until exhaustion (graphs on the right) are presented for animals treated with HF or HF + R400 (top) or chow diet (C) or chow diet and RSV at 400 mpk (C + R400) (bottom). N = ~8 animals/group. * = $P < 0.05$. Values represent means ± SEM.

according to their correlation to RSV treatment, and then the position of each gene-set member was identified, and a maximum enrichment score (MES) for each gene set was calculated. Amongst the top 30 gene sets, which were significantly enriched in RSV-treated mice, were ribosomal mRNA processing, striated muscle contraction, electron transport chain, OXPHOS, and ATP synthesis (Table S1). Three representative GSEA-scoring plots and their corresponding heat maps are shown (Figure 5A). Of particular note is their increase in gene expression under RSV treatment. Individual genes in the enriched pathways were related to muscle contraction (e.g., troponins) as well as enhanced oxidative metabolic status, including components of the respiratory apparatus (e.g., NDUFB8), oxidative enzymes (e.g., CoxVa), and ATPases (e.g., ATP5G3). These could provide a slow but stable, long-lasting supply of ATP, which would explain the increased muscle endurance associated with RSV. In addition, sets of genes supporting organelle biogenesis such as those encoding RNA-processing enzymes and ribosomal subunits were also enriched (Figures 5 and S2). Thus, this global molecular fingerprint of RSV identified coordinated changes in the expression of groups of genes functionally involved in mitochondrial biogenesis and function underpinning the enhanced oxidative capacity of the muscle.

To further evaluate the hypothesis that mitochondrial activity was affected by RSV treatment, we measured the expression of PGC-1α and several of its targets by Q-RT-PCR in gastrocnemius muscle. PGC-1α mRNA was significantly induced upon RSV treatment, which also translated into an increase in PGC-1α protein (Figures 5C and 5D). We also noted an increase in PGC-1β, which has several overlapping functions with that of PGC-1α in inducing genes related to OXPHOS (Lin et al., 2002c). The estrogen-related receptor α (ERRα), which mediates many of the downstream effects of activated PGC-1α on mitochondrial function and is itself a target of PGC-1α (Huss et al., 2002; Schreiber et al., 2003; Schreiber et al., 2004; Tcherepanova et al., 2000), was markedly increased by RSV, as was the ERRα/PGC-1 target, nuclear respiratory factor-1 (NRF-1) (Mootha et al., 2004; Patti et al., 2003). Mitochondrial transcription factor A (Tfam), a nuclear encoded mitochondrial transcription factor that is indispensable for the expression of key mitochondrial-encoded genes (Larsson et al., 1998) and a target of NRF-1, was also increased. In addition to the transcription

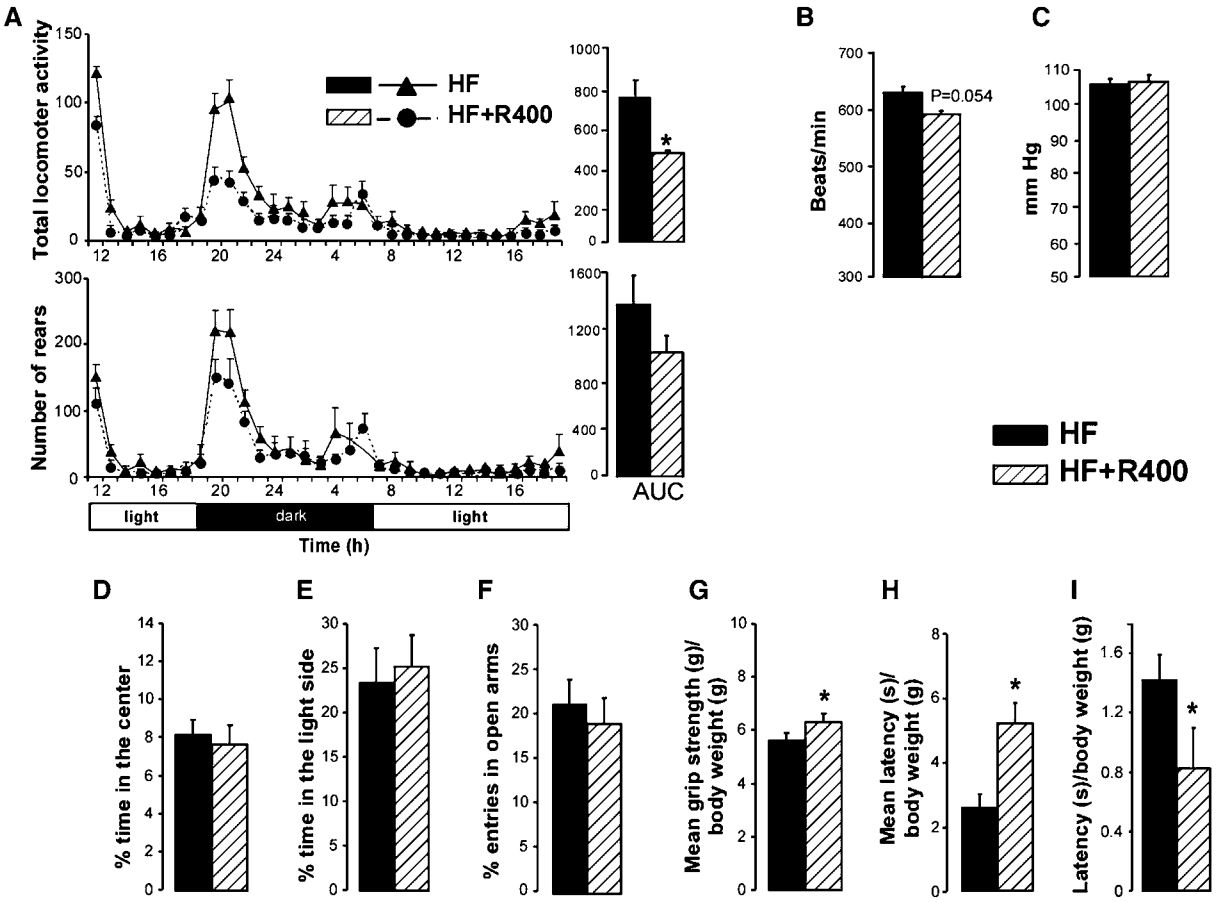


Figure 4. The Increase in Endurance and EE by RSV Is Not Explained by Increased Spontaneous Locomotor Activity or Altered Behavior

C57Bl/6J mice were fed an HF diet or HF diet and RSV 400 mpk (HF + R400). N = 8–10 animals/group.

(A) Circadian activity, measured as the total ambulatory locomotor activity (top graph) and the number of rears (bottom graph). The mean AUC are shown on the right.

(B and C) Heart rate as beats/min (B) and blood pressure in mm Hg (C).

(D–I) Behavior tests to evaluate mouse anxiety, including open field (D), light/dark box (E) and elevated-plus-maze (F), and sensorimotor function, including grip strength (G), rotarod (H), and string test (I). * = $P < 0.05$. Values represent means ± SEM.

factors, an array of additional downstream targets of PGC-1α (Lin et al., 2005), including genes involved in fatty-acid oxidation (medium chain acyl-CoA dehydrogenase, MCAD), uncoupling, and protection against ROS (uncoupling protein 3, UCP-3), and fiber-type markers (myoglobin and troponin 1) were induced by RSV.

As predicted from the electron microscopy and the cold test results, we noted a significant increase in gene expression in pathways related to energy homeostasis (Figure S3A) in BAT. PGC-1α, peroxisome proliferator-activated receptor α (PPARα), and UCP-1 mRNA levels were all induced by RSV. Like ERRα, PPARα induces genes that facilitate β-oxidation of fatty acids (Schoonjans et al., 1997), and UCP-1 is largely responsible for the uncoupling of respiration from ATP synthesis resulting in the production of heat in the BAT (Ricquier, 2005). Interestingly, however, mitochondrial changes were not evident in the heart, as reflected by a lack of changes in gene expression of PGC-1α and related target genes (Figure S3B), which corroborates the insignificant effects on heart physiology. We also surveyed the liver and found no changes in expression of gluconeogenic genes but a tendency for increased expression in genes related to OXPHOS (Figure S3B).

RSV Induces PGC-1α Activity through SIRT1

In spite of the RSV-mediated induction in PGC-1α mRNA and protein expression (Figure 5D), PGC-1α can also be regulated at the posttranslational level, as modifications, such as acetylation, significantly impact on its activity (Rodgers et al., 2005). Therefore, we compared PGC-1α acetylation in gastrocnemius muscle, BAT, and heart between mice that were fed an HF diet in the presence or absence of RSV (Figure 6A). In gastrocnemius muscle and BAT, we observed that the ratio of acetylated nuclear PGC-1α to total nuclear PGC-1α protein was significantly decreased in RSV-treated mice, suggesting that PGC-1α

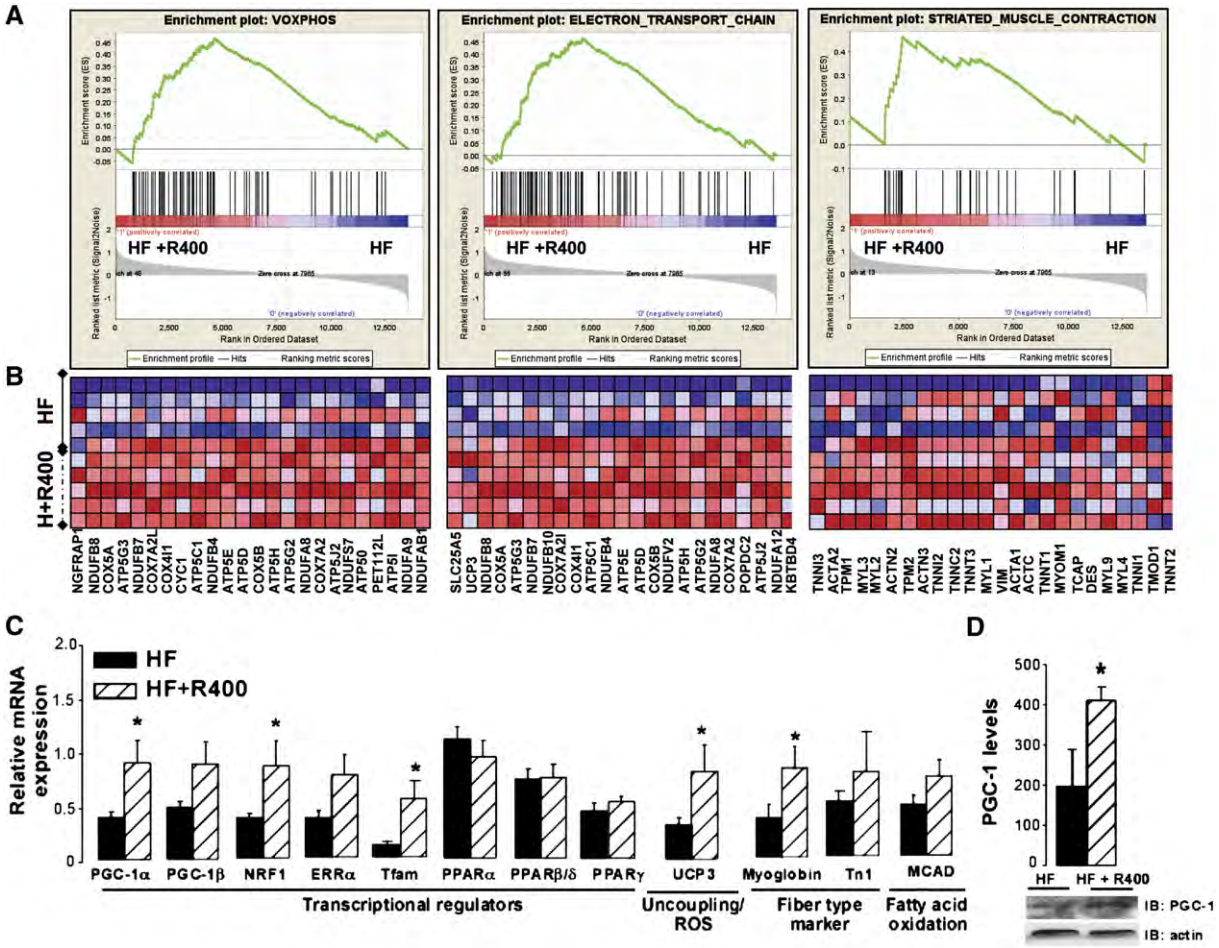


Figure 5. The Gene-Expression Profile of Skeletal Muscle from RSV-Treated Mice Is Enriched in Pathways Related to Mitochondrial Biogenesis and Function

(A) Gene-set enrichment analysis (GSEA) of gene-expression profile in gastrocnemius muscles isolated from HF-fed male C57Bl/6J mice treated with or without RSV (400 mpk, HF + R400). N = 5 animals/group. Three plots are shown where the FDR was <10% for both oxidative phosphorylation pathway and electron transport chain and <33% for striated muscle contraction. The top part of each plot shows the progression of the running enrichment score and the maximum peak therein. The middle part shows the genes in the gene set as "hits" against the ranked list of genes. The lower part shows the histogram for the ranked list of all genes in the expression data set. See Experimental Procedures and Results for more detailed description.

(B) The corresponding heat maps show the expression values for the top subset of genes of each pathway, which contributes most to the enrichment score in the five mice of each group. Results are transformed into colors, where red indicates a high and blue a low expression value.

(C) Relative mRNA expression levels of key genes related to mitochondrial function as measured by Q-RT-PCR in the gastrocnemius muscle of HF or HF + R400 mice. N=5-6 animals/group. Data represent mRNA levels relative to cyclophilin and are given as means ± SEM.

(D) Quantification and representative western blot of PGC-1 protein in nuclear extracts from the gastrocnemius muscle of RSV treated and nontreated HF fed animals. Values represent arbitrary density units ± SEM (n = 3 animals/group), and actin serves as loading control. * = $P < 0.05$.

activity was also increased (Figure 6A). In contrast, no effect on PGC-1α acetylation was observed in heart of the RSV-treated HF mice (Figure 6A), indicating a certain tissue specificity in RSV's effects.

To test whether RSV's effects on mitochondrial function are mediated by SIRT1 and PGC-1α, we coinfected C2C12 myotubes with an adenovirus expressing PGC-1α, together with either a specific short hairpin RNA (shRNA) directed against SIRT1 or a control shRNA. This strategy effectively reduced endogenous SIRT1 levels while still maintaining high PGC-1α expression (Figure 6B, top panel). Importantly the knockdown of the SIRT1 protein largely blocked the RSV-induced increase in MCAD, cytochrome C (CytC), and ERRα expression, (Figure 6B, bottom panels). This experiment also demonstrated the dependence of RSV's effect on PGC-1α, since no significant increases in mRNA expression of CytC, MCAD, or ERRα were observed in RSV-treated C2C12 cells in the absence of PGC-1α (Figure 6B).

To further consolidate our hypothesis that SIRT1 deacetylates and hence activates PGC-1α, we compared the efficacy of RSV to induce gene expression in cells infected

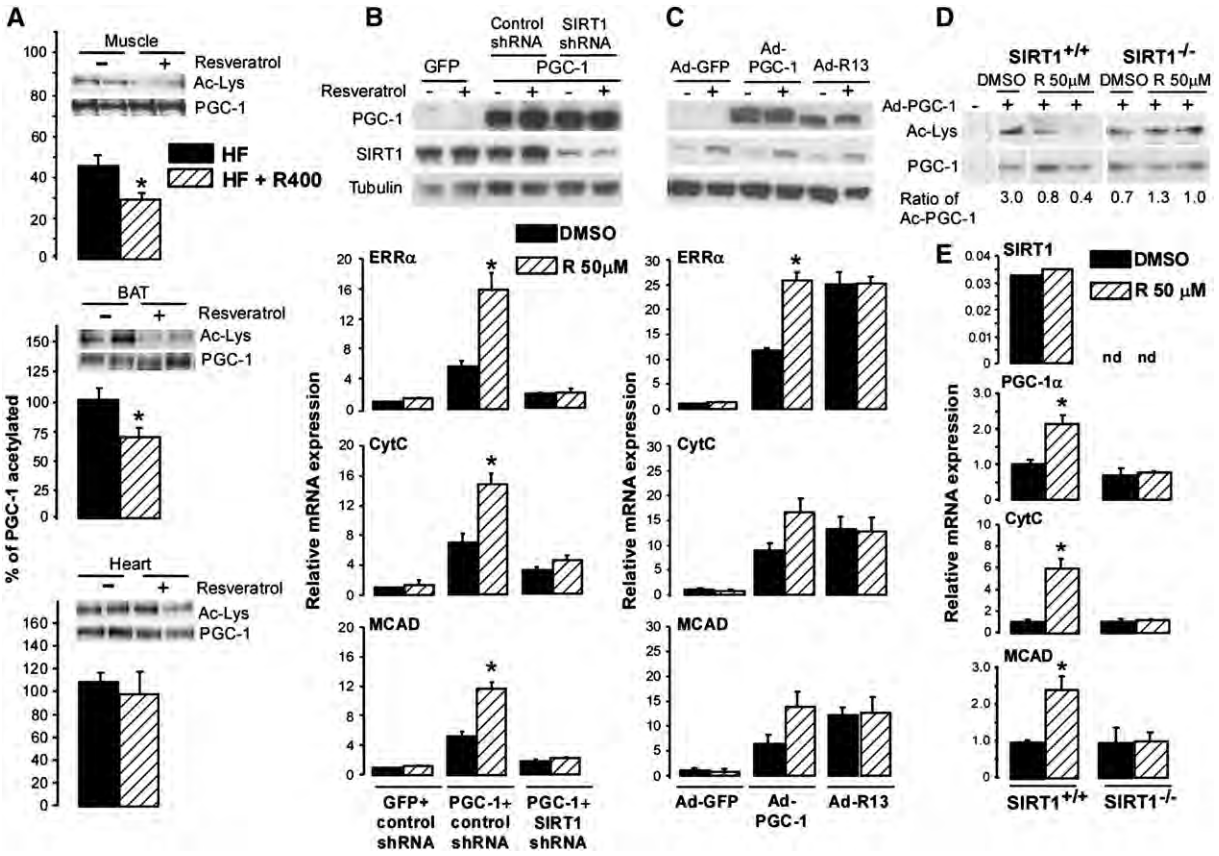


Figure 6. Muscle PGC-1α Is a Molecular Target of RSV In Vivo

(A) Representative western blots and quantification showing the relative amount of acetylated versus total PGC-1 protein, for gastrocnemius muscle, BAT, and heart. PGC-1 was immunoprecipitated (IP) from nuclear extracts and then immunoblotted with either an antiacetylated lysine antibody to determine the extent of PGC-1 acetylation (Ac-Lys) or a PGC-1 antibody to determine the total amount of PGC-1. N > 3 animals/group.

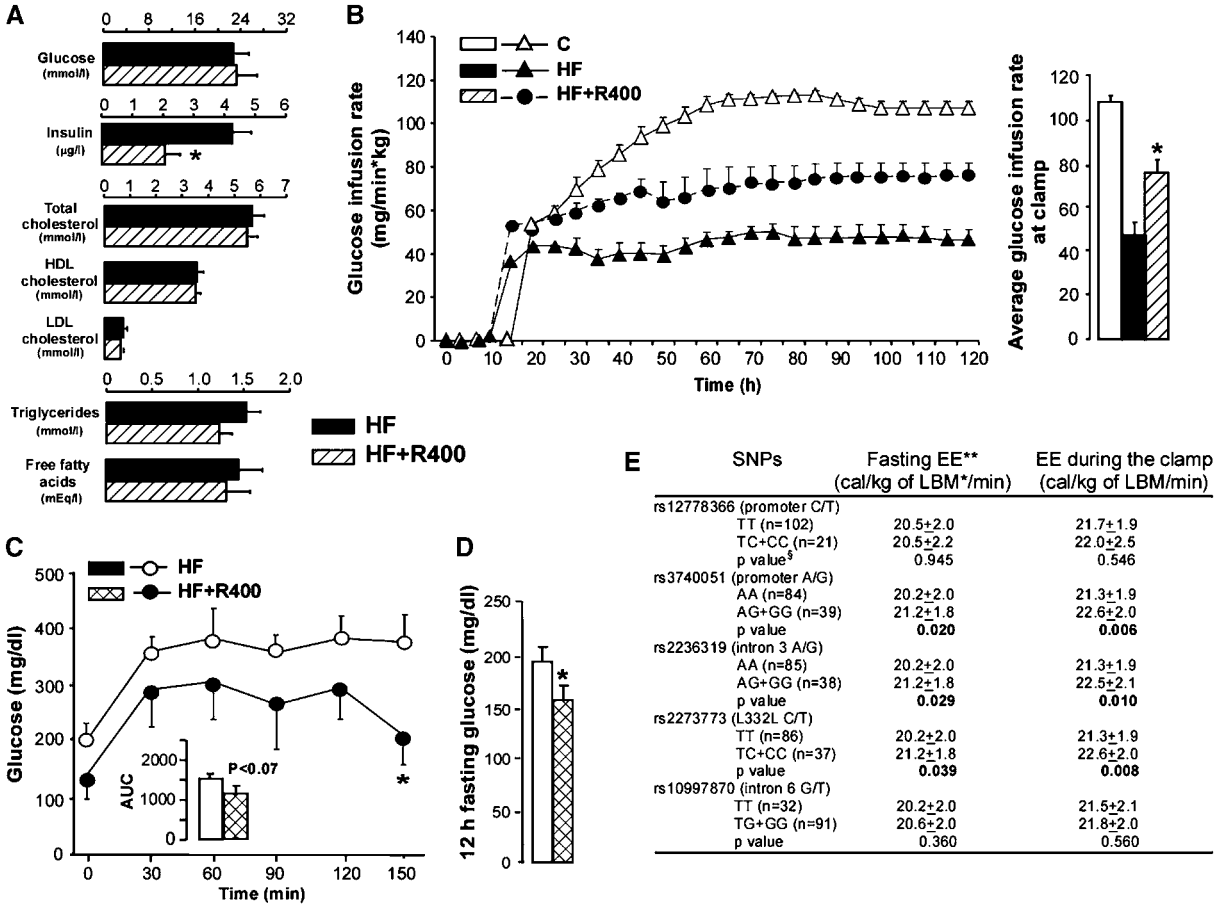
(B and C) C2C12 myotubes were coinfected with an adenovirus expressing either PGC-1α (Ad-PGC-1) or GFP and a SIRT1 shRNA or a control shRNA or a PGC-1α acetylation mutant (Ad-R13). Following 24 hr incubation with DMSO or RSV (R, 50 μM), cells were harvested for protein and RNA extraction. A representative western blot showing the protein expression levels of PGC-1 (wild-type or acetylation mutant) and SIRT1 in these cells is shown. Tubulin was used as a loading control. The mRNA expression levels of ERRα, CytC, and MCAD were determined by Q-RT-PCR. Values represent the mRNA levels relative to the housekeeping gene 36B4, * = $P < 0.05$.

(D) $SIRT1^{-/-}$ and $SIRT^{+/+}$ MEFs were infected with Ad-PGC-1 and treated with DMSO or RSV (R 50 μM) for 24 hr. PGC-1 was immunoprecipitated from lysates with a flag M2 antibody. Acetylated PGC-1 (Ac-Lys) was revealed by an antiacetylated-lysine antibody and total PGC-1 levels by an HA antibody. A representative western blot is shown and the quantification of the ratio of acetylated to nonacetylated PGC-1, n = 2 animals/group.

(E) Gene-expression levels are as measured in $SIRT1^{-/-}$ and $^{+/+}$MEFs following a 24 hr incubation with either DMSO or RSV (R, 50 μM). mRNA levels are relative to the 36B4 gene, * = $P < 0.05$ (n = 3). n.d. = not detected. Values represent means ± SEM.

with an adenovirus that either encoded the wild-type (WT) PGC-1α or the R13-PGC-1α protein in which 13 of the potential lysine acetylation sites were mutated into arginine (Rodgers et al., 2005) (Figure 6C). Since the capacity for acetylation is impaired for the R13-PGC-1α protein, it was no surprise that expression of the R13-PGC-1α mutant alone induced the PGC-1α target genes, ERRα, CytC, and MCAD, to a higher level as compared to WT PGC-1α. Importantly, addition of RSV failed to further induce the expression level of these PGC-1α target genes in the R13-PGC-1α infected C2C12 cells (Figure 6C), which is in sharp contrast to cells infected with WT PGC-1α and consistent with the dependence of RSV on SIRT1-mediated deacetylation of PGC-1α to activate PGC-1α transcriptional programs.

Finally, we sought in vivo support for the dependency of RSV effects on SIRT1 by using mouse embryonic fibroblasts (MEFs) isolated from $SIRT1^{+/+}$ and $SIRT^{-/-}$ mice (Chua et al., 2005). In contrast to WT MEFs, in $SIRT1^{-/-}$ MEFs, RSV did not decrease PGC-1 acetylation (Figure 6D), and there was no significant effect of RSV on expression of CytC, MCAD, and PGC-1α (Figure 6E), results entirely consistent with the crucial role of SIRT1 in mediating RSV's activity. The demonstration that RSV treatment results in deacetylation of PGC-1α and modulation of the expression of PGC-1α target genes in the muscle and



E

SNPs	Fasting EE** (cal/kg of LBM*/min)	EE during the clamp (cal/kg of LBM/min)
rs12778366 (promoter C/T)		
TT (n=102)	20.5±2.0	21.7±1.9
TC+CC (n=21)	20.5±2.2	22.0±2.5
p value§	0.945	0.546
rs3740051 (promoter A/G)		
AA (n=84)	20.2±2.0	21.3±1.9
AG+GG (n=39)	21.2±1.8	22.6±2.0
p value	**0.020**	**0.006**
rs2236319 (intron 3 A/G)		
AA (n=85)	20.2±2.0	21.3±1.9
AG+GG (n=38)	21.2±1.8	22.5±2.1
p value	**0.029**	**0.010**
rs2273773 (L332L C/T)		
TT (n=86)	20.2±2.0	21.3±1.9
TC+CC (n=37)	21.2±1.8	22.6±2.0
p value	**0.039**	**0.008**
rs10997870 (intron 6 G/T)		
TT (n=32)	20.2±2.0	21.5±2.1
TG+GG (n=91)	20.6±2.0	21.8±2.0
p value	0.360	0.560

Figure 7. RSV Increases Insulin Sensitivity and the Association of the Sirt1 gene with Energy Expenditure in Humans

(A) Average glucose, insulin, and lipid levels in HF-fed C57Bl/6J mice treated with or without RSV (HF + R400) for 16 weeks. N = 8–10 animals/group.

(B) Evolution of the glucose infusion rate (GIR) during the hyperinsulinemic euglycemic clamp on C57Bl/6J mice treated with chow (C) or HF diet or HF + R400. N = 4 animals/group. The average GIR at clamp is shown in the bar graph.

(C and D) Eight week-old male KKAy mice were treated with HF diet or HF diet plus RSV at a dose of 400 mpk (HF+R400) for 8 weeks. N = 5 animals/group. OGTT was performed, and the AUCs are shown in the inset bar graph (C). Fasting (12 hr) and nonfasting plasma glucose in KKAy mice treated with HF or HF + R400 (D). Values represent means ± SEM. * = $P < 0.05$.

(E) The association of SNPs of the *Sirt1* gene with EE as measured in normal weight offspring of probands with type 2 diabetes (n = 123). *LBM, lean body mass, ** EE, energy expenditure mean ± SD, p values adjusted for age, gender, and familial relationship.

BAT (Figure 6A), in combination with the absence of an effect of RSV on gene expression, when SIRT1 expression is reduced or eliminated (Figures 6B and 6E) and/or when the acetylation sites specifically targeted by SIRT1 were mutated in PGC-1α (Figure 6C), is highly suggestive that RSV relies to a large extent on SIRT1 activation and PGC-1 deacetylation to achieve its effects on PGC-1α-dependent gene expression in vivo.

Improved Insulin Sensitivity in RSV-Treated Mice

Since genomic profiling of human diabetic muscle revealed a coordinated decrease in expression of genes related to OXPHOS (Mootha et al., 2003), we determined whether the effects of RSV on mitochondrial metabolism translated into changed insulin sensitivity. Although fasting glucose levels were not altered by RSV, fasting insulin levels were significantly reduced, suggesting an insulin sensitization (Figure 7A). We thus performed a hyperinsulinemic euglycemic clamp study in these mice. In line with the fact that diet-induced obesity decreases insulin sensitivity, a significant decrease in the glucose infusion rate (GIR) was observed in HF compared to chow-fed mice (Figure 7B). Importantly, however, the GIR in RSV-treated HF mice was significantly higher as compared to HF control animals, indicating that RSV improves insulin sensitivity in a diet-induced obesity model (Figure 7B). No major impact on blood lipid levels was observed after RSV (Figure 7A). We also assessed the effects of RSV in a genetic mouse model of diabesity, the KKAy mouse. KKAy mice were treated with an HF diet without or with RSV (at 400 mpk for 10 weeks). Although in this model RSV did not significantly affect weight gain, glucose tolerance,

as assessed by an oral glucose tolerance test (OGTT, 2 g glucose/kg), was significantly improved by RSV (Figure 7C). This was paralleled by a significant decrease in fasting glucose levels, suggesting that RSV possesses intrinsic antidiabetic effects that are independent of its effects on body weight (Figure 7D).

Genetic Variation in the Human *Sirt1* Gene Is Associated with EE

To determine whether common alleles in *Sirt1* might contribute to heritable phenotypic variation in EE in humans, we investigated the effects of five genetic variants in the *Sirt1* gene on EE as profiled in a cohort of healthy, normal-weight (body mass index < 26.0 kg/m^2), nondiabetic offspring of type 2 diabetic patients (Ferrannini et al., 1988). Three out of five SNPs tested (i.e., rs3740051 [promoter A/G], rs2236319 [intron 3 A/G], and rs 2273773 [L332L C/T]) were significantly associated with whole body EE as evaluated either during fasting or during a hyperinsulinemic clamp (Figure 7E). These data indicate that in humans, *Sirt1* genetic polymorphisms covary with the degree of EE, which provides an independent genetic argument that bolsters the direct involvement of SIRT1 in modulating EE that we uncovered by manipulating its activity pharmacologically with RSV in mice.

DISCUSSION

Our data demonstrate that the SIRT1 activator, RSV, induces PGC-1α activity by facilitating SIRT1-mediated deacetylation. The effects of RSV on PGC-1α target gene expression were dependent on the presence of the WT PGC-1α protein and were lost in cases where the acetylation sites in PGC-1α that are targeted by SIRT1 were mutated or when SIRT1 expression was disrupted in either C2C12 myotubes by RNAi or in MEFs isolated from SIRT1-deficient mice. The effects of RSV were seen in both muscle and BAT and resulted in an increase in mitochondrial function, which translated into an increase in EE, improved aerobic capacity, and enhanced sensorimotor function. Importantly, mice on an HF diet were consequently protected from the development of obesity and remained insulin sensitive when they were treated with RSV. Our observations therefore extend the function of the SIRT1-PGC-1α axis beyond control of liver gluconeogenesis (Rodgers et al., 2005) to adaptive thermogenesis in the BAT and muscle function. Although most of our conclusions are based on cellular studies and pharmacological interventions in mice, the novel association between genetic variations in the *Sirt1* gene and energy homeostasis in man reveals a significant place for our work in the context of human pathophysiology.

In the BAT, RSV treatment induced striking mitochondrial morphological changes and also increased UCP-1 expression levels and thus poised the mitochondria for uncoupling of respiration (Puigserver et al., 1998). This effect is consistent with the observed increase in cold tolerance and goes a long way in explaining their increase in EE and resistance to weight gain. Surprisingly, though, we did not observe similar changes in mitochondrial biogenesis by RSV in the heart, despite the coexpression of PGC-1α and SIRT1 (Figure S3). PGC-1α expression, PGC-1α acetylation, and heart function were not altered by RSV. As cardiac-specific PGC-1α overexpression in mice ultimately results in cardiomyopathy and death (Lehman et al., 2000), the absence of an effect of RSV on mitochondrial biogenesis in the heart is interesting. Although in the liver, changes in expression of genes related to OXPHOS showed a tendency to increase, other known PGC-1 target genes related to gluconeogenesis were unaffected by RSV. Therefore, we suspect that cell-specific associations between PGC-1α and other transcription factors or cofactors may exist to modulate tissue-specific transcriptional consequences of RSV.

A striking feature of the myofiber is its ability to transform and remodel in response to environmental demands (Booth et al., 2002). The most notable is exercise training, which transforms the metabolic status of the myofiber to one of increased oxidative metabolism and switches the fiber from one of a fast twitch type 2 to a slow twitch type 1 (Booth et al., 2002). In this study, RSV treatment of HF-fed mice induced a similar myofiber remodeling but in the absence of increased physical activity. The myofibers from RSV-treated mice were enriched in mitochondria, exhibited enhanced oxidative capacity, and displayed a higher resistance to fatigue because of the concerted activation of a genetic program geared for aerobic metabolism. Although we were unable to prove that these progressive changes in oxidative capacity by RSV lasted long enough to induce a complete type 1 fiber transformation, we did see advanced improvement in motor function, which is a component of the integrated physiological response required to improve exercise performance. Comparable changes in muscle fiber types have been recapitulated in genetically engineered mouse models that trigger calcium regulatory pathways (Wu et al., 2002), mimic PPARβ/δ activation (Wang et al., 2004), or enhance PGC-1α activity (Lin et al., 2002a). The fact that RSV induces a muscle fiber type switch in the absence of genetic engineering underscores its powerful pharmacological activities. RSV could hence be viewed as a performance-enhancing drug, which, in contrast to other pharmacological mediators, such as anabolic steroids, improves performance by changing myofiber specificity rather than by increasing muscle mass.

Different cells and tissues have distinct sensitivities and requirements of mitochondrial function. Neurons appear particularly vulnerable to mitochondrial dysfunction, as testified by the many neurodegenerative diseases, including Alzheimer's and Huntington's diseases, which have been associated with abnormal mitochondrial activity and dynamics (Chan, 2006). Interestingly, we noted a significant improvement in motor coordination and traction force, as well as enhanced aerobic performance, in RSV-treated mice, suggesting a potential beneficial neuronal effect of RSV. In the brain, PGC-1α deficiency in mice leads

to certain behavioral abnormalities, including profound hyperactivity with neurodegeneration, reminiscent of Huntington's disease (Leone et al., 2005; Lin et al., 2004). Interestingly, we noted a significant decrease in spontaneous locomotor activity in RSV-treated mice, which is converse to the hyperactive phenotype of the PGC-1$\alpha^{-/-}$ mice, thus pointing once more to a potential connection between mitochondrial activation and PGC-1α, this time in the CNS. In fitting with these potential neuroprotective effects has been the recent observations that RSV rescued neuronal dysfunction induced by the polyglutamine tracts in the Huntington protein in *C. elegans* (Parker et al., 2005) and significantly delayed the age-dependent decay of locomotor activity and cognitive performances in the short-lived vertebrate, *N. furzeri* (Valenzano et al., 2006). A potential connection with SIRT1 becomes apparent in the remarkable protection against axonal degeneration afforded by SIRT1 activation in the Wallerian degeneration slow mice, an effect that can be reproduced in vitro on dorsal root ganglion cultures by RSV (Araki et al., 2004).

Mitochondrial function can impact on whole-body metabolism. This is most evident in the muscle, a metabolically flexible tissue that switches between carbohydrate and lipid as substrates in order to meet the energy demands (Kelly and Scarpulla, 2004). Indeed, impaired mitochondrial function that directs fatty acids toward storage, as opposed to oxidation, may contribute considerably to intramyocellular lipid accumulation, which has been linked to insulin resistance in obesity and type 2 diabetes in humans (Patti et al., 2003; Petersen et al., 2004; Virkamaki et al., 2001). In line with this, RSV significantly improved both muscle oxidative capacity and sensitivity to insulin in HF-fed mice. Although the RQ, reflective of whole-body substrates use, was unchanged under RSV treatment, gene-expression analysis in the gastrocnemius supported an increase in fatty-acid oxidation since MCAD (Figure 5C) expression was increased and glucose utilization reduced as PDK4 levels were increased (data not shown) (Kim et al., 2006). Complimenting the effects on tissues metabolizing fat, such as muscle and BAT, was the effect of RSV on storage tissues such as WAT, where it reduced both fat pad mass and adipocyte size. Consistent with such wide-spread effects of RSV on fat and muscle was the previous work showing increased fat mobilization by genetically manipulating SIRT1 activity (Picard et al., 2004), as was the capacity of SIRT1 to modulate muscle-cell differentiation (Fulco et al., 2003).

Admittedly, RSV is reported to have pleiotropic properties, including the activation of signaling pathways involving AMPK, thyroid hormone, and estrogen (Baur and Sinclair, 2006; Baur et al., 2006). However, in the muscle-microarray analysis, we did not observe enrichment of gene expression in pathways related, for example, to estrogen or thyroid signaling. Together, with our data demonstrating that the muscle gene-expression changes are critically dependent on the presence of SIRT1, our data confirm the fact that SIRT1 is the main target of RSV's metabolic actions (Howitz et al., 2003). At this point, we cannot determine whether PGC-1α is the only target of RSV-activated SIRT1. However, evidence supporting the importance of PGC-1α in mediating effects of RSV on mitochondrial gene expression in muscle cells is the fact that RSV's effects were not observed unless the wild-type PGC-1α protein was overexpressed and that these effects are lost in cases where the acetylation sites, targeted by SIRT1, were mutated in PGC-1α. Furthermore, the effects of RSV in the muscle and BAT recapitulate those observed by stimulating PGC-1α activity and are hence consistent with the convergence between SIRT1 and PGC-1α activation described in the hepatocyte (Rodgers et al., 2005). Despite this, we cannot exclude unequivocally that PGC-1α is the sole target of SIRT1, as SIRT1 interacts with and deacetylates other substrates (Blander and Guarente, 2004), including potential regulators of metabolism and mitochondrial function such as FOXO1 (Brunet et al., 2004; Motta et al., 2004) and p53 (Matoba et al., 2006). Finally, it is possible that the consequence of RSV activation of SIRT1 is different in other tissues, since unlike the stimulation of PGC-1α activity seen here in muscle and previously reported in liver (Rodgers et al., 2005), in the PC12 adrenal cell line, PGC-1α activity was inhibited by SIRT1 (Nemoto et al., 2005).

Since mitochondria are recognized organelles for aerobic production of high-energy phosphates and bear a central role in cellular metabolism, especially in tissues with high metabolic intensity, it is not surprising that their dysfunction has been associated with cardiovascular, metabolic, and neurodegenerative diseases. Our studies genetically and pharmacologically associate SIRT1 with PGC-1α and EE and warrant the further evaluation of SIRT1 activators as a strategy to prevent and/or treat these common disorders. This could be particularly appealing in the metabolic arena, where physical activity and dietary restriction, the cornerstones of clinical management of the metabolic syndrome, are known to enhance mitochondrial activity. It is tempting to speculate that the basis of the French paradox and the beneficial effect of RSV on life span could be attributed in part to the prevention of chronic cardiovascular, metabolic, and neurodegenerative diseases, important determinants of mortality in the industrialized world. This claim is supported by data in a concurrent study, which demonstrated that long-term RSV administration extended life spans of mice (Baur et al., 2006).

EXPERIMENTAL PROCEDURES

In Vivo Analysis

Four to eight week male C57Bl/6J mice from Charles River (L'Arbresle, France) and 8 week male KKAy mice from Clea (Tokyo, Japan) were housed in specific pathogen-free conditions with a 12 hr light-dark cycle and had free access to water and food. RSV (Orchid, Chennai, India) was mixed with either powdered chow (DO4, UAR, France) or HF diet (D12327, Research diet, New Brunswick, USA) at a concentration of 4 g/kg of food to provide a 400 mpk dose, and pellets were then reconstituted. Control groups received pellets without drug. Body weight and caloric intake were monitored throughout the experiments.

The protocols used to assess behavioral, cardiac, and metabolic phenotypes included the following: body composition by DEXA; EE by indirect calorimetry (13 hr, food and water) and 4°C cold test (6 hr); circadian activity by metabolic cage monitoring (32 hr); anxiety by open field, elevated-plus-maze, and light/dark test; locomoter function by rotarod, string and grip strength test; blood pressure and heart rate by tail-cuff system; cardiac anatomy and systolic and diastolic function by echocardiography; glucose sensitivity by an oral glucose tolerance test (16 hr fasted, 2 g glucose/kg mouse), and hyperinsulinemic euglycemic clamp (4 hr fast, 18 mU insulin/kg/min, clamped at 5.5 mmol/L for 60 min); and endurance test by variable speed belt treadmill and incremental speed protocol (range from 18 cm/s to 40 cm/s on habituated 2 hr fasted mice). These tests were performed as outlined in the standard operating procedures (SOP) linked to the EMPReSS website http://empress.har.mrc.ac.uk and as described in the Supplemental Experimental Procedures.

Ex Vivo Analysis

O_2 consumption was measured in glycolytic fibers isolated from gastrocnemius muscle, using the technique as described (N'Guessan et al., 2004). See Supplemental Experimental Procedures for a brief description.

Histological and Biochemical Analysis

Histological analysis, including HE and SDH staining and electron microscopy (EM), were performed as outlined on the EMPReSS website (http://empress.har.mrc.ac.uk) and described in the Supplemental Experimental Procedures. Mitochondria in EM images were quantified using Image J version 1.36b.

Citrate synthase activity in gastrocnemius muscle extracts was determined spectrophotometrically (Ceddia et al., 2000). Fecal lipids, including triglyceride and cholesterol content, were measured enzymatically, using commercially available kits and manufacturers protocol (WAKO, Richmond, VA), following a Folch extraction. Blood plasma was analyzed for insulin by ELISA (Crystal Chem, Downers Grove, IL), glucose by glucose oxidase kit (Sigma, Lyon, France) and free fatty acids, triglycerides, HDL, LDL, AST, ALT, and total cholesterol using enzymatic assays (Boehringer-Mannheim, Mannheim, Germany) on an Olympus automated analyzer.

In Vitro Analysis

SIRT1$^{-/-}$ and $^{+/+}$ MEFs (Chua et al., 2005) and the C2C12 mouse myoblast cell line were maintained in culture as described previously (Rodgers et al., 2005). Following C2C12 myotube differentiation, cells were infected with adenovirus expressing either Flag-HA-PGC-1α, Flag-HA-R13, or Sirt1 shRNA (Rodgers et al., 2005). MEFs and C2C12 were treated for 24 hr with 50 μM RSV or DMSO.

DNA, RNA, and Protein Analysis

Total DNA was extracted as described in the Supplemental Experimental Procedures, and quantitative (Q) PCR was performed using mitochondrial DNA and genomic DNA-specific primers.

RNA was extracted using the TRIzol® reagent (Invitrogen, Carlsbad, CA). Q-RT-PCR was performed as described (Watanabe et al., 2004). Primer details are listed online (Table S2). Affymetrix mouse 430_2 microarray analysis was performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Data were analyzed by Affymetrix MAS 5.0 software and GSEA (http://www.broad.mit.edu/gsea) (Mootha et al., 2003; Subramanian et al., 2005).

Nuclear protein fractions from gastrocnemius muscle were prepared as described online. Protein extracts were separated by SDS-PAGE and immunoblotted using antibodies against PGC-1 (H-300, Santa Cruz, CA), SIRT1 (anti-Sir2, Upstate, Norcross, GA), tubulin (Upstate) and actin (Santa Cruz, CA). PGC-1 acetylation was analyzed by immunoprecipitation of PGC-1 from nuclear lysates (50 μg) with anti-PGC-1 antibody (1 μg) followed by western blot using an acetyl-lysine antibody (Cell Signaling, Danvers, MA) (Rodgers et al., 2005).

Clinical Genetic Study

The collection of subjects and the study protocol have been published (Salmenniemi et al., 2004), and a brief summary is available online. The study protocol was approved by the Ethics Committee of the University of Kuopio, and all subjects gave an informed consent. The mean age and BMI of the subjects was 34 years and 23 kg/m^2, respectively. All subjects underwent an OGTT. Indirect calorimetry was performed in the fasting state and during hyperinsulinemia (40 mU/m^2/min insulin infusion for 120 min) as described (Salmenniemi et al., 2004). The rates of EE were calculated according to Ferrannini et al. (1988). Selection of the SNPs of *Sirt1* was based on linkage disequilibrium and haplotype block analysis of the HapMap project data (http://www.hapmap.org; Public Release #20/Phase II, January 24, 2006; population: Utah residents with ancestry from northern and western Europe).

Statistics

Statistical analyses were performed with the Student's t test for independent samples (nonparametric), and data are expressed as means ± SEM unless specified otherwise. P value > 0.05 was considered as statistically significant.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/127/6/1109/DC1/.

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Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1α

Zachary Gerhart-Hines[1,2], Joseph T Rodgers[1,2], Olivia Bare[3], Carles Lerin[1,2], Seung-Hee Kim[1,2], Raul Mostoslavsky[4], Frederick W Alt[4], Zhidan Wu[3] and Pere Puigserver[1,2,*]

[1]Dana-Farber Cancer Institute and Department of Cell Biology, Harvard Medical School, Boston, MA, USA, [2]Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, USA, [3]Novartis Institutes for Biomedical Research Inc., Cambridge, MA, USA and [4]Howard Hughes Medical Institute, Department of Genetics, The Children's Hospital, CBR Institute for Biomedical Research, Harvard Medical School, Boston, MA, USA

In mammals, maintenance of energy and nutrient homeostasis during food deprivation is accomplished through an increase in mitochondrial fatty acid oxidation in peripheral tissues. An important component that drives this cellular oxidative process is the transcriptional coactivator PGC-1α. Here, we show that fasting induced PGC-1α deacetylation in skeletal muscle and that SIRT1 deacetylation of PGC-1α is required for activation of mitochondrial fatty acid oxidation genes. Moreover, expression of the acetyltransferase, GCN5, or the SIRT1 inhibitor, nicotinamide, induces PGC-1α acetylation and decreases expression of PGC-1α target genes in myotubes. Consistent with a switch from glucose to fatty acid oxidation that occurs in nutrient deprivation states, SIRT1 is required for induction and maintenance of fatty acid oxidation in response to low glucose concentrations. Thus, we have identified SIRT1 as a functional regulator of PGC-1α that induces a metabolic gene transcription program of mitochondrial fatty acid oxidation. These results have implications for understanding selective nutrient adaptation and how it might impact lifespan or metabolic diseases such as obesity and diabetes.

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Introduction

Mammals have adapted to different environmental conditions and food by coordinating changes in tissue-specific metabolic pathways to maintain energy and nutrient homeostasis. Among the changes in food availability, nutrient deprivation triggers a whole rearrangement of glucose and lipid metabolism in key metabolic tissues such as skeletal muscle and liver. As glucose is the main fuel substrate for neuronal and red blood cells, peripheral tissues undergo a major shift from glucose to fatty acid oxidation, and gluconeogenic precursors such as lactate and alanine are delivered from muscle to the liver to synthesize glucose (Storlien *et al*, 2004). In muscle cells, a major regulatory metabolic enzyme that allows this nutrient shift is pyruvate dehydrogenase kinase-4 (PDK4) that inactivates pyruvate dehydrogenase (PDH) by phosphorylation and prevents the entry of pyruvate into the TCA cycle. PDK4 enzymatic activity is regulated by nutrient deprivation (Sugden *et al*, 1993). At the transcriptional level, several transcription factors including FOXO1, ERRα and PPARα are activators of PDK4 gene expression (Pilegaard and Neufer, 2004). Interestingly, PGC-1α, a common transcriptional coactivator of these factors, also induces PDK4, consistent with a key role of PGC-1α in fatty acid utilization (Wende *et al*, 2005). Furthermore, as PGC-1α induces OXPHOS genes involved in the final step of electron transport chain and ATP synthesis, it allows complete mitochondrial oxidation of fatty acids (Wu *et al*, 1999; Koves *et al*, 2005).

Nutrient or caloric restriction results in major tissue-specific metabolic changes that ultimately cause an increase of longevity in many different organisms (Bordone and Guarente, 2005; Sinclair, 2005). A key component of this response is Sir2, an NAD^+-dependent protein deacetylase that mediates increases of lifespan in response to restriction of nutrients (Bordone and Guarente, 2005). Genetic evidence for a role of Sir2 in this nutrient/aging pathway has been provided in model organisms such as yeast (Kaeberlein *et al*, 1999), worms (Tissenbaum and Guarente, 2001) and flies (Rogina and Helfand, 2004). Interestingly, in yeast, caloric restriction by lowering glucose levels has been associated with increased rates of respiration in an Sir2-dependent pathway to allow a more efficient use of fuel substrates (Lin *et al*, 2000). In mammals, SIRT1 controls different tissue-specific metabolic processes. For example, the ability of SIRT1 to repress PPARγ confers increases of lipolytic rates in white adipose tissue (Picard *et al*, 2004) and insulin secretion in β-pancreatic cells (Bordone *et al*, 2006). Moreover, we have previously shown that SIRT1 is increased in fasted liver to deacetylate and activate PGC-1α to promote hepatic glucose output (Rodgers *et al*, 2005). Together, these metabolic adaptations transcriptionally controlled by SIRT1 might be important for the effects of nutrient restriction in organismal survival and longevity.

PGC-1α is a metabolic coactivator that by interacting with transcription factors induces mitochondrial biogenesis and respiration (Lin *et al*, 2005; Finck and Kelly, 2006). In skeletal muscle cells, PGC-1α is sufficient to convert to type I fibers

*Corresponding author. Dana-Farber Cancer Institute, Department of Cell Biology, Harvard Medical School, One Jimmy Fund Way/ Smith-936C, Boston, MA 02115, USA
Tel.: +1 617 582 7977; Fax: +1 617 632 4770;
E-mail: pere_puigserver@dfci.harvard.edu

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that are rich in mitochondria and highly oxidative (Lin *et al*, 2002). In addition, interaction of PGC-1α with PPARα and ERRα transcription factors potently induces fatty acid oxidation genes such as MCAD and CPT-1b (Vega *et al*, 2000; Huss *et al*, 2002; Mootha *et al*, 2004; Schreiber *et al*, 2004). Interestingly, PGC-1α and mitochondrial OXPHOS targets are downregulated in human skeletal muscle of type II diabetic patients that might be linked to increased intramyocellular triglyceride accumulation and insulin resistance (Mootha *et al*, 2003; Patti *et al*, 2003). In fact, there is a strong correlation between the presence of intramyocellular lipid in skeletal muscle and liver and progression of type II diabetes (Guan *et al*, 2002; Kelley *et al*, 2002; Petersen *et al*, 2004). Therefore, a knowledge of the molecular mechanisms by which cells increase the rates of fatty acid oxidation in response to low nutrients is important to understand the pathophysiology of these metabolic diseases and their connection to aging biology.

We report here the identification of SIRT1 as the main deacetylase of PGC-1α that positively regulates mitochondrial and fatty acid utilization genes. Moreover, SIRT1 is required for increased rates of fatty acid oxidation in response to low glucose, providing a new metabolic regulator that allows mammalian cells to switch from glucose to fatty acid oxidation in nutrient deprivation conditions.

Results

SIRT1 deacetylates PGC-1α in skeletal muscle cells

Skeletal muscle undergoes a major metabolic reprogramming under nutrient deprivation conditions (Shuldiner and McLenithan, 2004). PGC-1α has been previously shown to regulate different metabolic pathways in muscle cells, leading to a more oxidative capacity towards lipid utilization (Lin *et al*, 2005). In liver cells, PGC-1α acetylation decreases in fasting conditions mainly through activation of SIRT1 deacetylase (Rodgers *et al*, 2005). In order to determine whether changes in PGC-1α acetylation also occurred in skeletal muscle, PGC-1α was immunoprecipitated from muscle tissue in fed and fasted conditions. Figure 1A shows that fasting strongly induced PGC-1α deacetylation. To investigate whether SIRT1 could target PGC-1α in cultured muscle cells, we treated C_2C_{12} with nicotinamide, an SIRT1 inhibitor. As shown in Figure 1B, nicotinamide induced PGC-1α acetylation and was blocked with ectopic expression of SIRT1. GCN5 acetyltransferase is part of a major protein complex associated with PGC-1α and acetylates PGC-1α (Lerin *et al*, 2006). Consistent with these results, PGC-1α was also acetylated by GCN5 in muscle cells (Figure 1B). Interestingly, treatment with nicotinamide and GCN5 strongly synergized to acetylate PGC-1α and again expression of SIRT1 largely decreased PGC-1α acetylation. To demonstrate further that SIRT1 is the main PGC-1α deacetylase, we used SIRT1−/− mouse embryonic fibroblasts (MEFs) (Chua *et al*, 2005). As shown in Figure 1C, in SIRT1+/+ MEFs, PGC-1α was deacetylated and treatment with nicotinamide induced its acetylation. In contrast, in SIRT1−/− MEFs, PGC-1α was constitutively acetylated and nicotinamide did not further increase it. Importantly, in these cells, deacetylation of PGC-1α was totally rescued by expression of SIRT1 (Figure 1D).

To demonstrate that SIRT1 localized to PGC-1α target genes in skeletal muscle cells, we performed ChIP assays (Figure 1E). SIRT1 was bound to promoters of PGC-1α targets such as cytochrome *c*, PDK4 and PGC-1α. Ectopic expression of PGC-1α induced a 7–10-fold recruitment of SIRT1 to these promoters. Together, these results indicate that PGC-1α is deacetylated by SIRT1 in skeletal muscle cells and SIRT1 localized to promoters that are controlled by PGC-1α, suggesting a regulatory role of SIRT1 on the expression of these genes.

SIRT1 regulates mitochondrial and fatty acid metabolism

In skeletal muscle cells, PGC-1α activates mitochondrial gene expression in response to energy demands (Wu *et al*, 1999; Lin *et al*, 2002). As SIRT1 was localized to promoters controlled by PGC-1α, we investigated whether SIRT1 regulated PGC-1α-induced mitochondrial gene expression in myotubes infected with adenoviruses expressing PGC-1α and SIRT1 shRNA (Figure 2G). As expected, PGC-1α increased a repertoire of mitochondrial gene expression, including regulatory genes ERRα, genes of the TCA cycle (IDH3α), respiratory chain (cyt-*c*, COXVa) and fatty acid utilization (MCAD, CPT-1b and PDK4). Remarkably, PGC-1α-increased mitochondrial and fatty acid utilization gene expression was largely prevented by knocking down SIRT1 (Figure 2A and B). Mitochondrial transcriptional regulator ERRα, but not NRF-1, followed exactly the same pattern (Figure 2C). However, the glycolytic gene pyruvate kinase did not change under these conditions (Figure 2D). These fluctuations in gene expression translated to an increase in fatty acid oxidation induced by PGC-1α that was prevented by SIRT1 knockdown (Figure 2E). Interestingly, in these conditions, shRNA SIRT1 prevented PGC-1α-decreased glucose oxidation (Figure 2F). These data indicate that SIRT1 is required, at least in part, for maximum expression of a large set of PGC-1α target genes and increased fatty acid oxidation.

The effects of knocking down SIRT1 on gene expression without ectopic PGC-1α expression were very minor in C_2C_{12} muscle cells (Figure 2). As these cells express very low levels of PGC-1α, we used primary skeletal muscle cells that express a significant level of PGC-1α. We tested whether SIRT1 was required to maintain mitochondrial and fatty acid oxidation gene expression. Primary myotubes transduced with an adenovirus expressing SIRT1 shRNA resulted in an efficient knockdown of SIRT1 (Figure 3E). The expression levels of mitochondrial and fatty acid utilization genes (Figure 3A and B) were significantly reduced in the cells transduced with SIRT1 shRNA. Interestingly, PGC-1α and its target mitochondrial transcriptional regulators, ERRα and mtTFA, were also downregulated (Figure 3C). These gene expression changes functionally translated to decreases in TCA cycle measured by citrate synthase activity (Figure 3D). These results indicate that SIRT1 controls a broad set of genes involved in mitochondrial oxidative function in muscle cells.

To have genetic evidence to further support the role of SIRT1 regulating mitochondrial and fatty acid oxidation functions, we analyzed the same set of genes in MEFs lacking SIRT1. SIRT1−/− MEFs displayed a decrease in expression of genes associated with mitochondrial (Figure 4A), fatty acid utilization (Figure 4B) and mitochondrial transcriptional regulators compared with wild-type cells (Figure 4C). To

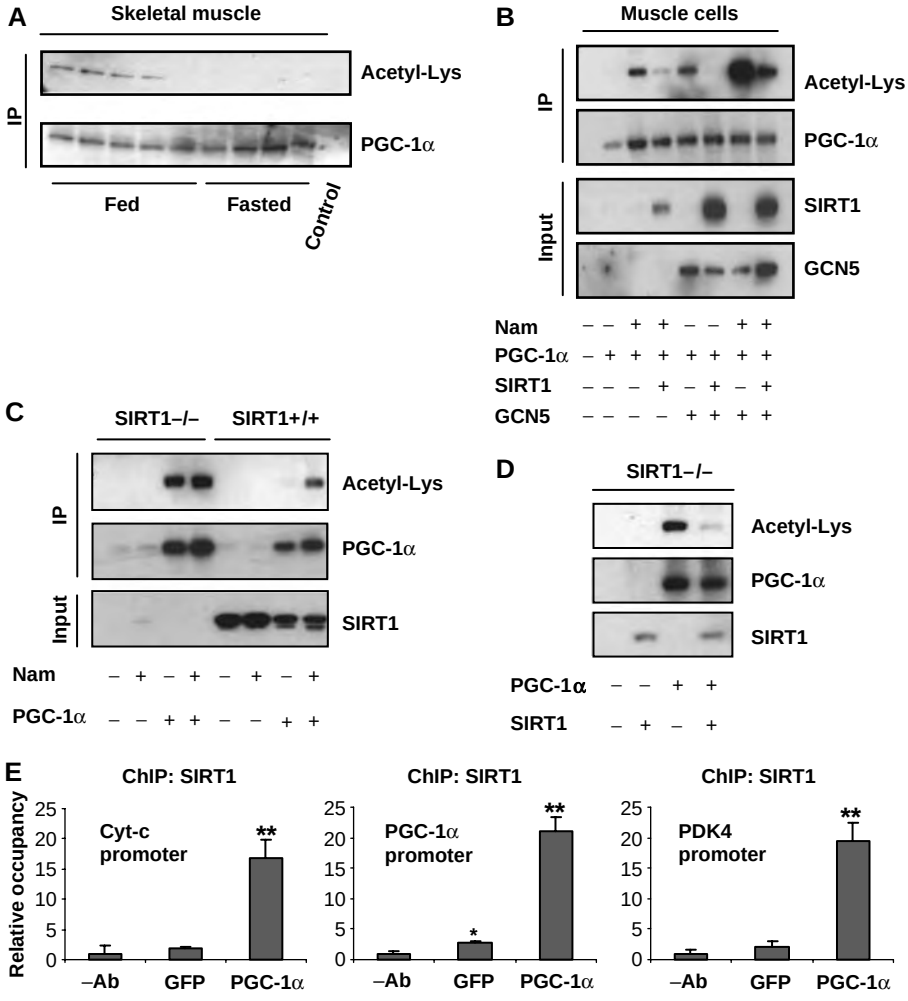


Figure 1 SIRT1 deacetylates PGC-1α in skeletal muscle cells. (**A**) Fasting-induced PGC-1α deacetylation in skeletal muscle. Mice were either fed or fasted for 16 h and skeletal muscle was used to immunoprecipitate PGC-1α and Western blot was performed to detect PGC-1α levels and acetylation. (**B**) Nicotinamide and GCN5 induce PGC-1α acetylation. C_2C_{12} myotubes were treated with 5 mM nicotinamide for 12 h and/or infected with the indicated adenoviruses for 48 h. After treatment, cells were harvested and protein cell extracts were used for PGC-1α immunoprecipitation and Western blot analysis using the specified antibodies. (**C**) PGC-1α is constitutively acetylated in SIRT1−/− MEFs. MEFs were treated with nicotinamide and/or infected with adenoviruses encoding PGC-1α as described in (B). (**D**) SIRT1 rescues PGC-1α deacetylation in SIRT1−/− MEFs. Adenoviruses expressing PGC-1α and SIRT1 were infected in MEFs as described in (B). (**E**) SIRT1 is bound to PGC-1α-targeted gene promoters. C_2C_{12} myotubes were infected with adenoviruses encoding either GFP or PGC-1α. Forty-eight hours after infection, cells were harvested and ChIP analysis using SIRT1 antibody was performed as described in Materials and methods. Values in (E) represent the mean of two experiments performed in triplicate. Error bars represent s.e.m. Statistical analyses were performed using Student's *t*-test. $^{*}P<0.05$ and $^{**}P<0.005$.

directly show that these effects were entirely dependent on SIRT1, we ectopically expressed SIRT1 in SIRT1−/− MEFs. Importantly, this decreased gene expression pattern was entirely rescued by SIRT1 in a dose-dependent manner (Figure 4D). Moreover, these changes in gene expression resulted in a decreased rate of fatty acid oxidation in SIRT1−/− cells (Figure 4E). Taken together, these results indicate that SIRT1 positively regulates complete mitochondrial and fatty acid oxidation, and at least in skeletal muscle cells, SIRT1 largely contributes to the effects of PGC-1α in regulating expression of genes linked to these metabolic pathways.

GCN5 and nicotinamide regulate mitochondrial and fatty acid metabolism in skeletal muscle cells

We have recently identified GCN5 as the main PGC-1α acetyltransferase and a negative regulator of PGC-1α biological functions (Lerin *et al*, 2006). We therefore investigated whether GCN5, by opposing SIRT1-positive effects, would negatively regulate PGC-1α function on mitochondrial and fatty acid utilization genes. Indeed, in C_2C_{12} myotubes, GCN5 largely abolished PGC-1α-induced mitochondrial, fatty acid utilization and mitochondrial transcriptional regulator gene expression (Figure 5A). As C_2C_{12} muscle cells express low levels of endogenous PGC-1α, we again used primary skeletal myotubes to analyze the effects of GCN5 expression. The same pattern of gene expression was observed in these cells, but without ectopically expressing PGC-1α (Supplementary Figure S1). To support that these effects of GCN5 were mediated through acetylation of PGC-1α, we used the SIRT1 inhibitor, nicotinamide. Consistent with the effects of GCN5, treatment of C_2C_{12} or primary skeletal muscle cells with nicotinamide caused a decrease in expression of PGC-1α targeted mitochondrial and fatty acid utilization

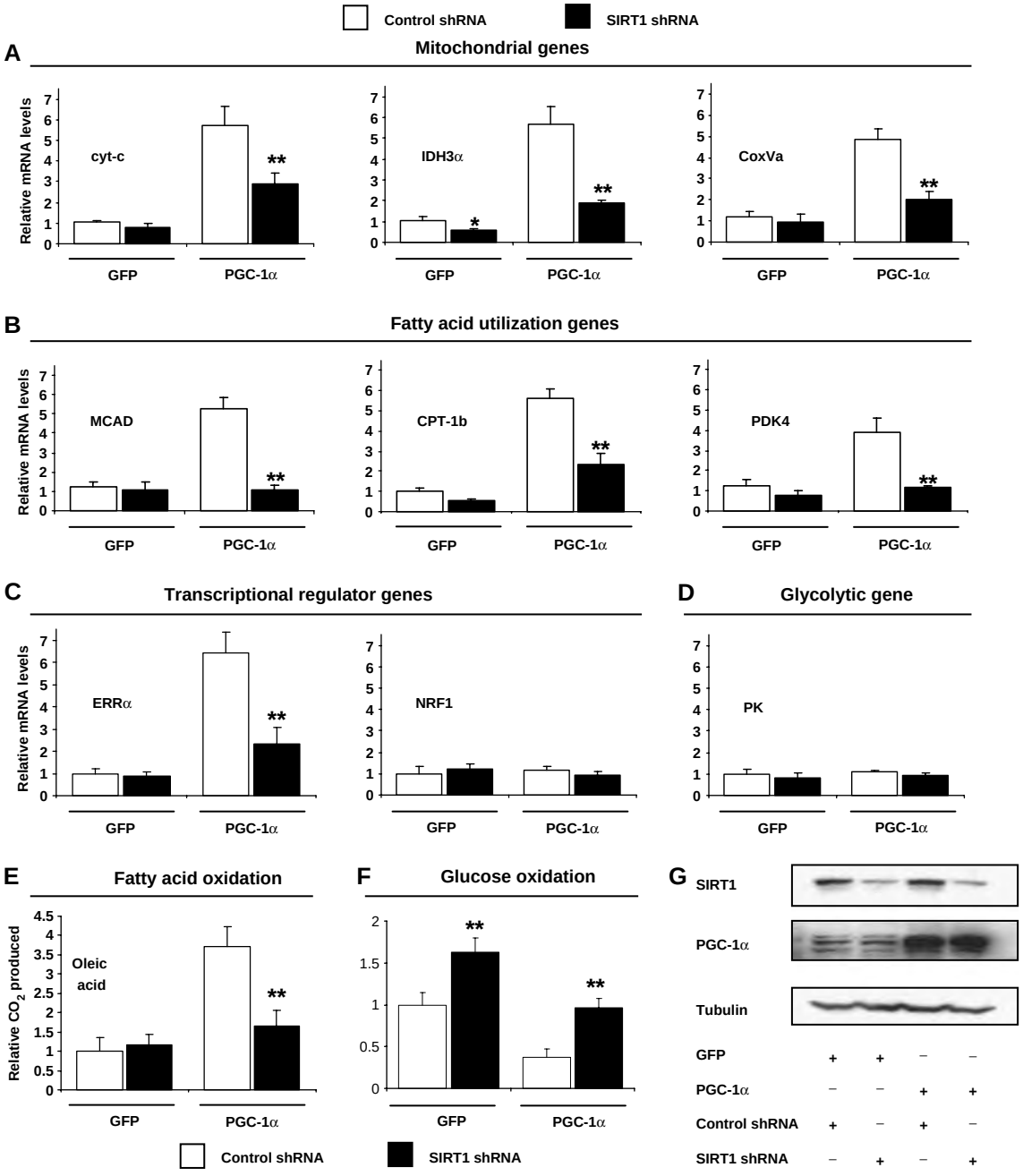


Figure 2 SIRT1 regulates PGC-1α-mediated mitochondrial and fatty acid metabolism in C_2C_{12} skeletal muscle cells. (**A**) SIRT1 knockdown decreases PGC-1α-induced mitochondrial, (**B**) fatty acid utilization, (**C**) ERRα, but not (**D**) pyruvate kinase gene expression. C_2C_{12} myotubes were infected for 3 days with the indicated adenoviruses. After harvesting, RNA was extracted and used for measuring indicated gene expression using quantitative RT–PCR analysis. (**E**) PGC-1α-induced oxidation rates of fatty acids are reduced by SIRT1 knockdown. C_2C_{12} myotubes were treated as in (A). Oleic acid oxidation rates were measured as described in Materials and methods. (**F**) PGC-1α-decreased oxidation rates of glucose are prevented by SIRT1 knockdown. Glucose oxidation rates were measured in C_2C_{12} myotubes as described in Materials and methods. (**G**) Knockdown of SIRT1 in C_2C_{12} myotubes. Cells were infected with the different adenoviruses as in (A–F). Protein cell extracts were used for Western blot analysis that was performed with the indicated antibodies. Values represent the mean of 2–3 experiments performed in duplicate. Error bars represent s.e.m. Statistical analyses were performed using Student's *t*-test. $^{*}P<0.05$ and $^{**}P<0.005$, shRNA control versus shRNA SIRT1.

genes (Figure 5B and Supplementary Figure S2). To demonstrate that these effects were dependent on SIRT1, we again used SIRT1−/− MEFs. As shown in Figure 5C, nicotinamide decreased expression of cyt-*c* and MCAD by approximately three-fold in SIRT1+/+ MEFs; however cells that lack SIRT1 did not decrease these genes in response to nicotinamide. Moreover, and consistent with the effects of transcription on these genes, PGC-1α potently induced fatty acid oxidation that was blocked by expression of GCN5 or nicotinamide treatment (Figure 5D). Together, these results suggest that acetylation of PGC-1α is a regulatory chemical modification that controls the oxidative function of this transcriptional coactivator.

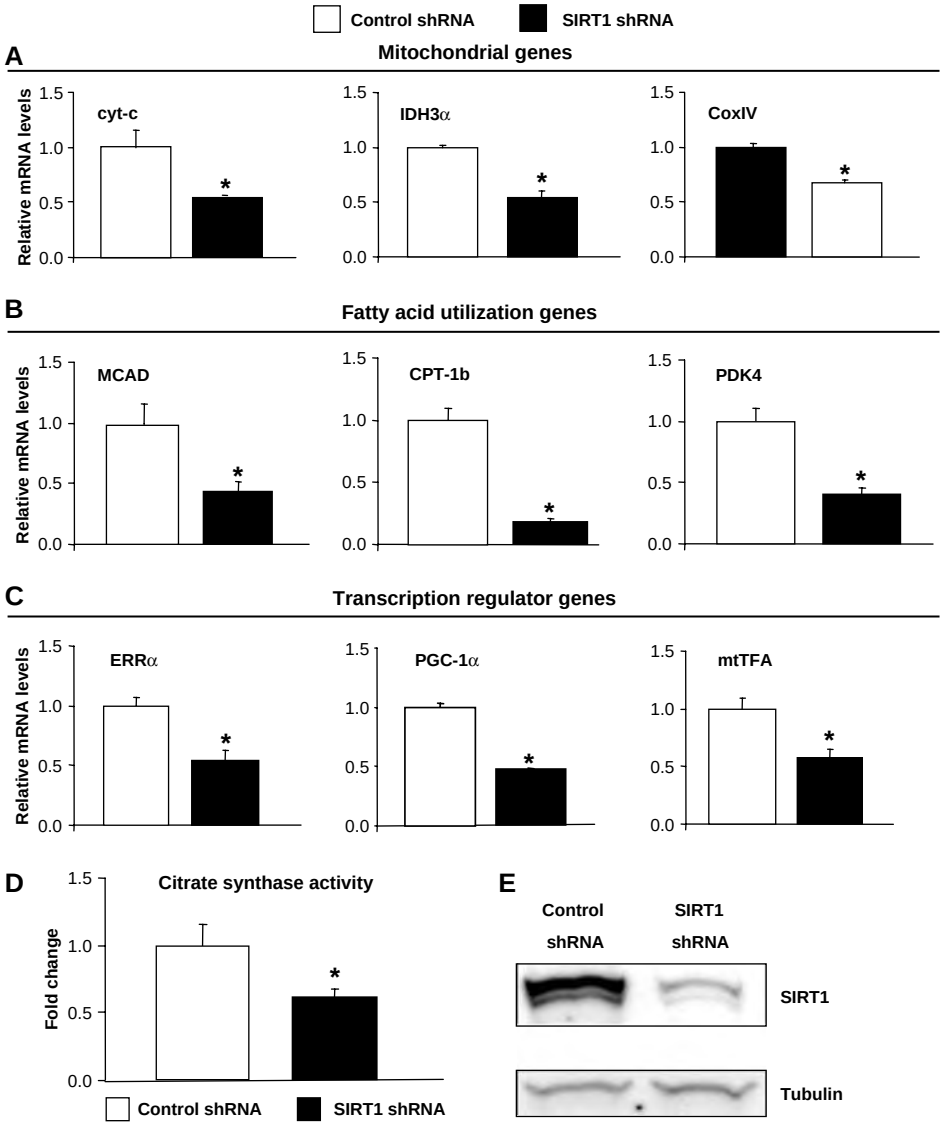


Figure 3 SIRT1 regulates mitochondrial and fatty acid oxidation genes in primary skeletal muscle cells. (**A**) SIRT1 knockdown decreases mitochondrial genes, (**B**) fatty acid utilization genes and (**C**) transcriptional regulator gene expresson in primary myotubes. Mouse primary myotubes were transduced with adenovirus expressing either SIRT1 shRNA or control shRNA for 71 h. Total RNA was extracted and used for measuring indicated gene expression using quantitative RT–PCR. (**D**) SIRT1 knockdown decreases citrate synthase enzymatic activity in primary myotubes. (**E**) Knockdown of SIRT1 in primary skeletal myotubes. Cells were infected with the indicated adenoviruses and protein cell extracts were used to perform Western blot analysis for SIRT1 and tubulin as a control. Values represent the mean of 2–3 experiments performed in duplicate. Error bars represent s.e.m. Statistical analyses were performed using Student's *t*-test. $*P<0.05$. shRNA control versus shRNA SIRT1.

SIRT1 is required to switch on fatty acid oxidation in response to low glucose concentrations

SIRT1 and GCN5 are modulators of PGC-1α and changes of amounts and/or activities of these two enzymes will define the ability of PGC-1α to regulate metabolic genes. However, an important question is what are the stimuli or signals that are involved in this physiological process. In hepatocytes, we have shown that SIRT1 is regulated through a nutrient pathway with changes in NAD^+ and pyruvate in food-deprivation conditions (Rodgers *et al*, 2005). Moreover, Figure 1A shows fasting-induced PGC-1α deacetylation in skeletal muscle. In order to investigate whether SIRT1 was a target of a nutrient response in skeletal muscle cells and regulated fatty acid oxidation—a metabolic pathway that is highly induced during nutrient deprivation—we first analyzed whether changes in glucose concentration might control fatty acid oxidation rates. As shown in Figure 6A, decreases in glucose concentrations induced fatty acid oxidation up to three-fold in C_2C_{12} myotubes. Interestingly, this decrease in glucose concentration resulted in an increase of NAD^+, a substrate and activator of SIRT1, as well as an increase in the ratio of free NAD^+/NADH measured by lactate and pyruvate concentrations (Figure 6B and Supplementary Figure S3). These data suggest that this change in NAD^+ levels could be sensed via SIRT1 and activate its enzymatic activity. To test this, we determined acetylation levels of PGC-1α in high and low glucose concentrations. Figure 6C shows that PGC-1α acetylation is largely decreased by lowering levels of glucose, which is consistent with an increase in NAD^+ and SIRT1 deacetylase activity. To demonstrate that SIRT1 mediated

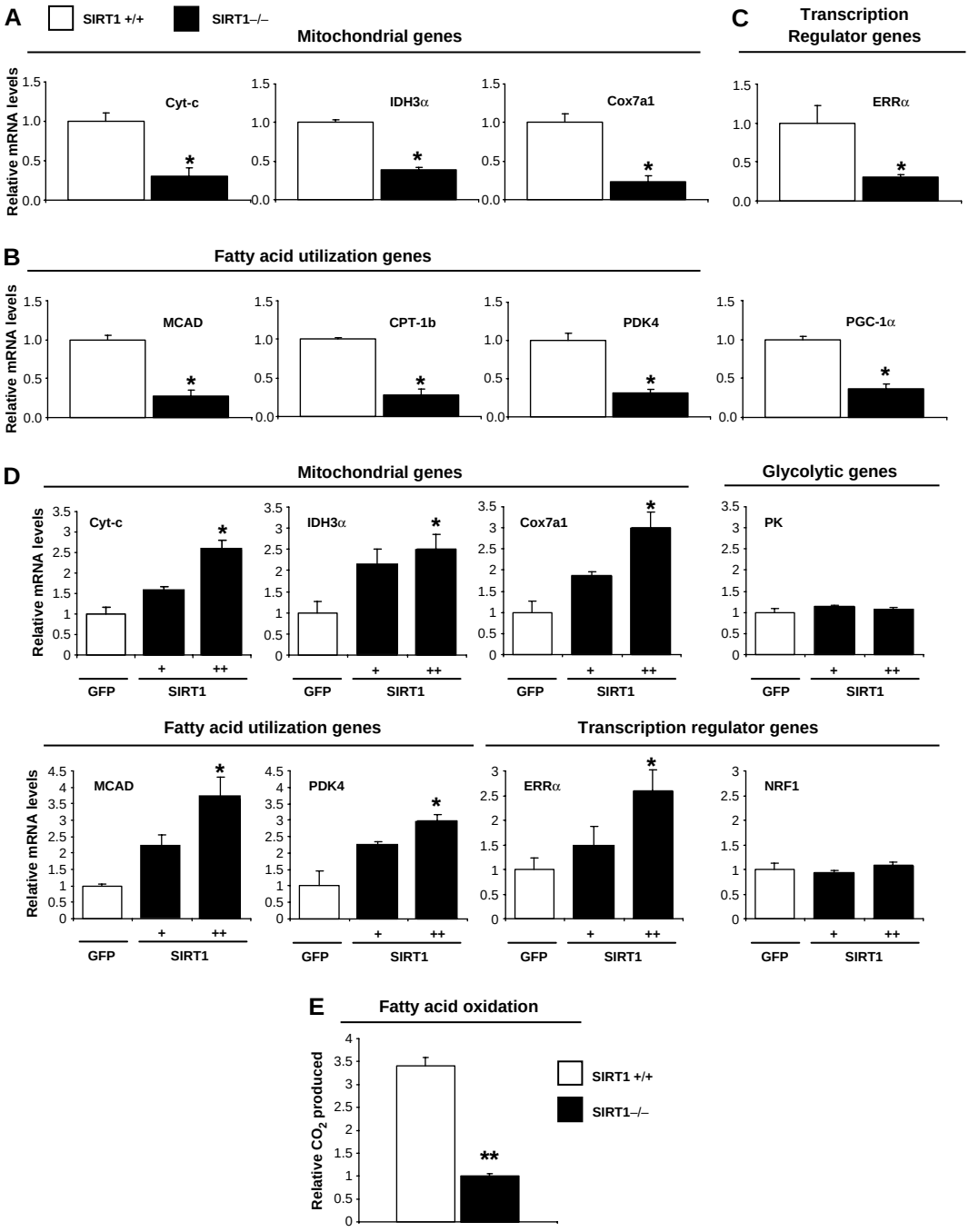


Figure 4 SIRT1 regulates mitochondrial and fatty acid metabolism in MEFs. Decreased mitochondrial (**A**), fatty acid utilization (**B**) and mitochondrial transcriptional regulator (**C**) gene expression in SIRT1−/− MEFs. MEFs were cultured until confluence, harvested and RNA was extracted to measure mitochondrial genes by quantitative RT–PCR. (**D**) Ectopic expression of SIRT1 rescues mitochondrial and fatty acid utilization gene expression. SIRT1−/− MEFs were infected with different amounts of adenoviruses encoding for SIRT1. After 3 days of infection, total RNA was isolated and indicated gene expressions were measured by quantitative RT–PCR. (**E**) Decreased rates of fatty acid oxidation in SIRT1−/− MEFs. Cells were treated as described in (A), but were used to measure oleic acid oxidation rates as described in Materials and methods. Values represent the mean of 2–3 experiments performed in duplicate. Error bars represent s.e.m. Statistical analyses were performed using Student's *t*-test. $^{*}P<0.05$ and $^{**}P<0.005$.

low glucose-induced fatty acid oxidation through regulation of gene expression, we used SIRT1−/− cells (Figure 6D). In SIRT1+/+ cells, low glucose induced expression of mitochondrial, fatty acid utilization and mitochondrial transcriptional regulator gene expression. Notably, this induction was totally blunted in SIRT1−/− MEFs. Furthermore, we tested the requirement of SIRT1 in C_2C_{12} myotubes expressing PGC-1α (Figure 6E). As expected, low glucose induced expression of the same set of PGC-1α-targeted genes; however knockdown of SIRT1 blocked this induction. As a control, PGC-1β did not change with these treatments. Consistent with this gene expression pattern, Figure 6F shows that SIRT1−/− MEFs completely lack the ability to induce fatty acid oxidation in response to low glucose. Furthermore, knockdown of

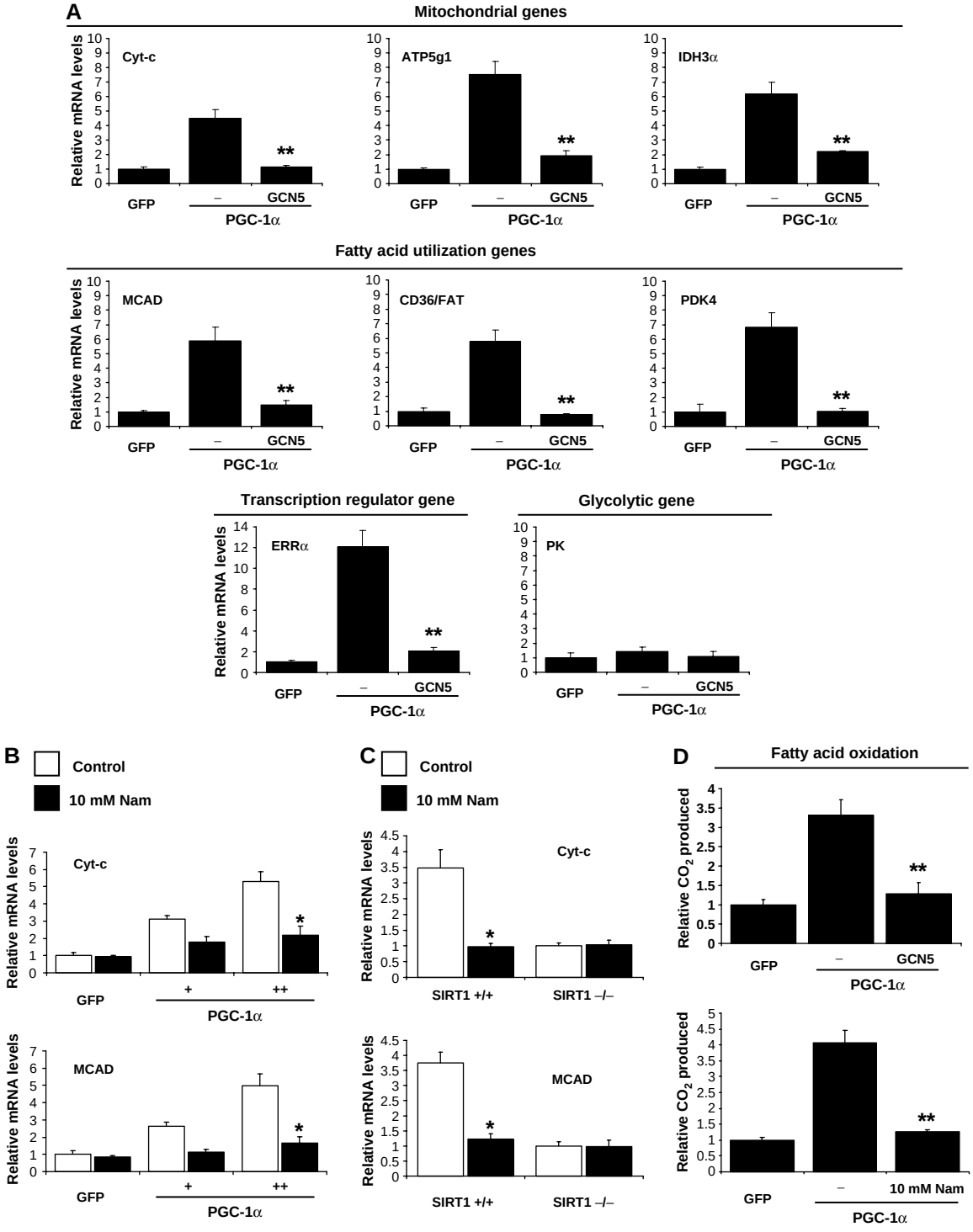
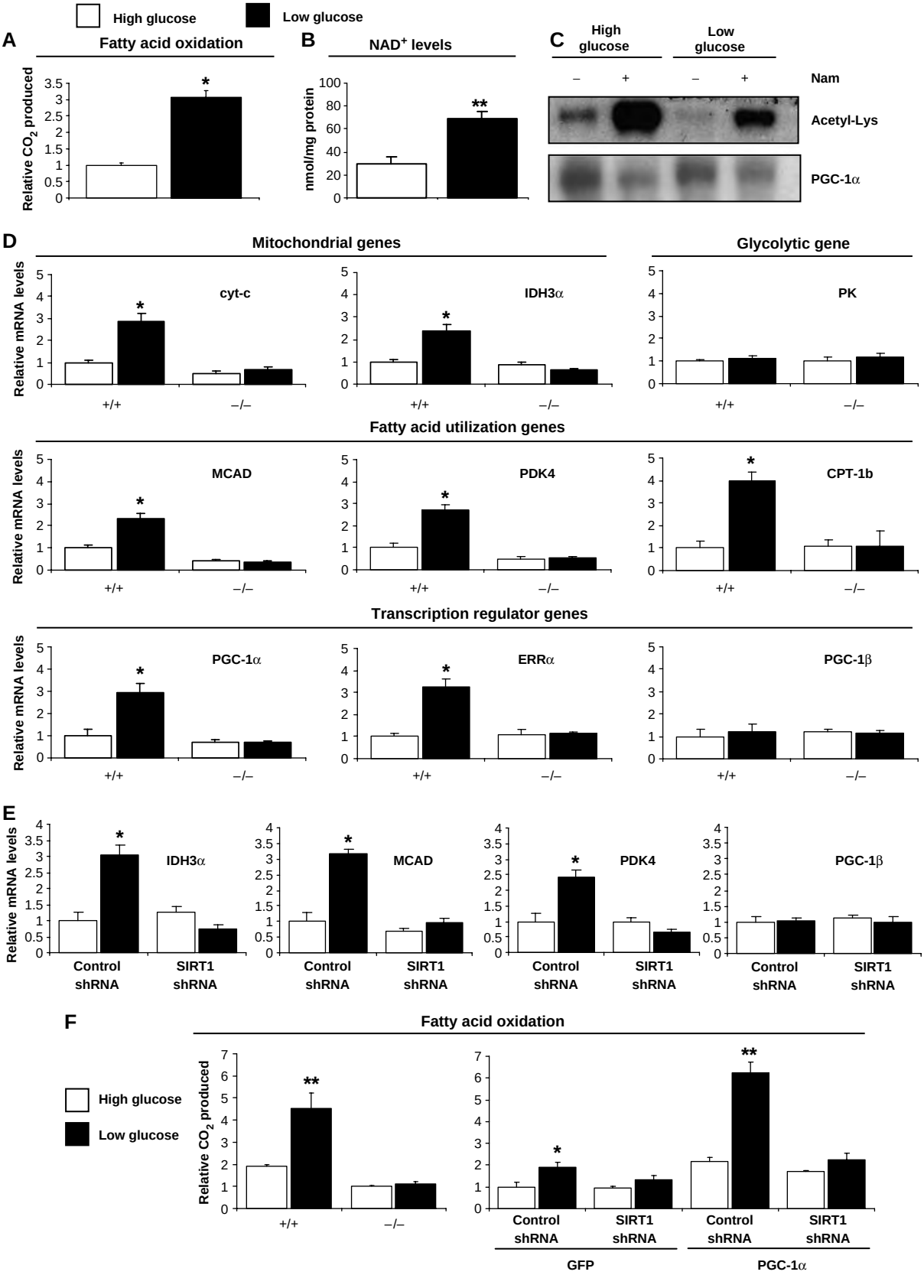


Figure 5 GCN5 regulates mitochondrial and fatty acid metabolism in skeletal muscle cells. (**A**) GCN5 downregulates PGC-1α-induced mitochondrial gene expression. C_2C_{12} myotubes were infected for 3 days with the indicated adenoviruses. Total RNA was extracted and analyzed for mitochondrial, fatty acid utilization and transcriptional regulator gene expression using quantitative RT–PCR analysis. (**B**) Nicotinamide downregulates PGC-1α-induced mitochondrial and fatty acid oxidation gene expression in C_2C_{12} myotubes and (**C**) MEFs. C_2C_{12} cells were infected with the indicated adenoviruses. At day 3 after infection, cells were treated for 12 h with 10 mM nicotinamide. Confluent MEFs were also treated for 12 h with nicotinamide and harvested for RNA analysis. (**D**) GCN5 and nicotinamide prevented PGC-1α-increased fatty acid oxidation rates. Cells were treated as in (A–C), but oleic acid oxidation assays were performed as described in Materials and methods. Values represent the mean of 2–3 experiments performed in duplicate. Error bars represent s.e.m. Statistical analyses were performed using Student's *t*-test. $^*P<0.05$ and $^{**}P<0.005$.

SIRT1 in C_2C_{12} myotubes also prevented the induction of fatty acid oxidation under low glucose concentrations, both in GFP- and PGC-1α-expressing cells (Figure 6F). Taken together, these results indicate that changes in glucose concentrations that occur in physiological situations such as starvation or caloric restriction induce an autonomous switch to increase oxidation of fatty acids. Importantly, this metabolic response entirely required SIRT1 to efficiently trigger fatty acid oxidation.

Discussion

Here we show the requirement of SIRT1 to induce a complete metabolic program of mitochondrial fatty acid oxidation in response to nutrient deprivation. We propose the following model of how nutrients can be sensed in cells to trigger a transcriptional metabolic response. In low levels of glucose, mammalian cells rearrange metabolic pathways that cause



increases in NAD^+ levels, which are sensed by the protein deacetylase SIRT1. Once activated, SIRT1 targets and deacetylates the transcriptional coactivator PGC-1α at promoter regions to induce gene expression of mitochondrial and fatty acid oxidation to maintain the bioenergetic state of the cell. This model provides a molecular mechanism by which fluctuations in nutrients target two key transcriptional regulators, SIRT1 and PGC-1α that control expression of mitochondrial oxidative genes, allowing cells to survive and adapt in periods of nutrient deprivation (Figure 7).

The ability of cells to switch from glucose to fatty acid oxidation in response to low concentrations of glucose correlates with changes in NAD^+ levels. Absence or decreased levels of SIRT1 prevent this metabolic response. We propose that this is mainly due to a reduction in expression of genes involved in mitochondrial and fatty acid utilization, however the essential and required target genes are unknown. A key enzyme in this switch is PDK4, which acts by inhibition of PDH activity, thereby preventing the entry of pyruvate into the TCA cycle (Sugden *et al*, 1993). In this scenario, oxidation of free fatty acids is the main supply of acetyl-CoA and NADH to the TCA and respiratory electron transport chain respectively. The fact that PDK4 is tightly controlled by SIRT1 and PGC-1α indicates that it might be a crucial target through which these transcriptional regulators control this nutrient response. In addition to PDK4 and genes of fatty acid oxidation such as CPT1b and MCAD, SIRT1 and PGC-1α also coordinate increases of genes of mitochondrial respiration. This increase is important because if the rate of fatty acid oxidation is not coupled to the use of NADH by mitochondrial respiration, it would inhibit the TCA cycle and compromise synthesis of ATP as energetic substrate for the cell. In fact, uncompleted rates of fatty acid oxidation in skeletal muscle could generate lipid intermediates that are linked to insulin resistance states (Koves *et al*, 2005). In this situation, increases in NADH levels could also potentially inhibit the activity of SIRT1 as has been previously proposed (Lin *et al*, 2004). Interestingly, this might suggest the existence of a negative feedback loop by increases of NADH to slow down rates of fatty acid oxidation through inhibition of SIRT1. Therefore, it is crucial in conditions of nutrient deprivation that cells maintain the ability to shift to oxidation of fatty acids and to couple it to mitochondrial respiration. Nicotinamide, an inhibitor and product of SIRT1 catalytic activity, also affected gene expression regulated by PGC-1α and prevented the low glucose response to induce oxidation of fatty acids. As nicotinamide is an endogenous inhibitor of SIRT1, it could also play a role in this metabolic response. In fact, in yeast, Pnc1p, an enzyme that synthesizes NAD^+ from nicotinamide and ADP-ribose, is induced by caloric restriction, suggesting that cellular nicotinamide concentrations might modulate lifespan (Anderson *et al*, 2003). In addition, in neuronal cells, caloric restriction also regulates endogenous levels of nicotinamide (Qin *et al*, 2006).

The levels of PGC-1α acetylation are controlled by the acetyl transferase GCN5 and SIRT1 deacetylase. It is currently not clear how or whether GCN5 is regulated or if it is constitutively active. We have shown previously that SIRT1 protein levels are increased in hepatocytes during starvation, ultimately leading to deacetylation of PGC-1α, which correlated with increases in NAD^+ and pyruvate (Rodgers *et al*, 2005). Interestingly, in myotubes, we did not observe any effect of pyruvate on SIRT1 protein levels (data not shown). It is conceivable that selective tissue transcription factors that interact with PGC-1α might account for these differences. However, in response to low concentrations of glucose, increased NAD^+ levels correlated with PGC-1α deacetylation suggesting that SIRT1 enzymatic activity was increased. The extent to which the observed changes in NAD^+ directly affect SIRT1 enzymatic activity in cells is unknown. It is possible that in addition to NAD^+, other alternative regulatory mechanisms or signaling pathways that are activated by low glucose concentrations might also regulate the activity of SIRT1 on PGC-1α. Such putative mechanisms are currently under exploration. We have previously mapped up to 13 acetylation sites on different structural domains of PGC-1α (Rodgers *et al*, 2005). At this point, it is not entirely clear which acetylation residues are required for the metabolic effects. Interestingly, acetylation of PGC-1α by GCN5 drives PGC-1α to nuclear foci that are transcriptionally inactive (Lerin *et al*, 2006), which could conceivably be the same mechanism that functions in myotubes.

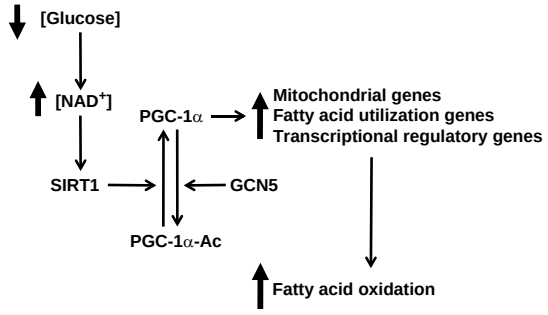


Figure 7 Model of glucose-dependent control of fatty acid oxidation through SIRT1/PGC-1α. Decreases in glucose elevate intracellular levels of NAD^+ that will activate SIRT1deacetylase enzymatic activity. SIRT1 interacts and deacetylates PGC-1α, which induces an upregulation of genes linked to mitochondrial function and fatty acid utilization. Finally, this change in PGC-1α-targeted gene expression results in the activation of complete oxidation of fatty acids. See text for further details.

Figure 6 SIRT1 is required for the switch to fatty acid oxidation in response to low glucose concentrations. (**A**) Low glucose concentration induced increase in rates of fatty acid oxidation and (**B**) increased in cellular NAD^+ levels. C_2C_{12} myotubes were treated with 25 mM (open bars) or 5 mM (filled bars) glucose for 12 h. Palmitic acid oxidation was measured and in another set of experiments, deproteinized cell extracts were used to determine NAD^+ levels, as described in Materials and methods. (**C**) Low glucose concentration induces deacetylation of PGC-1α. C_2C_{12} myotubes were infected with PGC-1α adenoviruses and treated for 12 h with 25 and 5 mM of glucose with or without nicotinamide (10 mM). (**D**) SIRT1 is required to increase mitochondrial and fatty acid utilization genes in response to low glucose in MEFs and (**E**) C_2C_{12} myotubes. C_2C_{12} cells were infected with the adenoviruses encoding PGC-1α and shRNA control or shRNA SIRT1. Twelve hours before harvesting, MEFs or C_2C_{12} cells were treated with different concentrations of glucose and indicated gene expression was analyzed. (**F**) SIRT1 is required for induction of mitochondrial fatty acid oxidation in response to low glucose. C_2C_{12} myotubes and MEFs were treated as in (D). Oxidation rates of oleic acid were analyzed as described in Materials and methods. Values represent the mean of 2–3 experiments performed in duplicate. Error bars represent s.e.m. Statistical analyses were performed using Student's *t*-test. $^*P<0.05$ and $^{**}P<0.005$.

In summary, we show here that SIRT1 is an important functional regulator of PGC-1α, and that under conditions of nutrient restriction, SIRT1 regulates PGC-1α target genes and is essential in responding to increases of complete fatty acid oxidation. This is a crucial metabolic adaptation that will not only allow cells to survive periods of low nutrients, but will also integrate muscle cells in the physiological response of food deprivation to spare glucose for neuronal and red blood cells. It is also possible that this metabolic adaptation might impact the molecular mechanisms by which caloric restriction modulates lifespan. As SIRT1 is an enzyme, it encourages the possibility of screening drugs that would modulate its activity on PGC-1α and activate mitochondrial oxidative genes that are dysregulated in diseases such as diabetes and neurodegeneration.

Materials and methods

Plasmids

PGC-1α, GCN5, SIRT1, control and *SIRT1* shRNA adenoviruses were constructed using the pAd-Easy system as previously described (Rodgers *et al*, 2005).

Animal experiments

C57B1/6 mice were fed *ad libitum* or fasted for 16 h. Skeletal muscle whole-cell homogenates were prepared with RIPA buffer and used for Western blot analysis as previously described (Rodgers *et al*, 2005).

Cell culture and adenoviral infections

C_2C_{12} skeletal muscle cells were cultured in DMEM with 10% calf serum. Differentiation was induced at ~90–100% confluency by switching the cell media to DMEM supplemented with 2% horse serum (HS) (differentiation media) for 72 h. Following differentiation into myotubes, C_2C_{12} cells was infected with adenovirus for 4 h in differentiation media. The media was then replaced with fresh DMEM/2%HS for an additional 48 h. The cells were then washed with PBS and incubated with indicated treatment for 12 h before collection in RIPA buffer (protein analysis) or Trizol (RNA analysis).

MEFs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Upon reaching 70% confluency, cells were infected for 24 h in DMEM with 10% FBS. The media was then replaced with fresh DMEM/10% FBS for an additional 48 h. The cells were washed with PBS and incubated with the indicated treatment for 12 h before collection in RIPA buffer (protein analysis) or Trizol (RNA analysis).

Primary muscle cells were isolated and cultured from 2 to 3-week-old FVB mice as described previously (Sabourin *et al*, 1999). To induce differentiation, myoblasts were grown to 80% confluence and then switched to the differentiation medium, DMEM, containing 5% HS. Myotubes were transduced with adenovirus for 24 h and media were then replaced with fresh DMEM with 5% horse serum for an additional 48 h. Cells were washed with PBS and lysed in RIPA buffer. Protein lysates were subjected to Western blot analysis to determine the expression level of SIRT1 protein. Total RNA was extracted from the cells and subjected to quantitative RT–PCR analysis to determine gene expression. The Taqman primer/probe sets were purchased from ABI (assay on demand) and 18S was used for normalization. Citrate synthase activity was measured in the cells as described previously (Moyes *et al*, 1997).

Gene expression analysis

Total RNA prepared from either C_2C_{12} cells or from MEFs was extracted with Trizol (Invitrogen). Complementary DNA generated by Superscript II enzyme (Invitrogen) was analyzed by quantitative reverse transcriptase-mediated PCR using an iQ SYBR Green Supermix (Bio-Rad). All data were normalized to tubulin expression. The oligonucleotide primers used are available upon request.

PGC-1α acetylation assays

PGC-1α lysine acetylation was analyzed by immunoprecipitation of PGC-1α followed by Western blot using acetyl-lysine antibodies (Cell Signalling and Technology) as previously described (Rodgers *et al*, 2005). C_2C_{12} cells or MEFs were infected with adenovirus expressing Flag-tagged PGC-1α as described above. After the indicated 6 h or 12 h treatment, PGC-1α was immunoprecipitated using anti-Flag beads (Sigma) and examined for acetylation.

Fatty acid and glucose oxidation and NAD^+ measurements assays

Cellular oleic and palmitic acid oxidation rates were determined in C_2C_{12} cells and MEFs using modifications of protocols previously described (Garcia-Martinez *et al*, 2005; Wende *et al*, 2005). C_2C_{12} cells were cultured in 12-well dishes, then differentiated and infected as described above. The cells were rinsed with PBS and incubated with MEM supplemented with 0.5% HS and either 500 μM oleic or palmitic acid for 12 h. Cells were then incubated for an additional 3 h with fresh DMEM/0.5% HS that was supplemented with [1-^{14}C]oleic or [1-^{14}C]palmitic acid (3.0 mCi/mmol). The oxidation reactions were terminated and CO_2 was released from the media by the addition of 3 M perchloric acid. Filter paper saturated with phenylethylamine was placed over each well to capture CO_2. Following a 3 h incubation with gentle shaking at 25°C, $^{14}CO_2$ resulting from oxidized fatty acid was quantified by scintillation counting of the filter paper. Each experiment was performed in triplicate and the results were normalized to total protein.

Glucose oxidation rates were measured using methods previously described (Wende *et al*, 2005).

NAD^+ nucleotide concentration was directly measured as described (Lin *et al*, 2001). In brief, a confluent 6 cm dish of C_2C_{12} myotubes was homogenized in 400 μl of acid extraction buffer to obtain the NAD^+ concentration. Homogenates were neutralized with 200 μl of 0.4 M Tris buffer. The concentration of NAD^+ was measured fluorometrically after an enzymatic reaction using 1.5 μl of sample. For NAD^+/NADH measurements, C_2C_{12} myotubes were deproteinized and concentrations of lactate and pyruvate were determined as previously described (Rodgers *et al*, 2005).

Statistical analysis

Data are the means ± s.e.m. Statistical analysis was performed by a two-tailed unpaired Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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Fasting-dependent glucose and lipid metabolic response through hepatic sirtuin 1

Joseph T. Rodgers and Pere Puigserver*

Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and Department of Cell Biology, Dana–Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

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In the fasted state, induction of hepatic glucose output and fatty acid oxidation is essential to sustain energetic balance. Production and oxidation of glucose and fatty acids by the liver are controlled through a complex network of transcriptional regulators. Among them, the transcriptional coactivator PGC-1α plays an important role in hepatic and systemic glucose and lipid metabolism. We have previously demonstrated that sirtuin 1 (SIRT1) regulates genes involved in gluconeogenesis through interaction and deacetylation of PGC-1α. Here, we show *in vivo* that hepatic SIRT1 is a factor in systemic and hepatic glucose, lipid, and cholesterol homeostasis. Knockdown of SIRT1 in liver caused mild hypoglycemia, increased systemic glucose and insulin sensitivity, and decreased glucose production. SIRT1 knockdown also decreased serum cholesterol and increased hepatic free fatty acid and cholesterol content. These metabolic phenotypes caused by SIRT1 knockdown tightly correlated with decreased expression of gluconeogenic, fatty acid oxidation and cholesterol degradation as well as efflux genes. Additionally, overexpression of SIRT1 reversed many of the changes caused by SIRT1 knockdown and depended on the presence of PGC-1α. Interestingly, most of the effects of SIRT1 were only apparent in the fasted state. Our results indicate that hepatic SIRT1 is an important factor in the regulation of glucose and lipid metabolism in response to nutrient deprivation. As these pathways are dysregulated in metabolic diseases, SIRT1 may be a potential therapeutic target to control hyperglycemia and hypercholesterolemia.

fasting response | glucose metabolism | lipid metabolism | deacetylase | transcriptional coactivator

In response to nutrient deprivation mammals trigger many tissue-specific metabolic pathways to maintain organismal survival. In particular, the liver functions as a major metabolic buffering system that controls macro- and micronutrient homeostasis, allowing other tissues to function normally under physiological stresses (1–3). Among its many functions, production of glucose by the liver is an essential process that contributes to normalization of systemic glucose levels (4, 5), ensuring that glucose-dependent tissues such as brain and red blood cells will have access to an energy supply during periods of nutrient deprivation. However, chronic elevation of hepatic glucose production is also a key contributor in diabetes that exacerbates hyperglycemia in both the fed and fasted states (6, 7). The importance of hepatic glucose production is underscored by the fact that current antidiabetic drugs such as metformin decrease blood glucose levels through inhibition of gluconeogenesis in liver (8, 9).

The liver also plays an important role in lipid homeostasis. In the fasted state, oxidation of hepatic free fatty acids supplies energy for glucose production (10). Dysregulation of hepatic fatty acid β-oxidation and/or fatty acid synthesis enzymes leads to hepatic steatosis or fatty liver (11). Moreover, cholesterol is synthesized and degraded in the liver accordingly to the needs by other tissues (12). Cyp7A1 is the key rate-limiting enzyme controlling cholesterol degradation through hepatic synthesis of bile acids (13, 14). Efflux and influx through scavenge receptor B1 (SR-B1), low-density lipoprotein (LDL) receptor, and ABC transporters also plays an important role in regulating cholesterol levels (15–17).

The majority of rate-limiting enzymes in key pathways involved in glucose and lipid homeostasis are controlled at the transcriptional level (17). In the last several years, the peroxisome proliferator activated receptor coactivator (PGC)-1α/β and liver X receptor (LXR)/sterol response element-binding protein (SREBP) have been identified as key transcriptional regulators of many metabolic enzymes and pathways (18–20). The PGC-1α transcriptional network is the target of hormonal and nutrient signals. Positively regulated by glucagon through TORC2 and CREB activation, (21) and glucocorticoids. PGC-1α function is negatively regulated by insulin, directly (22) and through FOXO1 (23, 24). This regulation ultimately leads to coordinated changes in the expression of glucose and lipid metabolic genes (25). In the case of lipid metabolism, main regulatory transcription factors include the SREBP family and hormone nuclear receptors LXRs and peroxisome proliferator-activated receptors (PPARs). These factors are also subject to hormonal and nutrient regulation via insulin and cholesterol (12, 26, 27).

We have previously identified a nutrient regulation of glucose homeostasis through the NAD^+-dependent deacetylase sirtuin 1(SIRT1). Under low nutrient conditions, up-regulation of SIRT1 promotes hepatic glucose production through interaction and deacetylation of PGC-1α (28). Other groups have shown that SIRT1 represses peroxisome proliferator-activated receptor γ (PPARγ) function, increasing lipolysis in white adipose tissue and insulin secretion in pancreatic β-cells (29–31). Interestingly, a nutrient connection to SIRT1 has been previously established in lower eukaryotes, including yeast (32), worms (33), and flies (34). In these species, SIRT1 homologs were required to extend life span in response to calorie restriction, although its impact on metabolic pathways is unknown. A crucial unsolved question from our previous studies was to what extent SIRT1 was required to control hepatic glucose and lipid metabolic pathways and how this affects systemic nutrient homeostasis *in vivo*. Here, we

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Abbreviations: ABC, ATP binding cassette; CPT, carnitine palmitoyltransferase; Cyp7A1, cytochrome P450 type 7A1; FAS, fatty acid synthase; G6Pase, glucose 6-phosphatase; GK, glucokinase; GTT, glucose-tolerance test; HNF, hepatic nuclear factor; ITT, insulin-tolerance test; LDL, low-density lipoprotein; LDLr, LDL receptor; LPK, liver pyruvate kinase; LXR, liver X receptor; MCAD, medium chain acyl-CoA deyhydrogenase; PGC, peroxisome proliferator-activated receptor coactivator; Pepck, phosphoenolpyruvate carboxylase kinase; PTT, pyruvate-tolerance test; shRNA, small hairpin RNA; SIRT1, sirtuin 1; SR-B1, scavenge receptor B-1; SREBP, sterol response element binding protein.

*To whom correspondence should be addressed at: Dana–Farber Cancer Institute, Harvard Medical School, One Jimmy Fund Way, Boston, MA, 02115. E-mail: pere_puigserver@dfci.harvard.edu.

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report that hepatic knockdown of SIRT1 results in mild hypoglycemia, increased glucose tolerance, insulin sensitivity, and decreased hepatic glucose production. Furthermore, we found accumulation of free fatty acids and cholesterol in hepatic tissue as well as decreased serum cholesterol in SIRT1-deficient mice. These effects correlate with changes in gene expression of enzymes involved in gluconeogenesis, glycolysis, fatty acid oxidation/synthesis, and cholesterol degradation and efflux pathways. Importantly, many of these changes were reversed by hepatic overexpression of SIRT1 and were dependent on PGC-1α. Taken together our results implicate SIRT1 as an important regulator of hepatic glucose and lipid homeostasis in response to fasting.

Results

Impaired Glucose Homeostasis in Hepatic SIRT1 Knockdown Mice. Our previous work demonstrated that SIRT1 was required to induce gluconeogenic genes in response to a nutrient fasting signal in cultured liver cells (28). We therefore initiated studies in mice to further investigate the role of hepatic SIRT1 in systemic glucose homeostasis. Tail vein injection of adenoviruses-expressing SIRT1 small hairpin RNA (shRNA) resulted in a significant knockdown of SIRT1 protein levels. Expression levels of other proteins that physically and functionally interact with SIRT1 such as PGC-1α and FOXO1 were largely not affected [supporting information (SI) Fig. 5*A*]. Acetylation of PGC-1α was decreased in the fasted state, and knockdown of SIRT1 was sufficient to increase acetylation of endogenous PGC-1α in both fed and fasted mice (SI Fig. 5*B*). Acetylation of FOXO1, another SIRT1 target, also increased in response to SIRT1 knockdown (SI Fig. 5*E*). Conversely, overexpression of SIRT1 decreased PGC-1α acetylation (SI Fig. 5 *C* and *D*). Blood glucose levels were modestly but consistently lower in SIRT1 shRNA-infected mice compared with control shRNA mice, both in fed and fasted states (Fig. 1*A*). Hepatic knockdown of SIRT1 also lowered glycemia in fasted diabetic *db*/*db* mice (Fig. 1*B*). To further analyze whether hepatic SIRT1 plays a role in systemic glucose and insulin sensitivity, we performed glucose (GTT) and insulin (ITT) tolerance tests. Fig. 1*C* shows that in a GTT, mice infected with SIRT1 shRNA adenoviruses displayed significantly lower blood glucose concentrations. Consistent with these results, insulin had a greater effect reducing blood glucose levels in an ITT (Fig. 1*D*). To determine whether the reductions in blood glucose levels observed in mice with hepatic SIRT1 knockdown were due to a deficit in hepatic glucose production, we performed a pyruvate tolerance test (PTT). As shown in Fig. 1*E*, hepatic SIRT1 knockdown mice displayed lower blood glucose concentrations at every time point after pyruvate administration. We next analyzed the effects of hepatic SIRT1 overexpression on glucose metabolism. SIRT1 overexpression caused moderate hyperglycemia in short-term fasting (5 h) and a slight hyperglycemic trend upon longer fasting (Fig. 1*F*). Mice with hepatic SIRT1 overexpression were less glucose tolerant in a GTT (Fig. 1*G*). However, in a PTT, SIRT1 overexpression did not increase blood glucose levels (Fig. 1*H*). Taken together, these data indicate that modulation of SIRT1 protein causes changes in blood glucose levels that correlate with effects on glucose and insulin sensitivity. Moreover, our data suggests that these effects are likely due, at least in part, to effects on hepatic glucose production.

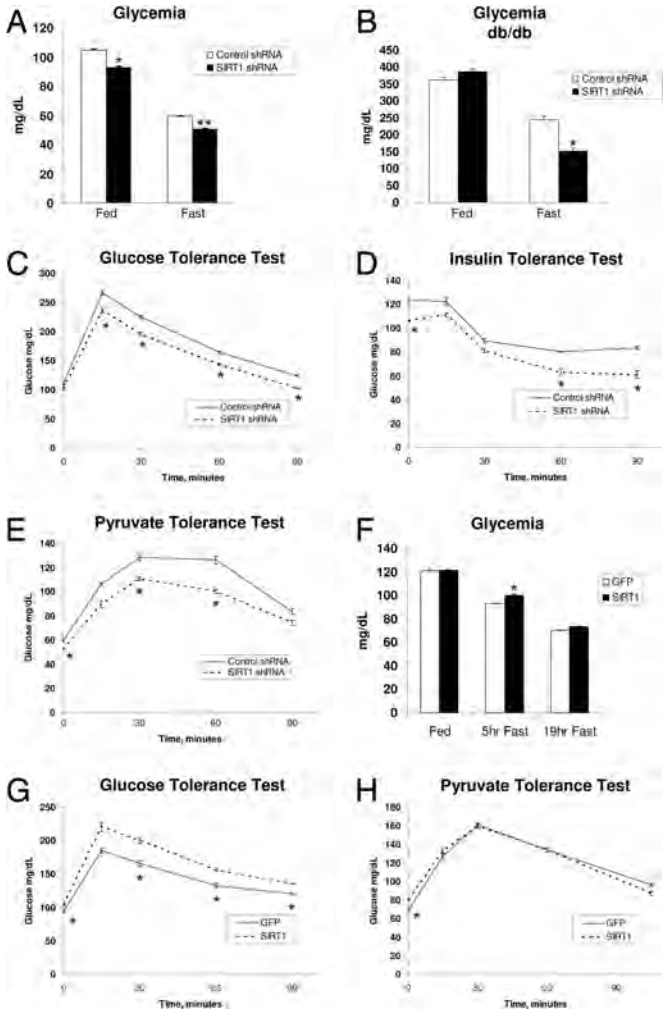

Fig. 1. Hepatic SIRT1 controls glucose metabolism. (*A*) Fed and fasted blood glucose levels of control and SIRT1 shRNA-infected mice. Data are presented as the average ± SEM of two independent experiments. Shown are control shRNA-infected mice [fed (n = 12), 20-h fasted (n = 12)] and SIRT1 shRNA-infected mice [fed (n = 13), 20-h fasted (n = 12)]. (*B*) Blood glucose levels from *db*/*db* mice infected with control shRNA (n = 5) or SIRT1 shRNA (n = 5) during feeding and during a short fast. (*C*) GTT. Control shRNA-infected (n = 5) and SIRT1 shRNA-infected (n = 6) mice were fasted 5 h before i.p. injection of 2 g/kg dextrose. (*D*) ITT. Control shRNA-infected (n = 5) and SIRT1 shRNA-infected (n = 5) mice were fasted 5 h before i.p. injection of 0.6 unit/kg insulin. (*E*) PTT. Control shRNA-infected (n = 7) and SIRT1 shRNA-infected (n = 7) mice were fasted 18 h before i.p. injection of 2 g/kg sodium pyruvate. (*F*) Blood glucose levels from mice infected with GFP or SIRT1 overexpression adenovirus. Feeding GFP and SIRT1 (n = 12), following a short 5-h fast (n = 6) or following a 19-h fast (n = 12). (*G*) GTT from GFP-infected (n = 6) or SIRT1-infected (n = 6) mice fasted for 5 h before injection of 2 g/kg dextrose. (*H*) PTT from GFP-infected (n = 6) and SIRT1-infected (n = 6) mice fasted for 18 h before injection with 2 g/kg pyruvate. All tolerance tests were performed in at least two independent experiments with similar results. Data are presented as the average ± SEM. Significance was determined by Student's t test. *, $P < 0.05$; **, $P < 0.01$.

Hepatic SIRT1 Controls Expression of Gluconeogenic and Glycolytic Genes. Our previous work in cultured liver cells demonstrated that nutrient signaling through pyruvate increases SIRT1 levels. Furthermore, pyruvate treatment induced gluconeogenic genes [phosphoenolpyruvate carboxylase kinase (Pepck) and glucose 6-phosphatase (G6Pase)] but repressed glycolytic genes [glucokinase (GK) and liver pyruvate kinase (LPK)] in a SIRT1-dependent manner (28). As shown in Fig. 2*A*, infection with SIRT1 shRNA resulted in reduced expression of the gluconeogenic G6Pase and Pepck under fasting conditions. Interestingly, no changes in the expression of these genes were observed in the fed state. In contrast, SIRT1 knockdown increased glycolytic GK gene expression upon fasting, but no changes were detected in LPK gene expression (Fig. 2*A*). Under fasting conditions, hepatic overexpression of SIRT1 further caused an increase in

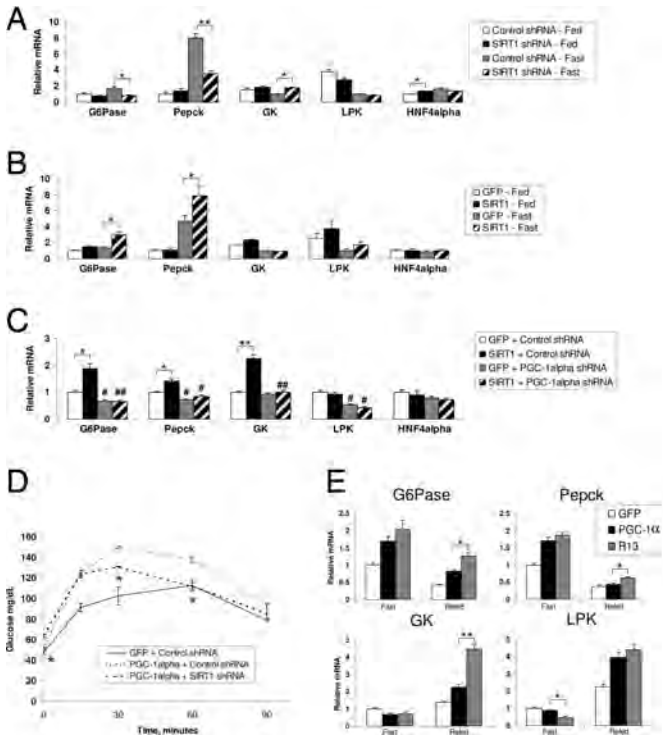

Fig. 2. Hepatic SIRT1 regulates genes involved in gluconeogenesis. (*A*) Quantitative RT-PCR analysis of expression of genes involved in liver glucose metabolism from mice in the fed state infected with control shRNA ($n = 5$) or SIRT1 shRNA ($n = 6$) or mice infected with control shRNA ($n = 5$) and SIRT1 shRNA ($n = 5$) and fasted for 20 h. (*B*) Fed, GFP ($n = 6$) or SIRT1 ($n = 6$); fasted for 19 h, GFP ($n = 6$) or SIRT1 ($n = 6$). (*C*) Double infection of GFP or SIRT1 overexpression and Control or PGC-1α shRNA (each bar, $n = 9$) following a 20-h fast. (*D*) PTT, PGC-1α overexpression, and SIRT1 knockdown. GFP + control shRNA-infected mice ($n = 4$), PGC-1α + control shRNA-infected mice ($n = 4$), and PGC-1α + SIRT1 shRNA-infected mice ($n = 4$) were fasted 18 h before i.p. injection of 2 g/kg sodium pyruvate. Significance indicated is between PGC-1α + control shRNA and PGC-1α + SIRT1 shRNA. (*E*) Quantitative RT-PCR analysis of gene expression of mice infected with GFP, PGC-1α overexpression, or R13 adenovirus fasted for 22 h (each bar, $n = 4$) or fasted for 22 h and refed overnight (16 h) (each bar, $n = 5$). All data are presented as the average ± SEM. Gene expression was normalized to 36b4 expression. Significance was determined by Student's *t* test. GFP/control shRNA vs. SIRT1/SIRT1 shRNA: *, $P < 0.05$; **, $P < 0.01$. Control shRNA vs. PGC-1α shRNA: #, $P < 0.05$; ##, $P < 0.01$.

G6Pase and Pepck mRNA levels but did not significantly change glycolytic gene expression (Fig. 2*B*). These results indicate that hepatic SIRT1 functions in the regulation of gluconeogenic gene expression. Notably, in both knockdown and overexpression studies, it is the regulation in response to fasting that is affected by SIRT1.

We next investigated whether PGC-1α was required for the effects of SIRT1 on glucose metabolism. We used a combination of adenovirus to simultaneously overexpress SIRT1 and knockdown PGC-1α (PGC-1α shRNA) (SI Fig. 6*A*). Consistent with previous reports (35, 36), knockdown of PGC-1α resulted in decreased expression of gluconeogenic genes (Fig. 2*C*) and reduced glycemia (SI Fig. 6*B*). Interestingly, knockdown of PGC-1α also blocked the induction of gluconeogenic genes (Fig. 2*C*), and the reduced glucose tolerance caused by SIRT1 overexpression (SI Fig. 6*C*), suggesting that SIRT1 requires PGC-1α for its regulation of hepatic glucose metabolism.

SIRT1 Controls PGC-1α-Dependent Increase of Hepatic Glucose Production. Because ectopic PGC-1α expression is sufficient to induce hepatic glucose production, we next tested whether these effects require SIRT1. To do this, we infected mice with a combination of adenoviruses, resulting in PGC-1α overexpression and SIRT1 knockdown (SI Fig. 7*A*). As expected, overexpression of PGC-1α in the liver resulted in increased blood glucose levels in a PTT. Hepatic knockdown of SIRT1 significantly reduced this increase by PGC-1α (Fig. 2*D*). Moreover, PGC-1α-induced Pepck gene expression was also reduced by SIRT1 shRNA (SI Fig. 7*B*) despite a large induction of PGC-1α protein (SI Fig. 7*A*). Together, these results suggest that SIRT1 is required, at least in part, for PGC-1α-dependent hepatic glucose production.

To determine whether acetylation of PGC-1α regulates its activity *in vivo*, we used a mutant PGC-1α construct in which 13 lysines have been mutated to arginine-R13 (SI Fig. 7*D*). This mutant is no longer acetylated when cells are treated with nicotinamide (28). As expected, PGC-1α overexpression caused mild hyperglycemia. Interestingly, the R13 mutant caused a larger increase in blood glucose levels compared with control and wild-type PGC-1α. This effect was particularly pronounced in the short-term fasting condition (SI Fig. 7*E*). We also observed that R13 was more transcriptionally active, inducing gluconeogenic genes, particularly in the refed situation (Fig. 2*E*). These data provide evidence that PGC-1α activity is, in part, regulated by SIRT1 and acetylation *in vivo*.

Defects in Fatty Acid and Cholesterol Metabolism in Mice with Altered Hepatic SIRT1 Expression. In addition to glucose metabolism, the liver has key roles in lipid homeostasis. We therefore investigated whether SIRT1 plays a role in hepatic lipid metabolism. SIRT1 knockdown or overexpression did not significantly alter serum triglyceride and free fatty acid levels (SI Fig. 8). However, SIRT1 knockdown very strongly increased intracellular hepatic free fatty acids (Fig. 3*A*) while having no effect on liver triglycerides (SI Fig. 8*A*). We also observed that SIRT1 had very pronounced effects on systemic and hepatic cholesterol. Hepatic knockdown of SIRT1 resulted in reduced systemic levels of total cholesterol in the fed and fasted state (Fig. 3*A*). Interestingly, SIRT1 overexpression reversed this effect, increasing systemic cholesterol most significantly in the fasted state (Fig. 3 *B* and *C*). In the fasted state, knockdown of SIRT1 also caused a significant accumulation of hepatic cholesterol (Fig. 3*A*) and overexpression modestly but significantly depleted liver cholesterol (Fig. 3 *B* and *C*). Knockdown of SIRT1 in *db*/*db* mice had a similar effect on systemic and hepatic lipid levels (SI Fig. 9). Interestingly, we note that unlike the effects on glucose metabolism, SIRT1 regulated cholesterol levels independently of PGC-1α. SIRT1 increased systemic cholesterol and decreased hepatic cholesterol just as potently when PGC-1α levels were reduced by shRNA (Fig. 3*C*).

Hepatic SIRT1 Controls Gene Expression of Enzymes Involved in Triglyceride, Fatty Acid, and Cholesterol Metabolic Pathways. The accumulation of liver free fatty acids by SIRT1 knockdown prompted us to analyze the gene expression of enzymes involved in fatty acids and triglyceride metabolism. As expected, fasting induced gene expression of fatty acid oxidation enzymes medium chain acyl-CoA deyhydrogenase (MCAD) and carnitine palmitoyltransferase-1a (CPT-1a) (Fig. 4*A*). Notably, mice with hepatic SIRT1 knockdown displayed lower expression of these enzymes compared with control mice. In both cases, the fasting induction was largely reduced by SIRT1 knockdown. Other genes involved in fatty acid and mitochondrial oxidation such as cytochrome c (Cyto-C) and L-FABP (data not shown) remained unchanged. In contrast, SIRT1 knockdown induced the expression of FAS, a key lipogenic gene in the fasted state (Fig. 4*A*). Expression of the transcription factor SREBP-1c/ADD1, a controller of lipogenic gene expression (12), followed a similar pattern to that of its target gene, FAS. Again, the fasting

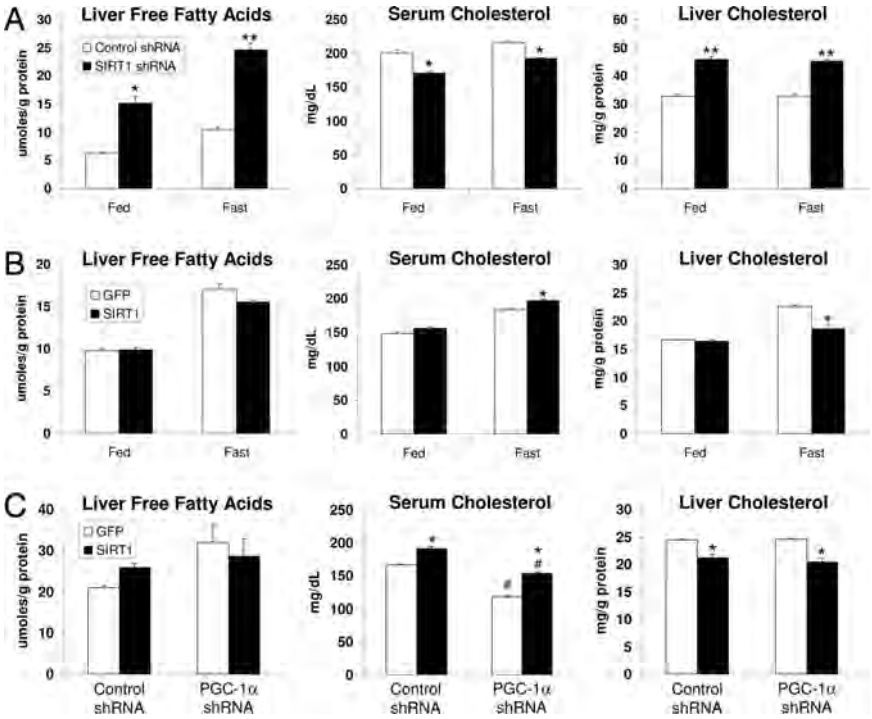

Fig. 3. Hepatic SIRT1 controls systemic and hepatic cholesterol and fatty acid homeostasis. (*A*) Liver free fatty acids (nonesterified fatty acids), serum total cholesterol, and liver cholesterol from control shRNA-infected fed ($n = 5$) and 20-h fasted ($n = 5$) mice and SIRT1 shRNA-infected fed ($n = 6$) and fasted ($n = 5$) mice. (*B*) Fed, GFP ($n = 6$) and SIRT1 ($n = 6$); 19-h fasted, GFP ($n = 10$) and SIRT1 ($n = 10$). (*C*) GFP or SIRT1 overexpression and control or PGC-1α shRNA double-infected mice (each bar, $n = 5$) following a 20-h fast. Liver measurements were normalized to protein content. Similar results for all measurements have been observed in at least two independent experiments. All data are presented as the average ± SEM. Significance was determined by Student's *t* test. GFP/control shRNA vs. SIRT1/SIRT1 shRNA: *, $P < 0.05$; **, $P < 0.01$. Control shRNA vs. PGC-1α shRNA: #, $P < 0.05$.

response, repression of these lipogenic genes, was impaired by SIRT1 knockdown. Because SIRT1 knockdown caused an accumulation of hepatic free fatty acids but no change in triglycerides, we analyzed expression of enzymes involved in triglyceride synthesis. Expression of DGAT2, an enzyme involved in fatty acid esterification to glycerol, was markedly reduced in SIRT1 knockdown mice (Fig. 4*A*). These results suggest that the accumulation of hepatic free fatty acids caused by SIRT1 knockdown could be due to changes in gene expression of enzymes involved in fatty acid oxidation and fatty acid and triglyceride synthesis. Although SIRT1 overexpression did not alter levels of liver free fatty acids (Fig. 3 *B* and *C*), it partially reversed the effect of SIRT1 knockdown, inducing MCAD, CPT-1a, and DGAT2 expression in the fasted state (Fig. 4*B*). This response depended on PGC-1α, as knockdown of PGC-1α blocked the SIRT1 induction of these genes in the fasted state (Fig. 4*C*).

Because hepatic SIRT1 knockdown caused accumulation of total cholesterol in liver (Fig. 3*A*) and SIRT1 overexpression reduced liver cholesterol (Fig. 3*B*), we analyzed gene expression of enzymes involved in cholesterol transport, synthesis, and degradation. Hepatic gene expression of HMG-CoA reductase, a rate-limiting enzyme in cholesterol synthesis, was not altered by SIRT1 knockdown or overexpression (data not shown). However, SIRT1 did regulate several genes involved in cholesterol transport and degradation. Hepatic cholesterol levels are also tightly regulated by efflux-mediating cell membrane ATP binding cassette (ABC) transporters ABCA1 and ABCG1 as well as SR-B1. Hepatic expression of ABCA1 and SR-B1 was down-regulated by SIRT1 shRNA (Fig. 4*D*). Conversely, SIRT1 overexpression resulted in a fasting induction of SR-B1 and a trend toward induction of ABCA1, although not statistically significant (Fig. 4*E*). Another route for cholesterol import into hepatocytes is transport through the LDL receptor (LDLr) (17). Consistent with an increase in hepatic cholesterol levels, SIRT1 knockdown resulted in a fasting induction of LDLr gene expression (Fig. 4*D*). Bile acid synthesis from cholesterol is yet another pathway involved in cholesterol clearance and catabolism (37). A rate-limiting enzyme involved in bile acid synthesis, Cyp7A1, was decreased in fasted livers of SIRT1 shRNA mice (Fig. 4*D*) and induced by SIRT1 overexpression in the fasted state (Fig. 4*E*). The decreased expression of genes acting in cholesterol efflux and degradation is consistent with the accumulation of hepatic cholesterol in SIRT1 knockdown livers. SIRT1 overexpression in the fasted state caused an induction of the efflux and degradation pathways resulting in decreased in hepatic cholesterol content.

We also analyzed expression of two important transcriptional regulators of cholesterol transport and metabolism: LXRα and PGC-1β (19, 38). SIRT1 knockdown caused a slight yet highly significant decrease in LXRα expression in both fed and fasted livers. However, SIRT1 knockdown decreased expression of PGC-1β only the fasted state (Fig. 4*D*). These data suggest that the effects of SIRT1 knockdown on serum and hepatic cholesterol may be mediated through down regulation of LXRα and PGC-1β mRNA. However, SIRT1 overexpression was not capable of reversing these effects on PGC-1β and LXRα gene expression (Fig. 4*E*). Finally, we tested whether the effects of SIRT1 on cholesterol metabolism genes required PGC-1α. In contrast to the effects of SIRT1 on cholesterol levels, which were independent of PGC-1α (Fig. 3*C*), the SIRT1-mediated increases in SR-B1 and Cyp7a1 were totally dependent on PGC-1α (Fig. 4*F*). These data indicate that expression of SR-B1 and Cyp7A1 is not essential for changes in cholesterol levels. Although it is not clear exactly which genes mediate the effects of SIRT1 on cholesterol levels, compensatory effects such as a

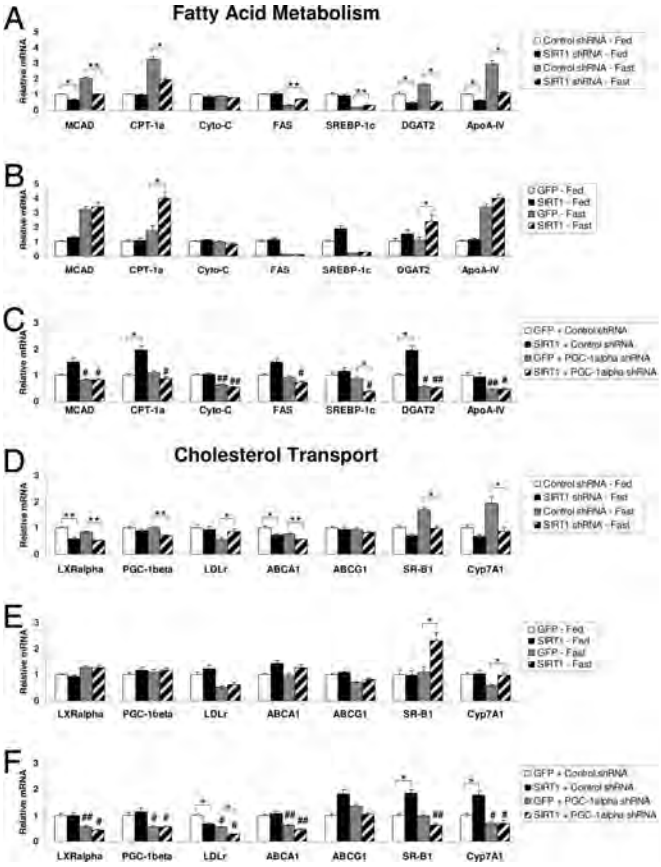

Fig. 4. SIRT1 controls hepatic expression of genes involved in lipid metabolism. Quantitative RT-PCR was used to analyze genes involved in fatty acid and cholesterol metabolism. (*A* and *D*) Fed, control shRNA ($n = 5$) or SIRT1 shRNA ($n = 6$); fasted for 20 h, control shRNA ($n = 5$) and SIRT1 shRNA ($n = 5$). (*B* and *E*) Fed, GFP ($n = 6$) or SIRT1 ($n = 6$); fasted for 19 h, GFP ($n = 6$) or SIRT1 ($n = 6$). (*C* and *F*) Double infection with GFP or SIRT1 overexpression and control or PGC-1α shRNA (each bar, $n = 9$) following a 20-h fast. All data are presented as the average ± SEM normalized to 36b4 expression. Significance was determined by Student's *t* test. GFP/control shRNA vs. SIRT1/SIRT1 shRNA: *, $P < 0.05$; **, $P < 0.01$. Control shRNA vs. PGC-1α shRNA: #, $P < 0.05$; ##, $P < 0.01$.

strong reduction of LDLr expression by PGC-1α knockdown may attempt to normalize cholesterol levels when efflux and degradation pathways are repressed (Fig. 4*F*).

Discussion

In response to nutritional challenges, metabolic gene expression needs to be precisely controlled to maintain glucose and lipid homeostasis. This control is accomplished by a network of transcription factors and coactivators, which are responsible for connecting the hormonal and nutrient signals to transcriptional regulation of metabolic pathways. In this context, we have found that SIRT1 has an important role mediating the liver's metabolic response to fasting. Hepatic knockdown of SIRT1 severely abrogates the fasting induction of gluconeogenic and fatty acid oxidation genes. Moreover, SIRT1 knockdown also reduced glycemia and serum cholesterol while causing accumulation of hepatic free fatty acids and cholesterol. We also found that SIRT1 contributes to the regulation of genes involved in triglyceride metabolism and cholesterol degradation and transport. Importantly, many of the effects we observed by SIRT1 knockdown were reversed with SIRT1 overexpression.

The coactivator PGC-1α is part of a transcriptional network that is involved in adaptation to nutrient stresses, through regulation of gluconeogenic and fatty acid β-oxidation gene expression (25, 39). We have previously shown that PGC-1α's ability to induce gluconeogenesis is largely regulated by acetylation (28, 40). Here, we extend these results by showing that *in vivo* knockdown and overexpression of SIRT1 is sufficient to alter endogenous acetylation of PGC-1α. We find that in the fasted liver the acetylation state of PGC-1α strongly correlates with repression/induction of gluconeogenic genes. Importantly, we show that when PGC-1α levels are reduced by shRNA knockdown, SIRT1 overexpression no longer reduces glucose tolerance or up-regulates gluconeogenic genes, suggesting that SIRT1 requires PGC-1α for these effects. Conversely, it seems that to a large extent that PGC-1α requires SIRT1 to stimulate glucose production. SIRT1 knockdown reduces the effect of PGC-1α overexpression in a PTT and on gluconeogenic gene expression. Moreover, the stimulatory effect of SIRT1 on PGC-1α is most likely mediated through deacetylation, as demonstrated by the greater induction of gluconeogenic genes and hyperglycemia caused by overexpression of the R13 PGC-1α acetylation mutant. These data combined suggest that the fasting induction, interaction, and deacetylation of PGC-1α by SIRT1 are an important regulatory component in the fasting induction of gluconeogenesis.

Oxidation of fatty acids and triglyceride synthesis are also important components of the liver's metabolic response to fasting and are activated by PGC-1α (19, 41). Consistent with SIRT1 activating PGC-1α upon fasting, expression of fatty acid oxidation and triglyceride genes were effected by SIRT1 knockdown and overexpression. Importantly, these changes in gene expression were also dependent on PGC-1α. These results further extend the role of SIRT1-PGC-1α to lipid metabolism in the fasted liver to control expression of key genes. One unexpected and new finding in our studies is the effect of SIRT1 on cholesterol metabolism and transport, an effect that seems to be independent of the fasting response. Intriguingly, whereas SIRT1 effects on systemic and hepatic cholesterol were PGC-1α independent, expression of genes that control cholesterol levels such as SR-B1 and Cyp7A1 required PGC-1α. A plausible explanation might involve compensatory mechanisms, such as decreased LDRr expression by SIRT1 and PGC-1α knockdown. Taken together, our results indicate that, although cholesterol levels are not dependent on PGC-1α, some of the effects on cholesterol metabolic gene expression require PGC-1α. It would be interesting to identify other factors that mediate the effects of SIRT1 in the liver and the extent to which PGC-1α is required for their activity.

We have previously reported induction of SIRT1 activity and protein in response to fasting in mouse liver and muscle and the role of pyruvate as an important nutrient signal in liver cells. Ablation of SIRT1 in cultured cells renders them unresponsive to nutrient fasting signals (28, 42). The fact that *in vivo* knockdown and overexpression of SIRT1 in liver had the most significant effect in the fasted state is consistent with our observations *in vitro*. This data provides evidence to suggest that SIRT1 is a nutrient sensor regulating metabolic pathways through deacetylation of PGC-1α and other targets. However, the specific nutrient and/or hormonal inputs that regulate SIRT1 are unknown at this time. SIRT1 may be able to respond to a broad range of signals through metabolic flux and NAD^+/NADH ratios. Interestingly, this biological function is consistent with the hypothesized role of SIRT1 homologs in calorie restriction observed in other species (43). Taken together, our results indicate that SIRT1 plays a key role in coordinating metabolic responses to calorie restriction/nutrient deprivation. Using an *in vivo* model, we find that SIRT1 is required for the maintenance of glucose and lipid homeostasis in the liver.

Methods

Animal Experiments. All animal experiments conformed to protocols approved by animal care and use committees at the Johns Hopkins School of Medicine and the Dana–Farber Cancer Institute. Experiments were performed in 6- to 8-week-old male BALB/c mice purchased from Harlan Laboratories (Indianapolis, IN). Adenovirus infections were performed by tail vein injection with 1×10^9 infectious particles per mouse (1.5×10^9 for double infections). Mice were killed 7–9 days after transduction. For detailed methods, see *SI Methods*.

GTT, ITT, and PTTs. For the GTT, after a 5-h fast, mice received an i.p. injection of 2 g/kg dextrose (Sigma, St. Louis, MO) dissolved in PBS. For the ITT, after a 5-h fast, mice received an i.p. injection of 0.6 unit/kg insulin (Sigma) in PBS. For the PTT, after an 18-h fast, mice received an i.p. injection of 2 g/kg sodium pyruvate (Sigma) dissolved in PBS. Blood glucose concentrations were measure via tail bleed before and at times indicated after injection. All glucose measurements were made using an Ascenscia Elite XL glucometer (Bayer, Wuppertal, Germany).

Metabolite Measurements. Serum true triglycerides and liver total triglycerides were measured using a colorimetric assay (TR0100; Sigma). Serum and liver free fatty acids (nonesterified fatty acids) were measured using the NEFA C kit (Wako, Osaka, Japan). Total serum and liver cholesterol was measured using cholesterol reagent (Pointe Scientific, Canton, MI). For detailed methods, see *SI Methods*.

For more information about PGC-1α acetylation, see *SI Appendices 1 and 2*.

Quantitative Real-Time PCR. All gene expression was measured by quantitative real-time PCR. Expression was determined by ΔC_T versus control 36B4. Primer sequences are available upon request.

Statistical Analysis. All data presented is the average ± SEM, unless indicated otherwise. Significance was determined using two-tailed unpaired Student's *t* test. $*$ denotes $P < 0.05$, and $**$ denotes $P < 0.01$.

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The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1α

Agnès Coste[a,1,2], Jean-Francois Louet[b,1], Marie Lagouge[a,1], Carles Lerin[c], Maria Cristina Antal[d], Hamid Meziane[d], Kristina Schoonjans[a,e], Pere Puigserver[c], Bert W. O'Malley[b,3], and Johan Auwerx[a,c,d,3]

[a]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, 67404 Illkirch, France; [b]Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030; [c]Dana Farber Cancer Institute and Department of Cell Biology Harvard Medical School, Boston, MA 02115; [d]Institut Clinique de la Souris, 67404 Illkirch, France; and [e]Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

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Transcriptional control of metabolic circuits requires coordination between specific transcription factors and coregulators and is often deregulated in metabolic diseases. We characterized here the mechanisms through which the coactivator SRC-3 controls energy homeostasis. SRC-3 knock-out mice present a more favorable metabolic profile relative to their wild-type littermates. This metabolic improvement in SRC-3$^{-/-}$ mice is caused by an increase in mitochondrial function and in energy expenditure as a consequence of activation of PGC-1α. By controlling the expression of the only characterized PGC-1α acetyltransferase GCN5, SRC-3 induces PGC-1α acetylation and consequently inhibits its activity. Interestingly, SRC-3 expression is induced by caloric excess, resulting in the inhibition of PGC-1α activity and energy expenditure, whereas caloric restriction reduces SRC-3 levels leading to enhanced PGC-1α activity and energy expenditure. Collectively, these data suggest that SRC-3 is a critical link in a cofactor network that uses PGC-1α as an effector to control mitochondrial function and energy homeostasis.

acetyltransferase | caloric restriction | cofactors | deacetylase | SIRT1

Transcriptional control is achieved through an interwoven and redundant molecular circuitry that involves individual transcription factors, the basal transcriptional machinery, and multiprotein coregulator complexes, which fine-tune metabolic homeostasis. Most of the coregulators interact directly with transcription factors and can either repress or enhance their transcriptional activities (1). Aberrant signaling by coregulators is known to generate abnormalities of cellular metabolism, and hence can contribute to abnormalities of systemic metabolic pathways and to the pathogenesis of several common disorders, such as obesity and type 2 diabetes (T2DM) (2). Steroid receptor coactivators (SRCs), also known as p160 proteins, which include SRC-1/NCoA-1, SRC-2/GRIP1/TIF2/NCoA2 and SRC-3/p/CIP/AIB1/ACTR/RAC3/TRAM-1, are transcriptional coactivators that interact with nuclear receptors and enhance their transactivation in a ligand-dependent manner (3–5). The main support for a role of the p160 proteins in the control of metabolism came from the characterization of various knock-out animal models. SRC-2$^{-/-}$ mice are protected against diet-induced obesity because of an enhanced energy expenditure (EE) in brown adipose tissue (BAT) (6) and in skeletal muscle (unpublished data). SRC-2$^{-/-}$ mice furthermore have an improved glucose tolerance and insulin sensitivity and a decreased adipocyte differentiation. In sharp contrast to SRC-2$^{-/-}$ mice, SRC-1$^{-/-}$ mice are prone to obesity due to reduced EE (6); SRC-3 is best known for its effects on cell proliferation and tumorigenesis (7, 8). The fact that SRC-3 decreases adipocyte differentiation (9, 10) and that the double SRC-1/3$^{-/-}$ mice exhibit an increased basal metabolic rate (10) suggested an impact of SRC-3 in metabolic control. The exact mechanisms through which SRC-3 affects metabolism, however, have not yet been fully characterized.

We here investigated the potential role of SRC-3 in energy homeostasis. As SRC-3$^{-/-}$ mice display clear signs of increased EE, subsequent to enhanced mitochondrial function in BAT and skeletal muscle, their body weight was lower and lipid and glucose homeostasis was improved. Mitochondrial activation in the absence of SRC-3 was caused by the deacetylation and increase in activity of the PPARγ coactivator-1α (PGC-1α), which coordinates mitochondrial function (11, 12). SRC-3 facilitates the acetylation and the consecutive inactivation of PGC-1α (13), through its effect on the expression of GCN5, the prime PGC-1α acetyltransferase (14). Furthermore, the expression of both SRC-3 and GCN5 is sensitive to fasting and high fat (HF) feeding, which subsequently, through its effect on PGC-1α-mediated EE, fine-tunes energy homeostasis. Taken together, these observations implicate SRC-3 as a critical component of a cofactor signaling network that converges on PGC-1α to control energy homeostasis.

Results

SRC-3$^{-/-}$ Mice Are Lean and Protected Against Diet-Induced Diabesity. To characterize the metabolic role of SRC-3, we monitored weight evolution of SRC-3$^{+/+}$ and SRC-3$^{-/-}$ mice for three months. After weaning, SRC-3$^{-/-}$ mice weighed already significantly less. However, they gained weight at an rate equivalent to the SRC-3$^{+/+}$ mice when fed chow (Fig. 1*A*). Strikingly, when fed an HF diet, SRC-3$^{-/-}$ mice gained less weight as compared to their WT littermates (Fig. 1*A*). These differences in body weight do not correlate with any change in caloric intake, spontaneous locomotor activity, or absorption [supporting information (SI) Fig. S1 *A* and *B* and data not shown] and are accounted for by a reduced fat mass accretion. Indeed, SRC-3$^{-/-}$ mice had a significantly lower body fat content than WT mice, as measured by dual energy x-ray absorptiometry (Fig. S1*C*) and as mirrored by the reduced weight of the epididymal white adipose tissue (WAT) and intrascapular BAT depots (Fig. S1*D*, ref. 10), whether they were fed chow or an HF diet.

The reduction in body weight in SRC-3$^{-/-}$ mice is accompa-

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The authors declare no conflict of interest.

[1]A.C., J.F.L., and M.L. contributed equally to this work.

[2]Present address: UMR 152-IRD/Université Paul Sabatier, 31062 Toulouse, France.

[3]To whom correspondence may be addressed. E-mail: johan.auwerx@epfl.ch and berto@bcm.tmc.edu.

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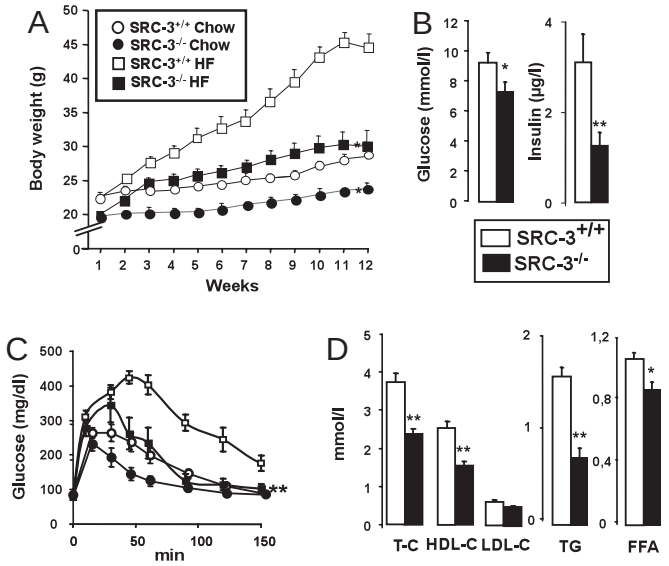

Fig. 1. SRC-$3^{-/-}$ mice are lean with an increased insulin sensitivity and an improved lipid profile compared to $SRC3^{+/+}$ mice (*A*) Body weight evolution of mice on either chow or HF diet ($n = 8$). (*B*) Serum glucose and insulin levels in mice fed an HF diet after a 6 h fasting ($n = 8$). (*C*) Serum glucose levels evolution during an i.p. glucose tolerance test performed after a glucose load (2g/kg) in chow or HF fed, overnight-fasted mice ($n = 8$). (*D*) Serum levels of total cholesterol (T-C), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG) and free fatty acids (FFA) in mice fed an HF diet ($n = 8$). *($P < 0.05$) and **, $P < 0.01$ indicates significant differences as compared to SRC-$3^{+/+}$ mice.

nied by an improved metabolic control as evidenced by the lower fasting plasma glucose and insulin levels (Fig. 1*B*). Furthermore, SRC-$3^{-/-}$ mice cleared glucose more effectively after i.p. glucose injection than SRC-$3^{+/+}$ animals, in chow or HF fed conditions (Fig. 1*C*). The rate of glucose clearance upon i.p. insulin injection was also higher in SRC-$3^{-/-}$ mice, further suggesting insulin sensitization (Fig. S1*E*).

Another indication of an improved metabolic profile was provided by the lower fasting cholesterol, triglycerides, and free fatty acids levels in SRC-$3^{-/-}$ relative to WT mice (Fig. 1*D*). In addition, whereas the liver weight of SRC-$3^{-/-}$ and SRC-$3^{+/+}$ mice under chow was indistinguishable, the livers of SRC-$3^{-/-}$ animals weighed less (Fig. S1*D*) and were smaller and less pale (Fig. S1*F*) when fed an HF diet, indicating an increased metabolic activity and a decreased lipid accumulation. Similar conclusions were derived from the analysis of hematoxylin and eosin (HE) stained liver sections of HF-fed animals and were confirmed by oil red O staining (Fig. S1*F*). Taken together these data suggest that the absence of SRC-3 improves both lipid and glucose homeostasis and induces a more favorable metabolic profile.

Energy Expenditure Is Enhanced in SRC-$3^{-/-}$ Mice. Since changes in caloric intake or locomotor activity (Fig. S1 *A* and *B*) could not explain the weight differences, we investigated whether the protective effects against obesity observed in SRC-$3^{-/-}$ mice were mediated by an increase in basal metabolism. The higher O_2 consumption and CO_2 production evident in SRC-$3^{-/-}$ mice were consistent with an increased EE. The respiratory quotient was furthermore lower in SRC-$3^{-/-}$ mice, suggesting an increased fatty acid oxidation (FAO) (Fig. 2*A*).

To identify the site(s) responsible for the increased EE, we performed a detailed histological analysis of key metabolic tissues. Transmission electron microscopy analysis of BAT sections showed significantly smaller lipid vacuoles and more mitochondria in SRC-$3^{-/-}$ mice (Fig. S2*A*). This amplification of the mitochondria was reflected by the increase in BAT mitochondrial DNA (mtDNA) content (Fig. 2*B*). Furthermore, the brown adipocytes size was reduced in SRC-$3^{-/-}$ mice, as assessed by an HE staining (Fig. S2*B*). Finally, consistent with the general increase in EE and with the BAT morphology, rectal temperature of SRC-$3^{-/-}$ mice remained significantly higher upon cold exposure, demonstrating an enhanced adaptive thermogenesis (Fig. S2*C*).

The number and the size of mitochondria were also increased in SRC-$3^{-/-}$ mice muscle (Fig. 2*C*). This was confirmed by immunohistochemical studies using an antibody directed against Optic Atrophy 1, a protein of the inner mitochondrial membrane

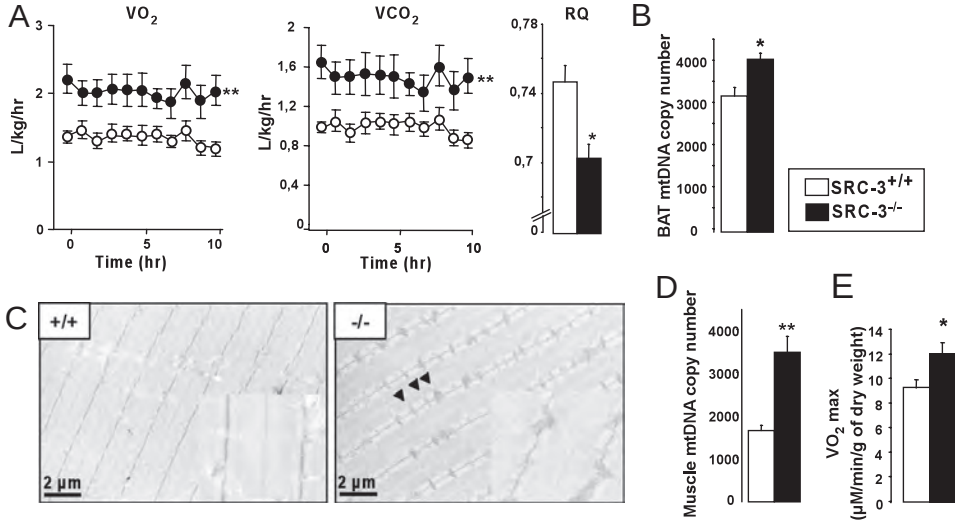

Fig. 2. Enhanced energy expenditure and endurance in SRC-$3^{-/-}$ mice relative to SRC-$3^{+/+}$ mice (*A*) O_2 consumption (VO_2) and CO_2 production (VCO_2) in mice fed regular chow. The respiratory quotient ($RQ = VCO_2/VO_2$) was calculated between 2 and 3 a.m. ($n = 8$). (*B*) Mitochondrial DNA (mtDNA) copy number in brown adipose tissue from mice fed an HF diet. (*C*) Representative pictures of gastrocnemius muscle sections from mice fed an HF diet, analyzed by electron microscopy. The inset shows an enlarged image of the muscle. Mitochondria are indicated by arrowheads. (*D*) Mitochondrial DNA copy number in the gastrocnemius muscle from SRC-$3^{+/+}$ and SRC-$3^{-/-}$ mice fed an HF diet. (*E*) Maximum O_2 consumption measured *ex vivo* in isolated gastrocnemius fibers of SRC-$3^{+/+}$ and SRC-$3^{-/-}$ mice fed a chow in the presence of increasing ADP concentration ($n = 5$). *, $P < 0.05$ and **, $P < 0.01$ indicates significant differences as compared to SRC-$3^{+/+}$ mice.

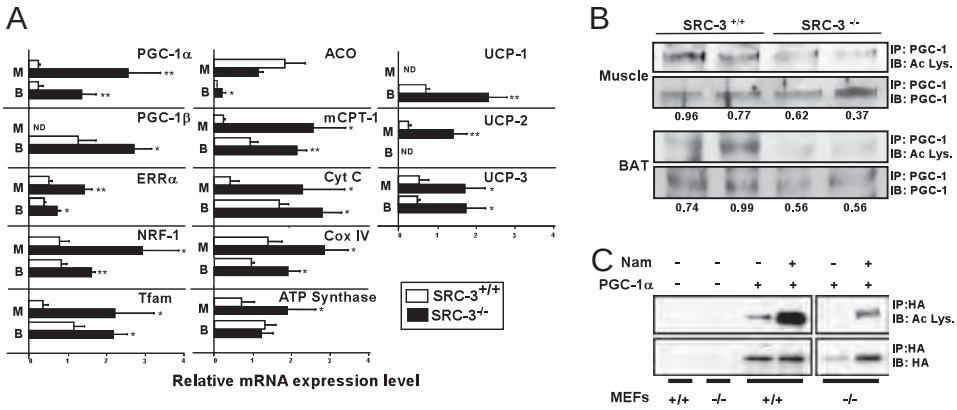

Fig. 3. SRC-3 affects PGC-1α acetylation and activity. (*A*) mRNA levels of PGC-1α, PGC-1β, ERRα, NRF-1, Tfam, mCPT-1, ACO, Cyt C, Cox IV, ATP synthase, UCP-1, UCP-2, UCP-3, SRC-1 and SRC-2 in gastrocnemius muscle (Muscle) and brown adipose tissue (BAT) of SRC-3$^{+/+}$ and SRC-3$^{-/-}$ mice determined by quantitative PCR ($n = 8$). (*B*) Acetylation level of PGC-1 in the gastrocnemius muscle and brown adipose tissue (BAT) of 2 representative SRC-3$^{+/+}$ and SRC-3$^{-/-}$ mice fed a high fat diet. PGC-1 was first immunoprecipitated (IP). Total and acetylated PGC-1 were then detected through an immunoblot (IB) using anti-PGC-1 and anti-acetylated lysine (Ac Lys.) antibodies respectively. The values under the blots represent the ratio of acetylated over total PGC-1. (*C*) Acetylation level of PGC-1α in SRC-3$^{+/+}$ and $^{-/-}$ MEFs. Where indicated, MEFs were infected with an adenovirus expressing PGC-1α (PGC-1α) and incubated with 20 mM nicotinamide (Nam). PGC-1α was first immunoprecipitated. Total and acetylated PGC-1α were detected through an immunoblot using anti-HA and anti-acetylated lysine antibodies respectively. *, $P < 0.05$ and **, $P < 0.01$ indicate significant differences as compared to SRC-3$^{+/+}$ mice.

(15), which showed a more intense staining of the SRC-3$^{-/-}$ mice muscle sections (Fig. S2*D*), as well as by the robust increase in muscle mtDNA content (Fig. 2*D*). In line with the morphological aspect of the muscle, the distance run to exhaustion on a treadmill was significantly increased in SRC-3$^{-/-}$ mice (Fig. S2*E*). Moreover, the maximum O_2 consumption monitored in isolated muscle fibers was significantly higher in SRC-3$^{-/-}$ mice, testifying to an increased oxidative capacity (Fig. 2*E*). These results together indicate a major contribution of BAT and muscle to the enhanced EE observed in SRC-3$^{-/-}$ mice.

SRC-3 Controls PGC-1α Activity by Modifying Its Acetylation Status. To further understand the molecular role of SRC-3 in the regulation of EE, we analyzed, in BAT and muscle, the expression of a set of representative genes known to be involved in mitochondrial function. With few exceptions, the expression of all of the transcription factors and genes that control FAO and oxidative phosphorylation (OXPHOS) that we analyzed was up-regulated in the BAT and muscle of SRC-3$^{-/-}$ compared to WT mice, confirming our phenotypic observations (Fig. 3*A*). Most notable was the change in the expression of PGC-1α, the master regulator of mitochondrial activity (11).

We also evaluated whether the genetic absence of SRC-3 could alter the acetylation levels of PGC-1α, one of the prime ways PGC-1α activity is regulated (13). Interestingly, the ratio of acetylated over total PGC-1α protein was significantly decreased in both muscle and BAT isolated from SRC-3$^{-/-}$ compared to SRC-3$^{+/+}$ mice (Fig. 3*B*). To further substantiate that the absence of SRC-3 decreases the acetylation of PGC-1α, we compared PGC-1α acetylation in SRC-3$^{+/+}$ and SRC-3$^{-/-}$ mouse embryonic fibroblasts (MEFs) that were infected with an adenovirus encoding for PGC-1α. Relative to SRC-3$^{+/+}$ MEFs, the acetylation level of PGC-1α in SRC-3$^{-/-}$ MEFs is significantly decreased (Fig. 3*C*). Even in the presence of nicotinamide, an inhibitor of the PGC-1α deacetylase SIRT1, PGC-1α acetylation was significantly lower in the SRC-3$^{-/-}$ MEFs (Fig. 3*C*).

To consolidate our hypothesis, we evaluated whether reduction of SRC-3 expression in C2C12 cells that were infected with an adenovirus expressing PGC-1α—and hence express comparable levels of PGC-1α—would decrease PGC-1α acetylation levels and subsequently induce the expression of PGC-1α target genes. As predicted, the siRNA-mediated knock-down of SRC-3 in these C2C12 myotubes decreased PGC-1α acetylation significantly (Fig. S3*A*). Furthermore the expression levels of mRNAs encoding for a set of genes involved in mitochondrial function were all induced concomitantly with the reduction of SRC-3 expression in these C2C12 cells (Fig. S3*B*). In combination, these *in vivo* and *in vitro* data prove that SRC-3 facilitates the acetylation of PGC-1α and controls its activity.

SRC-3 Facilitates PGC-1α Acetylation Through Promoting GCN5 Expression. As PGC-1α was recently reported to be acetylated by GCN5, resulting in its inactivation (14), and as SRC-3 is an important coactivator for gene expression, we investigated the possibility that SRC-3 could control GCN5 expression and hence affect PGC-1α acetylation. Interestingly, GCN5 gene expression was significantly decreased in both muscle and BAT of SRC-3$^{-/-}$ mice (Fig. 4*A*). The cell autonomous effects of SRC-3 on GCN5 expression were then investigated by both gain and loss of function approaches. In a cellular context, SRC-3 overexpression enhanced GCN5 mRNA expression (Fig. 4*B*); conversely, the reduction of its expression by siRNA-mediated knock-down reduced GCN5 mRNA levels (Fig. 4*C*), demonstrating that SRC-3 controls the expression of GCN5. This transcriptional regulation of GCN5 expression is the consequence of a direct and specific binding of SRC-3 on the promoter of GCN5 as indicated by the results of a ChIP assay (Fig. 4*D*). Finally, we could recapitulate the transcriptional effect of SRC-3 on the expression of a luciferase reporter construct under the control of a specific region of the GCN5 promoter that was identified by ChIP to bind the coactivator (Fig. 4*E*). All together, these data suggest that GCN5 expression is transcriptionally controlled by the coactivator SRC-3.

To validate the effect of SRC-3 on GCN5-mediated PGC-1α acetylation, C2C12 cells were infected with adenoviruses expressing PGC-1α, SRC-3 or GCN5 respectively (Fig. 4*F*). As expected, GCN5 induced PGC-1α acetylation in C2C12 cells, whereas SRC-3 by itself did not enhance PGC-1α acetylation. Interestingly, the acetylation level of PGC-1α was further enhanced in cells coinfected with SRC-3 and GCN5-expressing adenoviruses (Fig. 4*F*). In a similar experiment in HEK293 cells, SRC-3 also failed to acetylate PGC-1α directly, but again facilitated GCN5-mediated PGC-1α acetylation (Fig. S4). It is of note that in both cellular models, SRC-3 overexpression is also

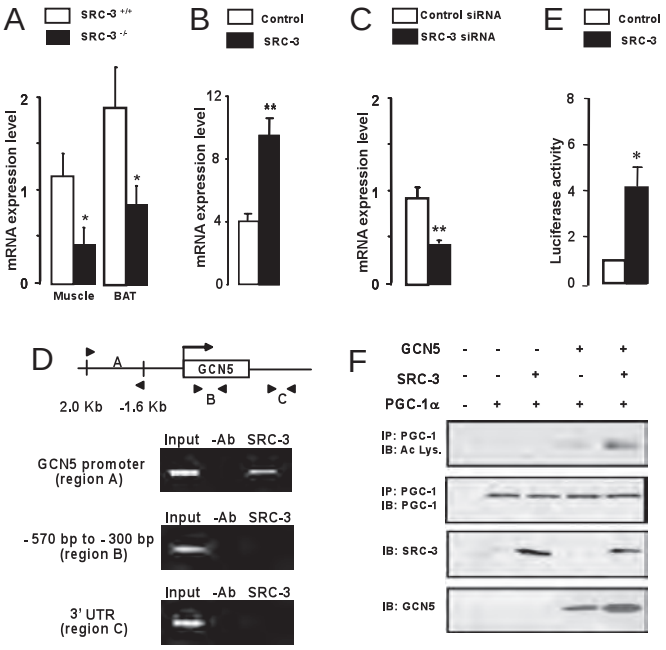

Fig. 4. SRC-3 promotes GCN5 expression (*A*) GCN5 mRNA level in gastrocnemius muscle and brown adipose tissue (BAT) of SRC-$3^{+/+}$ and SRC-$3^{-/-}$ mice fed a high fat diet ($n = 8$). (*B*) GCN5 mRNA level in C2C12 cells infected with an adenovirus expressing SRC-3 (*C*) GCN5 mRNA level in C2C12 cells transfected with either a control siRNA or a SRC-3 siRNA ($n = 6$). (*D*) ChIP assay showing the specific recruitment of SRC-3 on the promoter but not on the coding or 3′UTR of the mGCN5 gene. (*E*) Luciferase activity detected in extracts of C2C12 cells transfected with a GCN5 promoter luciferase reporter construct and cotransfected or not with SRC-3 ($n = 6$). (*F*) Acetylation level of PGC-1α determined from total extracts of C2C12 cells infected with a combination of adenoviruses, expressing PGC-1α, GCN5 and/or SRC-3. Western-blot analysis of SRC-3 and GCN5 are shown in the bottom panels, whereas the levels of acetylated (Ac-Lys) and total PGC-1α are depicted in the top panels. *, $P < 0.05$ and **, $P < 0.005$ are indicative of significant differences.

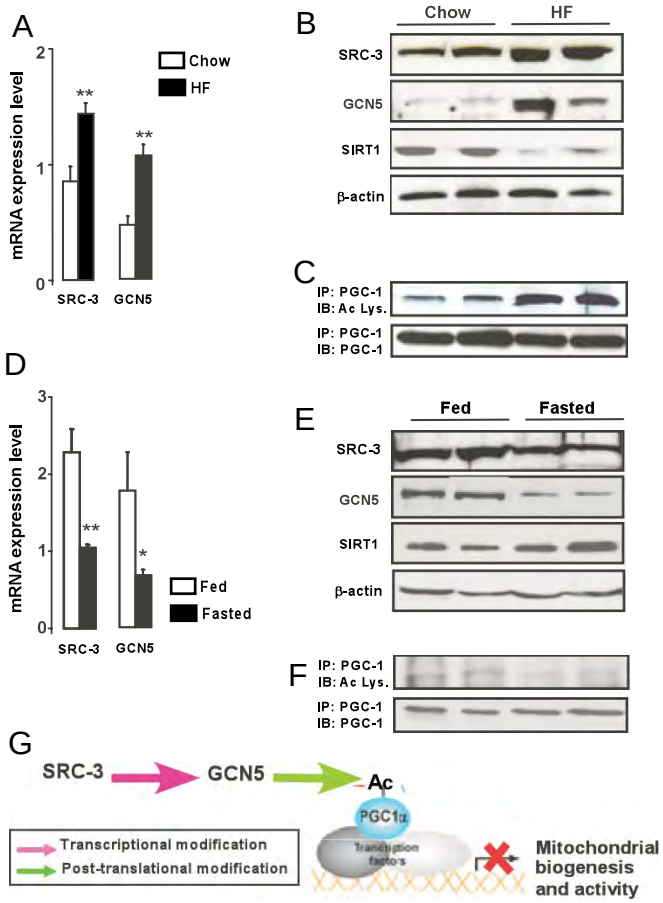

Fig. 5. Modulation of SRC-3 in diet-induced obese mice and under fasting condition. (*A*) SRC-3 and GCN5 mRNA levels in gastrocnemius muscle of C57BL/6J male mice fed either a chow or a high fat (HF) diet for 4 months ($n = 6$). (*B*) SRC-3 and GCN5 protein levels in animals treated as described in *A*. β-actin levels are shown as loading controls. (*C*) PGC-1 acetylation level determined from gastrocnemius muscle nuclear extracts of C57BL/6J male mice fed either regular chow or HF diet for 4 months. PGC-1 was immunoprecipitated (IP). Then total and acetylated PGC-1 were detected through an immunoblot (IB) using anti-PGC-1 and anti-acetylated lysine (Ac Lys.) antibodies respectively. (*D*) SRC-3 and GCN5 mRNA levels in gastrocnemius muscle of C57BL/6J male mice under fed or fasted (6 h) conditions ($n = 6$). (*E*) SRC-3 and GCN5 protein levels in animals treated as described in *D*. β-actin is used as loading control. (*F*) PGC-1 acetylation level determined from gastrocnemius muscle nuclear extracts of 2 C57BL/6J male mice under fed or fasted conditions. PGC-1 acetylation level is detected as described in C. (*G*) Schematic illustration of the role of SRC-3 on the expression of GCN5, on PGC-1α acetylation and subsequent activity. *, $P < 0.05$ and **, $P < 0.01$ indicate significant differences as compared to the control mice.

correlated with an increased GCN5 protein level, which fits with our previous observations showing that SRC-3 stimulates GCN5 expression (Fig. 4*F*, Lane 5).

SRC-3 and GCN5 Levels Are Reciprocally Regulated by HF Diet and Fasting. Since it was clear from the data that SRC-3, together with GCN5, is a critical component of the regulatory machinery that converges on PGC-1α to control energy homeostasis, we tested whether such a regulation has a physiological impact. We evaluated the expression of SRC-3 and GCN5 in C57BL/6J mice that were exposed either to a caloric excess, by feeding them an HF diet, or to a caloric deficit, by fasting them. Expression levels of both SRC-3 and GCN5 mRNA were robustly induced in the muscle after HF feeding (Fig. 5*A*), as were the protein levels (Fig. 5*B*, *Top*). Conversely, after a 6 h fast, SRC-3 and GCN5 mRNA and protein levels were decreased (Fig. 5 *D* and *E*). Interestingly, the coordinate regulation of SRC-3 and GCN5 expression upon HF feeding was associated with an increase in the ratio of acetylated over total PGC-1α protein (Fig. 5*C*), whereas the changes in the expression levels of both cofactors upon fasting reduced this ratio in the muscle (Fig. 5*F*). Strikingly, the expression of SIRT1, which is known to be regulated by caloric restriction (13) and to deacetylate and activate PGC-1α, showed almost a mirror image of that of SRC-3 and GCN5 (Fig. 5 *B* and *E*). This converse regulation of SIRT1 expression could clearly reinforce the changes in PGC-1α acetylation and activity, induced by the alterations in SRC-3 and GCN5 expression. Altogether, the changes in cofactor expression induced by caloric excess or restriction hence control PGC-1α acetylation and activity and govern the dynamic adaptation of energy homeostasis as summarized in Fig. 5*G*.

Discussion

Mitochondrial dysfunction in general and reduced PGC-1α activity in particular, has recently been linked with the pathogenesis of metabolic diseases as it increases metabolic and cardiovascular risk and precedes the development of T2DM (16–18). Conversely, an increase in physical activity and dietary restriction, the cornerstone of the therapy of obesity and the metabolic syndrome, are both inducing mitochondrial function (19). It is also clear in our study that enhanced PGC-1α activity contributes in a major fashion to the mitochondrial activation observed in BAT and muscle of SRC-$3^{-/-}$ mice. PGC-1α activity

is predominantly regulated by various posttranslational modifications, including changes in its acetylation status (for review see ref. 12). Consistent with this type of regulation, we demonstrate here that SRC-3 facilitates PGC-1α acetylation in muscle and BAT, through enhancing the expression of the PGC-1α acetyltransferase, GCN5 (14). How SRC-3 exactly controls GCN5 expression remain to be investigated. We hypothesize that SRC-3 could act through transcription factors such as C/EBPδ, for which response elements are present within the GCN5 promoter in the region where SRC-3 is binding. In addition, our data do not permit us to exclude the possibility that SRC-3 could also control GCN5 levels via posttranslational processes such as protein stabilization, rendering the interplay between both acetyltransferases even more complex.

Acetylation of PGC-1α is an important way to reduce PGC-1α activity and EE. Conversely, the type III histone deacetylase SIRT1 (20) was shown to promote EE, through deacetylation and activation of PGC-1α (13, 21). PGC-1α acetylation and activity seems to be controlled by a delicate balance between cellular acetyltransferases and deacetylases. For its deacetylase activity, SIRT1 is strictly dependent on cellular NAD^+ levels, which reflect cellular energy status. Also the activity of acetyltransferases, such GCN5, that use acetyl-CoA as substrate, is coupled to the metabolic state of the cells (22). Like changes in cellular NAD^+ levels, that affect SIRT1 deacetylase activity (20), variations in acetyl-CoA substrates for acetyltransferases (22), can therefore lead to changes in histone and protein acetylation, that impact on transcription (23). The relative activity of acetyltransferases and deacetylases therefore informs PGC-1α about the cellular energy status, which then adapts cellular energy production through its commanding role on mitochondrial biogenesis and function. Such additional cofactors act along with PGC-1α to influence energy balance (reviewed in ref. 2) and the p160 family of cofactors is a good case in point, as all of its member have been shown to impact on energy homeostasis. In fact, mice lacking the SRC-2 protein are protected against obesity due to enhanced adaptative thermogenesis, whereas SRC-1 deficient animals are prone to obesity because of reduced EE (6). Double SRC-1/3 germline mutant mice have also increased basal metabolic rate, although it was difficult to assign the effects to either SRC-1, SRC-3, or an epistatic interaction between the two proteins (10).

Interestingly, we show here that the expression of several acetyltransferases and deacetylases is highly regulated by the energy status. In fact, SRC-3 and GCN5 expression is induced during HF feeding, whereas their expression is reduced upon fasting. When energy is in excess, such as encountered during the consumption of an HF diet, the increased expression of SRC-3 and GCN5 promotes GCN5-mediated acetylation of PGC-1α, leading thereby to its inactivation, attenuating as such mitochondrial function and FAO. Under such conditions, EE cannot compensate for the higher energy intake, thus promoting the storage of excess energy in WAT. Our data are fully in line with a recent report indicating that an HF diet attenuates gene pathways necessary for OXPHOS and mitochondrial biogenesis and provides a mechanistic explanation for the observed phenotype in that study (24). Conversely, in situations of caloric restriction, mimicked by fasting in the current study, SRC-3 and GCN5 expression decreases, reducing the acetylation but enhancing the activity of PGC-1α, which facilitates the use of fatty acids stored in adipose tissues as a source of energy. Interestingly, as previously reported and confirmed in the present study, the expression levels of SIRT1, the major PGC-1α deacetylase, mirror those of SRC-3 and GCN5, resulting in a convergent regulation of PGC-1α acetylation by both acetyltransferases and deacetylases. From an evolutionary perspective, the regulation of PGC-1α acetylation by energy levels would favor the storage of energy during periods of feast, which could later be spend during times of famine. In our current industrialized societies, where food and energy are abundant, such a thrifty response is maladapted and could contribute to development of obesity and insulin resistance. It is therefore tempting to speculate that this converging cofactor network can be exploited to design new preventive and therapeutic strategies to combat obesity and associated metabolic disorders as T2DM.

Materials and Methods

Animal experiments: The generation of the SRC-$3^{-/-}$ mice has been described (23). All mice were maintained on a pure C57BL/6J background. Only male, aged-matched mice were used for phenotyping. Animals were maintained in standard conditions described in the supplemental method section. The methods used to assess the metabolic phenotype and for histological analysis were performed as outlined in the standard operating procedures linked to the EMPReSS website http://empress.har.mrc.ac.uk and as described (6, 21). Glucose, insulin, and lipids serum levels were measured as described (21).

***ex Vivo* O_2 Consumption Measurements.** The O_2 consumption was measured in isolated gastrocnemius muscle fibers as described (21).

RNA and DNA Analysis. RNA was extracted from tissues or cultured cells and quantified by quantitative RT-PCR as described in the *SI Methods*. MtDNA content was measured exactly as described (21).

Immunoblotting and Immunoprecipitations. Tissue homogenization and preparation of cytosolic and nuclear fractions were performed as described (25). Protein extracts were separated by SDS/PAGE. The list of the antibodies used is available in the *SI Methods*. PGC-1α acetylation was analyzed by immunoprecipitation of PGC-1 from nuclear lysates as described in *SI Methods* (26).

ChIP analyses in C2C12 cells were performed using an anti-SRC-3 antibody (BD Biosciences). The sets of primers used to amplify the different regions of the GCN5 mouse promoter and PCR conditions are available in *SI Methods*.

Cell Culture, Transient Transfections and Adenoviral Infections. these protocols can be found in the *SI Methods* section online.

Statistics. Statistical analyses were performed with the Student's test for independent samples (nonparametric), and data are expressed as means $\pm$ SEM. P value < 0.05 was considered as statistically significant.

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Concurrent regulation of AMP-activated protein kinase and SIRT1 in mammalian cells

Gabriela Suchankova [a], Lauren E. Nelson [a], Zachary Gerhart-Hines [b], Meghan Kelly [a], Marie-Soleil Gauthier [a], Asish K. Saha [a], Yasuo Ido [a], Pere Puigserver [b], Neil B. Ruderman [a,*]

[a] Section of Endocrinology and Departments of Medicine, Physiology and Biochemistry, Boston Medical Center, Boston, MA 02118, USA
[b] Dana-Farber Cancer Institute and Department of Cell Biology, Harvard Medical School, Boston, MA, USA

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ABSTRACT

We examined in HepG2 cells whether glucose-induced changes in AMP-activated protein kinase (AMPK) activity could be mediated by SIRT1, an NAD^+-dependent histone/protein deacetylase that has been linked to the increase in longevity caused by caloric restriction. Incubation with 25 vs. 5 mM glucose for 6 h concurrently diminished the phosphorylation of AMPK (Thr 172) and ACC (Ser 79), increased lactate release, and decreased the abundance and activity of SIRT1. In contrast, incubation with pyruvate (0.1 and 1 mM) for 2 h increased AMPK phosphorylation and SIRT1 abundance and activity. The putative SIRT1 activators resveratrol and quercetin also increased AMPK phosphorylation. None of the tested compounds (low or high glucose, pyruvate, and resveratrol) significantly altered the AMP/ATP ratio. Collectively, these findings raise the possibility that glucose-induced changes in AMPK are linked to alterations in SIRT1 abundance and activity and possibly cellular redox state.

AMP-activated protein kinase (AMPK) belongs to a family of highly conserved serine kinases that are regulated by nutritional and metabolic stresses that alter cellular energy state [1–3]. When activated, AMPK protects the cell against ATP depletion by stimulating processes such as fatty acid oxidation that promote ATP generation and inhibiting others, such as protein and lipid synthesis, that require ATP but are not acutely necessary for survival [1,4]. Although the activation of AMPK appears to be a direct consequence of an increase in the AMP-to-ATP ratio in many situations, studies in various tissues have shown that AMPK can be activated or inhibited by mechanisms that may not involve changes in adenine nucleotide levels [5,6]. In one such study, Itani et al. [7] demonstrated that the incubation of rat extensor digitorum longus (EDL) muscle with a high glucose medium (25 vs. 6 or 0 mM) for 4 h diminished AMPK activity without changing the whole-tissue concentrations of creatine phosphate or adenine nucleotides. The reduced AMPK activity correlated with an increased release of lactate by the EDL, raising the possibility that alterations in its redox state contributed to these changes.

Sirtuins are a family of redox-sensitive, NAD^+-dependent deacetylases that regulate gene expression by controlling the acetylation status of lysine residues on histones, transcription factors, and transcriptional coactivators [8]. Sir2 and its mammalian homolog SIRT1 are induced in response to nutrient deprivation and are thought to mediate the effects of caloric restriction on longevity [9,10]. Rodgers et al. [11] found an increase in SIRT1 abundance and activity in the livers of 24-h fasted mice, demonstrating increased deacetylation and activation of PGC-1α (PPAR-gamma coactivator-1α). Conversely, refeeding following a 24-h fast decreased hepatic SIRT1 abundance, as did incubation of cultured Fao rat hepatocytes in a high glucose medium (10 vs. 0 mM). We have previously shown that refeeding following a 48-h fast similarly reduces AMPK activity in rat liver [12], as does incubation of HepG2 cells with 25 vs. 5 mM glucose [3]. These findings suggest a link between SIRT1 and AMPK.

In this study, the linkage between SIRT1 and AMPK was examined more directly. We determined in HepG2 cells whether (1) glucose- and pyruvate-induced changes in AMPK activity (phosphorylation) are associated with alterations in SIRT1 abundance and activity, (2) SIRT1 activation and inhibition by pharmacological agents produce parallel changes in AMPK, and (3) observed alterations in AMPK activity occur in the presence or absence of changes in cellular energy state. The results suggest concurrent regulation of SIRT1 and AMPK in the absence of a change in whole cell energy state.

Materials and methods

Resveratrol was from Calbiochem (San Diego, CA). Pyruvate and nicotinamide were from Sigma (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and PBS were

* Corresponding author. Fax: +1 617 638 7094.
E-mail address: nrude@bu.edu (N.B. Ruderman).

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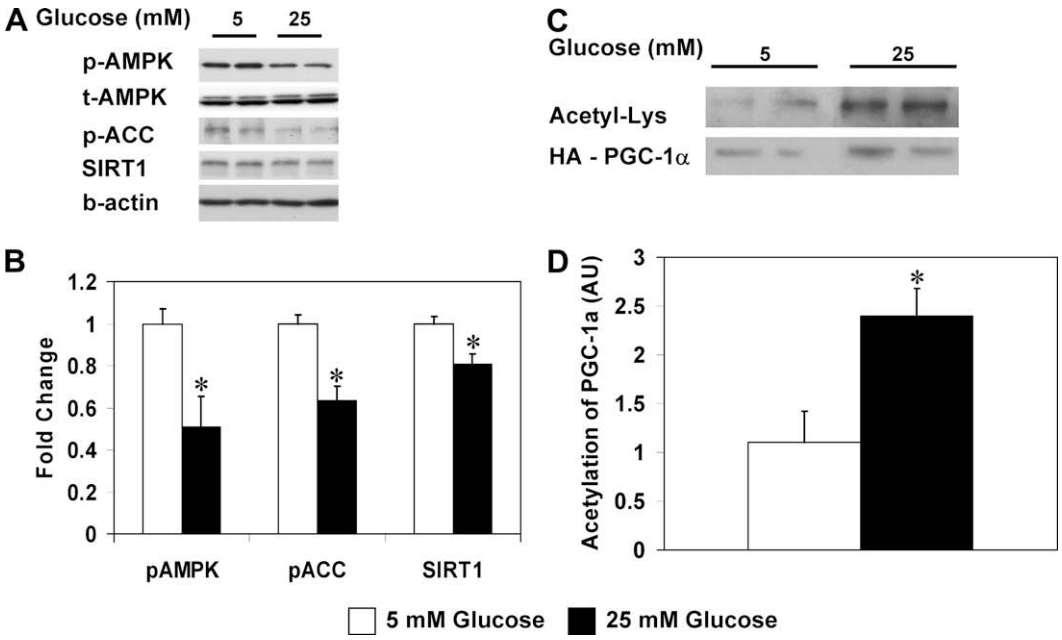

Fig. 1. Increasing the ambient glucose concentration decreases the phosphorylation of AMPK and ACC, and the abundance and activity of SIRT1 in HepG2 cells. HepG2 cells incubated in 5 or 25 mM glucose for 6 h. (A) Western blot analysis for p-AMPK (Thr 172), p-ACC (Ser 79), and SIRT1. (B) Quantification of representative blot shown in (A), results are means ± SE ($n = 5$); $^{*}p < 0.05$. (C) PGC-1α acetylation after 6 h incubation in 5 or 25 mM glucose. (D) Quantification of Acetyl-Lys/HA-PGC-1α. Results are means ± SE, ($n = 4$); $^{*}p < 0.05$.

from Gibco (Grand Island, NY). Rabbit monoclonal anti-phospho-Thr 172 AMPK and rabbit polyclonal anti-AMPKα subunit antibodies were from Cell Signaling. Anti-phospho-Ser 79 ACC1 was from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-SIRT1 (H-300) and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatments. HepG2 cells were cultured in DMEM containing 5 mM glucose supplemented with 10% FBS, 1% penicillin/streptomycin and subjected to assays after overnight serum and pyruvate depletion. C2C12 myocytes were cultured to 80% confluence in 5 mM glucose DMEM containing 1% GlutaMAX, 1% penicillin/streptomycin and 10% FBS, then differentiated to 80% myotubes in 5 mM glucose DMEM containing 2% Horse Serum, 1% GlutaMAX, and 1% penicillin/streptomycin.

Immunoblotting analysis. Samples (50 μg protein, as determined by Bio-Rad assay) were separated by SDS–PAGE, transferred to PVDF membrane, blocked with 5% milk in TBST, and incubated with primary antibody overnight at 4 °C. Bound antibodies were detected with HRP-linked secondary antibodies and visualized using enhanced chemiluminescence (Thermo Fisher) and autoradiography.

PGC-1α acetylation assay. PGC-1α lysine acetylation was determined in HepG2 cells transfected with an HA-tagged PGC-1α adenovirus, as described previously [13].

Other analyses. ATP, AMP, and ADP were measured spectrophotometrically as described previously [14]. Lactate release was determined spectrophotometrically using lactate dehydrogenase and NAD [15].

Experimental animals. Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained on a 12-h light cycle and fed ad libitum. Experiments were performed following an 18- to 20-h fast. All research on these animals were reviewed and performed in accordance with the requirements of the IACUC at Boston University Medical Center.

Muscle incubation. Rats were anesthetized with sodium pentobarbital (6 mg/100 g body weight i.p.). EDL muscle was isolated as described previously [14]. Muscles were initially equilibrated in Krebs–Henseleit solution containing 5 mM glucose for 20 min, followed by incubation ± resveratrol in DMSO for 2 h.

Statistics. Results are expressed as means ± SE. Student's *t*-test or ANOVA followed by Student–Newman–Keuls post-hoc analysis were used to determine significance ($p < 0.05$).

Results

Effect of incubation with different glucose concentrations on the phosphorylation of AMPK and ACC, and SIRT1 protein abundance and activity

Using a high glucose-induced model of insulin resistance in HepG2 cells [3], we first confirmed the finding of Zang et al. [3] that incubation of HepG2 cells in a high glucose medium (25 vs. 5 mM) for 6 h decreases the phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC) without altering AMPK abundance (Fig. 1A and B). We found that these alterations were not paralleled by changes in cellular energy state, as reflected by the AMP/ATP ratio (Table 1). On the other hand, they were associated with a decrease in SIRT1 protein abundance and a more than 2-fold increase in PGC-1α acetylation (Fig. 1A–D), suggesting that hyper-

Table 1
The effect of variations in glucose, pyruvate, and resveratrol concentration on cellular energy state in HepG2 cells.

	ATP	ADP	AMP	AMP/ATP
6 h Incubation, nmol/mg protein				
5 mM Glucose	9.4 ± 1.4	2.2 ± 0.2	0.39 ± 0.06	0.042 ± 0.009
25 mM Glucose	9.7 ± 0.8	3.10 ± 0.1	0.31 ± 0.03	0.035 ± 0.003
2 h Incubation, nmol/mg protein				
0 mM Pyruvate	11.9 ± 0.5	3.4 ± 0.2	0.37 ± 0.02	0.031 ± 0.001
1 mM Pyruvate	12.6 ± 0.2	3.6 ± 0.1	0.34 ± 0.02	0.027 ± 0.002
1 h Incubation, nmol/mg protein				
0 μM Resveratrol	11.3 ± 0.8	2.4 ± 0.2	0.28 ± 0.03	0.025 ± 0.002
50 μM Resveratrol	11.7 ± 1.1	2.6 ± 0.2	0.36 ± 0.06	0.033 ± 0.009
100 μM Resveratrol	11.9 ± 1.1	2.2 ± 0.2	0.39 ± 0.07	0.032 ± 0.005

Unless otherwise noted, incubations were carried out in 5 mM glucose DMEM. Adenine nucleotide levels were measured spectrophotometrically. Values are means ± SEM, $n = 9$ ($n = 3$/group on three independent experiments).

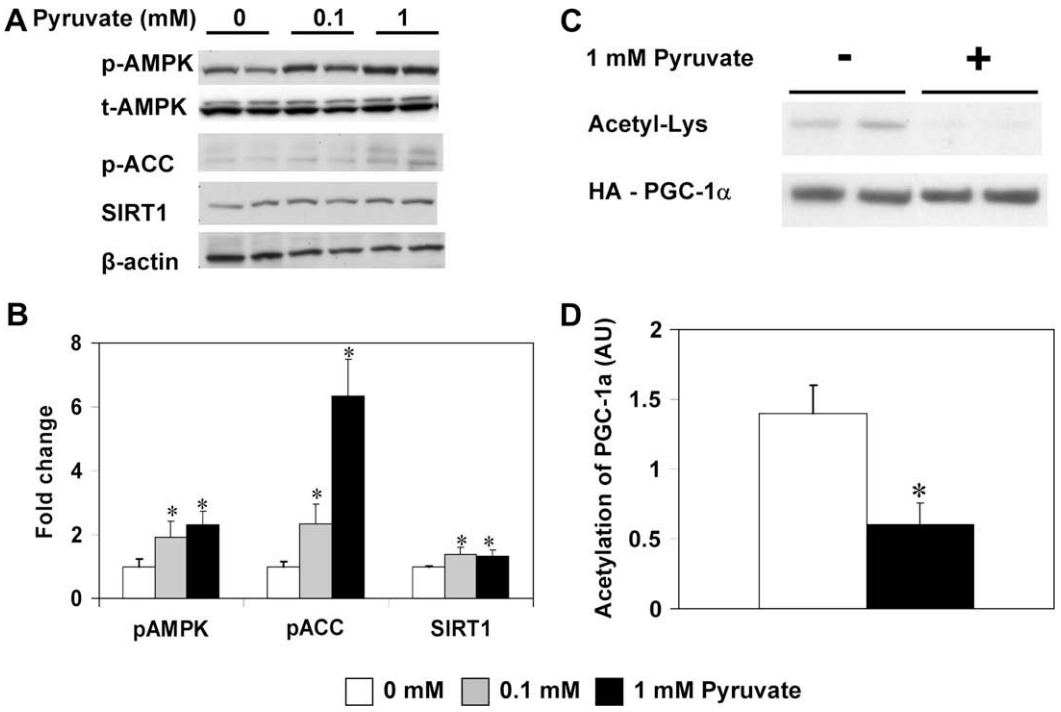

Fig. 2. Increasing pyruvate concentration increases p-AMPK, p-ACC, and SIRT1 abundance and activity. (A) Western blot analysis of HepG2 cells following 2 h incubation with the indicated concentrations of pyruvate. (B) Quantification of p-AMPK, p-ACC, and SIRT1 relative to β-actin. Results are means ± SE ($n = 6$); $^{*}p < 0.05$ vs. 0 mM. (C) PGC-1α acetylation after 2 h incubation ± 1 mM pyruvate. (D) Quantification of Acetyl-Lys/HA-PGC-1α. Results are means ± SE, ($n = 4$); $^{*}p < 0.05$.

glycemia decreased SIRT1 activity. In addition, the release of lactate was increased by 23% in the cells incubated with 25 vs. 5 mM glucose for 6 h ($n = 3$; $p < 0.01$).

The effects of pyruvate on AMPK and ACC phosphorylation, and SIRT1 protein and activity

The effects of incubating HepG2 cells in a 5-mM glucose medium with increasing concentrations of pyruvate are shown in Fig. 2. The addition of 0.1 or 1 mM pyruvate markedly enhanced the phosphorylation of AMPK, and even more so that of ACC at 2 h (Fig. 2A and B). In cells incubated with 1 vs. 0 mM pyruvate, these changes were associated with a 30% increase in SIRT1 protein abundance (Fig. 2B) and a 60% decrease in the acetylation of PGC-1α (Fig. 2C and D). Incubation with 1 mM pyruvate did not increase the AMP/ATP ratio (Table 1), suggesting that the increase in AMPK activity occurred without an alteration of cellular energy state.

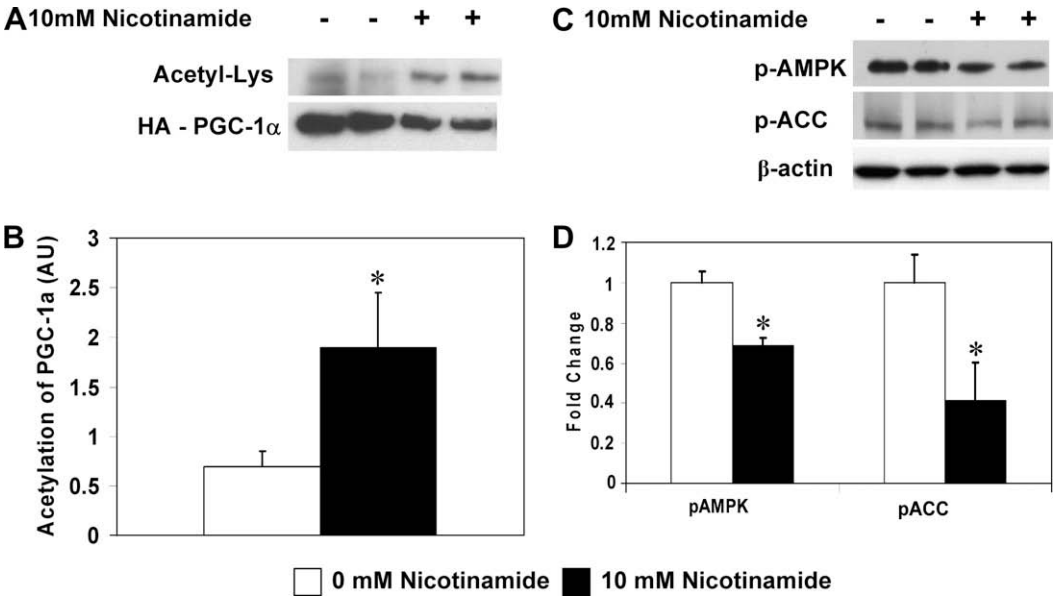

Fig. 3. Nicotinamide increases PGC-1α acetylation and decreases the phosphorylation of AMPK and ACC. (A) PGC-1α acetylation in HepG2 cells incubated ± 10 mM nicotinamide (NAM) for 6 h. (B) Quantification of Acetyl-Lys/HA-PGC-1α. Results are means ± SE, ($n = 4$); $^{*}p < 0.05$. (C) Western blot analysis of p-AMPK and p-ACC. (D) Quantification of representative blot shown in (C). Results are means ± SE, ($n = 4$); $^{*}p < 0.05$.

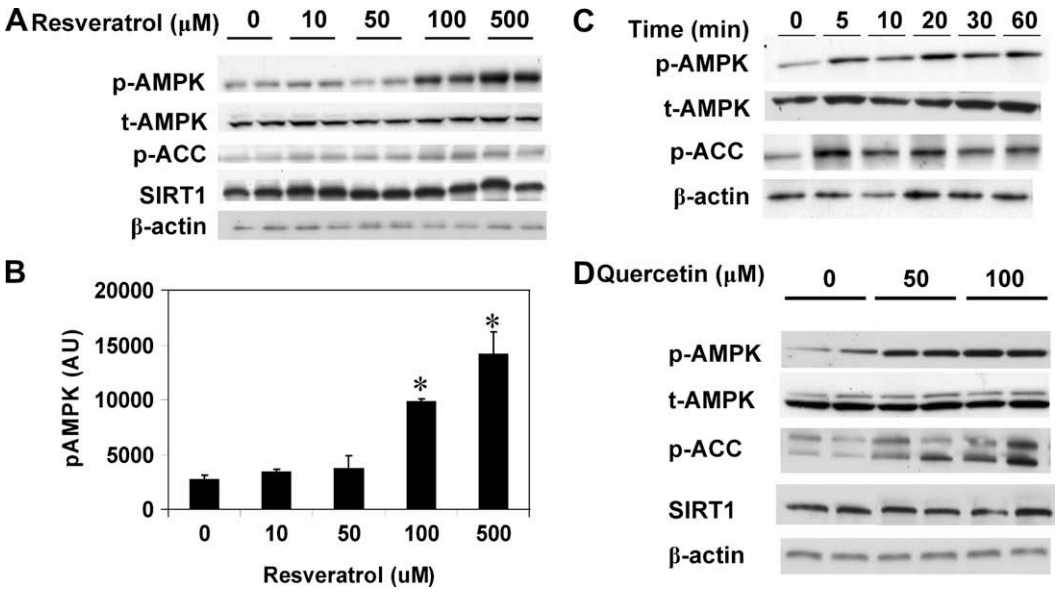

Fig. 4. Resveratrol and quercetin increase the phosphorylation of AMPK and ACC in HepG2 cells. (A and B) Dose–response of p-AMPK and p-ACC to 1 h resveratrol incubation. Results are means ± SE ($n = 2$); $^{*}p < 0.05$ vs. 0 μM resveratrol. (C) Time course of changes in p-AMPK and p-ACC caused by 100 μM resveratrol. (D) Western blot analysis showing enhanced p-AMPK and p-ACC following 1 h incubation with increasing concentrations of quercetin.

Nicotinamide suppresses SIRT1 activity and phosphorylation of AMPK and ACC

Nicotinamide and the reduced dinucleotide, NADH, are inhibitors of sirtuins [16], although their biological significance is incompletely understood. Rodgers et al. [11] have shown that nicotinamide induces PGC-1α acetylation in 293T cells, an effect that is overcome by SIRT1 overexpression. Here, we demonstrate that HepG2 cells exposed to 10 mM nicotinamide for 6 h show enhanced acetylation of PGC-1α (Fig. 3A and B) as well as diminished phosphorylation of AMPK and ACC (Fig. 3C and D). Thus, nicotinamide concurrently modulates the activities of SIRT1 and AMPK.

SIRT1 activators enhance the phosphorylation of AMPK in HepG2 and C2C12 cells, and in incubated rat EDL muscle

Resveratrol, a polyphenol found in foods such as grapes, red wine, and peanuts, has received increasing attention as a putative SIRT1 activator that can mimic the effects of caloric restriction on the aging process [17], the development of obesity and insulin resistance in fat-fed rodents [13,18], and possibly the prevalence of atherosclerotic cardiovascular disease in humans [19]. Fig. 4A and B demonstrate that resveratrol increases the phosphorylation of AMPK in a dose-dependent manner in HepG2 cells. The dose–response of ACC phosphorylation was less apparent, although at all concentrations resveratrol significantly increased p-ACC ($p < 0.05$). A time course experiment demonstrated that at a concentration of 100 μM, a marked increase in p-AMPK and p-ACC was evident within 5 min (Fig. 4C). No alteration in SIRT1 abundance was observed (Fig. 4A), in keeping with evidence suggesting that resveratrol binds to and activates SIRT1 by increasing its protein substrate affinity [20]. Similar effects of resveratrol were observed in C2C12 cells and incubated rat extensor digitorum longus (EDL) muscle, in which incubation with 50 and 100 μM resveratrol enhanced p-AMPK and p-ACC abundance (data not shown). Resveratrol had no effect on the cellular energy state of HepG2 cells after incubations of 1 h (Table 1) or 15 min (data not shown). Quercetin, a putative SIRT1-activating flavonoid, also enhanced the phosphorylation of AMPK and ACC in a dose-dependent manner in HepG2 cells (Fig. 4D). As with resveratrol, no change in the abundance of SIRT1 was observed.

Discussion

The results indicate that incubation in a high glucose medium (25 vs. 5 mM) diminishes the activity of AMPK in HepG2 cells, whereas incubation with pyruvate has the opposite effect. The observed changes in AMPK activity were not associated with alterations in cellular AMP/ATP ratios, but were linked to concomitant changes in SIRT1 abundance and activity. In line with this, we found that AMPK phosphorylation is diminished during incubation with nicotinamide, a SIRT1 inhibitor, whereas resveratrol, a putative SIRT1 activator [17], enhanced the phosphorylation of AMPK without altering cellular energy state. Similar findings were obtained with another putative SIRT1 activator, quercetin [17], although energy state was not determined. The effects of resveratrol on p-AMPK were also observed in C2C12 myotubes and incubated rat EDL muscle, indicating that they are not tissue- or cell-specific. Overall these results provide strong correlative evidence for a linkage between SIRT1 and AMPK.

The observation that increasing the ambient glucose concentration decreases SIRT1 abundance is in agreement with the findings of Rodgers et al. [11] in Fao rat hepatocytes. Collectively, these findings raise the question of how changes in the ambient glucose concentration influence SIRT1. The sirtuins depend on the availability of NAD^+ as both an activator and a substrate for deacetylase reactions. Hence, changes in the NAD^+/NADH ratio within a cell, i.e., the cellular redox state, may influence SIRT1 activity. Results from the present study show that incubation in a high glucose medium increases the amount of lactate released by HepG2 cells. If as assumed this reflects changes in the cytoplasmic and nuclear free NAD^+/NADH ratio [21], it raises the possibility that glucose-induced alterations in cellular redox state could influence the activity of SIRT1 and secondarily AMPK. This possibility is in line with the observation that in the fasted state, where both SIRT1 and AMPK are activated, hepatic pyruvate and NAD^+ content are increased whereas lactate content is decreased [11]. Furthermore, incubation with pyruvate, which acutely would increase the NAD^+/NADH

ratio, has been shown to increase SIRT1 abundance in Fao cells [11], and in the present study it increased SIRT1 and AMPK activity in HepG2 cells. Furthermore, we found that incubation with quercetin, a SIRT1-activating compound that has been shown to increase the acetoacetate to β-hydroxybutyrate ratio [22] (an indicator of the mitochondrial NAD^+/NADH ratio), also increased AMPK activity. Collectively these data suggest a correlation between redox state and the activities of SIRT1 and AMPK; however, direct measures of cellular redox state, such as the lactate/pyruvate ratio and cellular NAD and NADH, are needed to establish this more definitively. Still unanswered is the basis for the changes in SIRT1 abundance observed in this and other studies [11]. In this context, Zhang et al. [23] have proposed that the redox-sensitive transcriptional corepressor, CtBP, may regulate SIRT1 transcription.

The notion that changes in SIRT1 may regulate AMPK activity is also supported by other recent studies. Overexpression of SIRT1 has been found to increase the phosphorylation of AMPK and ACC both in vivo and in vitro [24], by a reaction dependent on the AMPK kinase LKB1 [24–26]. More specifically, we found that SIRT1 deacetylates lysine residue 48 of LKB1, and that this is associated with LKB1 movement from the nucleus to the cytoplasm, where it binds to STRAD (STE-related adapter) and MO25 (mouse protein 25) and is activated [26]. Likewise, Hou et al. [24] have found that pharmacologic and genetic inhibition of SIRT1 in HepG2 cells attenuates the increase in AMPK phosphorylation caused by polyphenol exposure, and it has been demonstrated that refeeding after a fast decreases SIRT1 [11], LKB1 and AMPK activity, and increases LKB1 acetylation in rodent liver [26].

The data presented in this report, together with several other recent studies [11,24,26], are consistent with a model in which alterations in the ambient glucose concentration influence SIRT1 and secondarily AMPK activity. In contrast, an alternative model to explain the effects of glucose concentration on SIRT1 has recently been proposed by Fulco et al. [27]. In brief they reported that glucose deprivation (5 vs. 25 mM) inhibited the differentiation of C2C12 myoblasts by leading to a decrease in cellular ATP and activation of AMPK, which in turn caused the activation of SIRT1. They also demonstrated that SIRT1 activation was enhanced by the AMPK activator AICAR, and that both glucose deprivation and AICAR induced the transcription of the NAD^+ biosynthetic enzyme Nampt (nicotinamide phosphoribosyltransferase), resulting in a decrease in the concentration of the SIRT1 inhibitor nicotinamide, an increase in the NAD^+/NADH ratio, and activation of SIRT1. These findings suggest that the upstream event in glucose regulation of AMPK is a change in the energy state, a finding also observed in pancreatic β-cells [28]. In contrast, in this as well as in earlier studies in incubated rat EDL muscle (30 min–2 h) [7] and human umbilical vein endothelial cells (2 h) [29], glucose deprivation led to AMPK activation in the apparent absence of a decrease in cellular energy state, although the possibility that a transient alteration in energy state occurred at earlier time points was not excluded. Thus, whether glucose regulates SIRT1 by effects on cellular energy or redox state or by both mechanisms remains to be determined. If both mechanisms operate, it would suggest the existence of a SIRT1/AMPK cycle that links cellular redox and energy states.

In summary, the results presented here support a strong association between SIRT1 and AMPK in mammalian cells, as demonstrated by concurrent regulation of both molecules in response to varying concentrations of glucose and pyruvate. Additionally, exposure to SIRT1 activators and inhibitors resulted in AMPK activation and inhibition, respectively. The absence of significant changes in energy state (where measured) suggest that modulation of SIRT1 by redox changes could mediate the effects of glucose and pyruvate on its activity and abundance; however, additional studies are needed to establish this with greater certainty.

Acknowledgments

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LETTERS

AMPK regulates energy expenditure by modulating NAD$^+$ metabolism and SIRT1 activity

Carles Cantó[1,2], Zachary Gerhart-Hines[3], Jerome N. Feige[1], Marie Lagouge[1], Lilia Noriega[1,2], Jill C. Milne[4], Peter J. Elliott[4], Pere Puigserver[3] & Johan Auwerx[1,2,5]

AMP-activated protein kinase (AMPK) is a metabolic fuel gauge conserved along the evolutionary scale in eukaryotes that senses changes in the intracellular AMP/ATP ratio[1]. Recent evidence indicated an important role for AMPK in the therapeutic benefits of metformin[2,3], thiazolidinediones[4] and exercise[5], which form the cornerstones of the clinical management of type 2 diabetes and associated metabolic disorders. In general, activation of AMPK acts to maintain cellular energy stores, switching on catabolic pathways that produce ATP, mostly by enhancing oxidative metabolism and mitochondrial biogenesis, while switching off anabolic pathways that consume ATP[1]. This regulation can take place acutely, through the regulation of fast post-translational events, but also by transcriptionally reprogramming the cell to meet energetic needs. Here we demonstrate that AMPK controls the expression of genes involved in energy metabolism in mouse skeletal muscle by acting in coordination with another metabolic sensor, the NAD$^+$-dependent type III deacetylase SIRT1. AMPK enhances SIRT1 activity by increasing cellular NAD$^+$ levels, resulting in the deacetylation and modulation of the activity of downstream SIRT1 targets that include the peroxisome proliferator-activated receptor-γ coactivator 1α and the forkhead box O1 (FOXO1) and O3 (FOXO3a) transcription factors. The AMPK-induced SIRT1-mediated deacetylation of these targets explains many of the convergent biological effects of AMPK and SIRT1 on energy metabolism.

AMPK is a critical regulator of mitochondrial biogenesis in response to energy deprivation[6]. Although the mechanisms by which AMPK modulates mitochondrial gene expression are not entirely elucidated, they seem to require the peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), either by increasing its expression[7] or direct phosphorylation[8]. Because PGC-1α is also activated by SIRT1-mediated deacetylation[9–12], we speculated that AMPK alters PGC-1α activity by changing its acetylation status. Treatment of C2C12 myotubes with the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR) decreased PGC-1α acetylation after 4 h of treatment (Fig. 1a). Unrelated AMPK activators (Supplementary Fig. 1a, b), such as resveratrol[13,14], metformin[3], dinitrophenol (DNP)[15] and A-769662[16], also decreased PGC-1α acetylation (Fig. 1b and Supplementary Fig. 1c). Furthermore, overexpression of a constitutively active form of AMPKα_1 led to a robust deacetylation of PGC-1α that could not be further enhanced by AICAR (Fig. 1c). In contrast, when a dominant negative form of AMPKα_i was overexpressed, AICAR was unable to deacetylate PGC-1α (Fig. 1c). The activation of AMPK hence triggers PGC-1α deacetylation in C2C12 myotubes.

To validate these observations *in vivo*, we examined PGC-1α acetylation in hindlimb muscles after a single AICAR injection. PGC-1α acetylation was markedly reduced by AICAR in extensor digitorum longus (EDL) and gastrocnemius, but not in soleus (Fig. 1d). The soleus is an oxidative muscle, where basal PGC-1α activity is presumably higher than in glycolytic muscles such as the EDL. Supporting this hypothesis, basal PGC-1α acetylation levels were lower in soleus than EDL (Supplementary Fig. 2a), explaining why the soleus is refractory to AICAR. AICAR-induced PGC-1α deacetylation in muscle correlated with an increase of PGC-1α target genes (Supplementary Fig. 2b). Consistent with PGC-1α acetylation levels, AICAR had, however, minor effects on mitochondrial gene expression in the soleus (Supplementary Fig. 2c).

We then tested whether AMPK activation through exercise decreases PGC-1α acetylation. An exhaustive single bout of treadmill running transiently activated AMPK (Supplementary Fig. 3a) and induced PGC-1α deacetylation with a maximal effect 3 h after ending exercise (Fig. 1e). As observed with AICAR infusion, the soleus was refractory to exercise-induced PGC-1α deacetylation (Fig. 1e) despite being effectively recruited, as indicated by the diminished glycogen levels and increased AMPK activity (Supplementary Fig. 3b, c). The decrease in PGC-1α acetylation in EDL and gastrocnemius, but not in soleus, translated to a marked induction of PGC-1α target genes, such as carnitine palmitoyltransferase 1b (*Cpt1b*), pyruvate dehydrogenase kinase 4 (*Pdk4*) or *Glut4* (also called *Slc2a4*; Supplementary Fig. 3d), whereas these genes only minimally responded in soleus (Supplementary Fig. 3e). Together, these results physiologically correlate AMPK activation, PGC-1α deacetylation and PGC-1α activity.

Because SIRT1 interacts with and deacetylates PGC-1α[9,11], we next evaluated whether SIRT1 mediates the AICAR-induced deacetylation of PGC-1α. Pre-treatment of C2C12 myotubes with nicotinamide (NAM), a type III histone deacetylase inhibitor[17], blocked AICAR-induced PGC-1α deacetylation (Supplementary Fig. 4). Furthermore, AICAR failed to decrease PGC-1α acetylation when SIRT1 expression was knocked down with a short hairpin RNA[9] (shRNA; Fig. 2a) or genetically ablated, as in *Sirt1*$^{-/-}$ mouse embryonic fibroblasts (MEFs)[18] (Fig. 2b). The lack of SIRT1 did not affect AICAR-induced AMPK phosphorylation in any of these models (Fig. 2c, d). The protein levels of SIRT2 and SIRT3, the closest homologues of SIRT1, were not affected by reduced SIRT1 expression (Supplementary Fig. 5), ruling out their implication in the blunted response to AICAR.

We next explored whether PGC-1α deacetylation influences AMPK-induced PGC-1α transcriptional activity. Because PGC-1α positively autoregulates its own promoter[19], we monitored PGC-1α activity by transiently transfecting a luciferase reporter under the control of the *Pgc1-α* (also called *Ppargc1a*) promoter, in the absence/presence of PGC-1α and a *Sirt1* shRNA. AICAR robustly increased PGC-1α action on its own promoter in both C2C12 myocytes (Fig. 3a) and *Sirt1*$^{+/+}$ mouse embryonic fibroblasts (Fig. 3b). This increase was

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, 67404 Illkirch, France. [2]Ecole Polytechnique Fédérale de Lausanne, CH1015 Lausanne, Switzerland. [3]Dana-Farber Cancer Institute and Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA. [4]Sirtris Pharmaceuticals Inc., Cambridge, Massachusetts 02139, USA. [5]Institut Clinique de la Souris, BP10142, 67404 Illkirch, France.

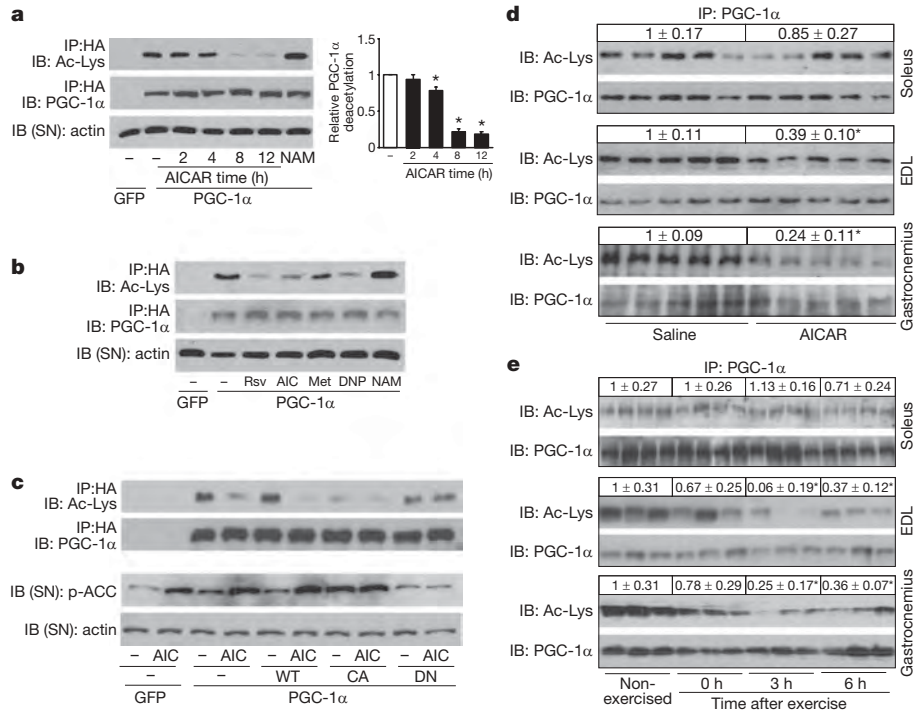

Figure 1 | Activation of AMPK triggers PGC-1α deacetylation in C2C12 myotubes and skeletal muscle. **a**, **b**, C2C12 myotubes infected with adenoviruses for GFP or Flag–HA-tagged PGC-1α (PGC-1α) were treated with vehicle (−), AICAR (0.5 mM, 2–8 h) or nicotinamide (NAM; 5 mM; 12 h). Then, acetyl-lysine levels were checked on PGC-1α immunoprecipitates (IP). The supernatant (SN) was blotted against actin as input control. Relative quantification of PGC-1α acetylation is shown on the right. **b**, As in **a**, but myotubes were treated for 8 h with vehicle (−), resveratrol (Rsv; 50 μM), AICAR (AIC), metformin (Met; 1 mM), DNP (0.5 mM) or NAM. **c**, C2C12 myotubes were infected with adenoviruses encoding GFP, PGC-1α and wild-type (WT), constitutively active (CA) or dominant negative (DN) forms of AMPKα$_1$. After AICAR treatment, total lysates were analysed as in **a**. **d**, PGC-1α acetylation was measured on total protein (soleus and EDL) or nuclear extracts (gastrocnemius) from muscles of mice treated with AICAR or saline. Relative acetylation levels are shown on top of the panels. **e**, Soleus, EDL and gastrocnemius were obtained from non-exercised or exercised mice at 0, 3 or 6 h after cessation of exercise, and analysed as in **d**. Values are expressed as mean ± s.e.m. Asterisks indicate statistical difference versus corresponding vehicle, saline or non-exercised group at $P < 0.05$.

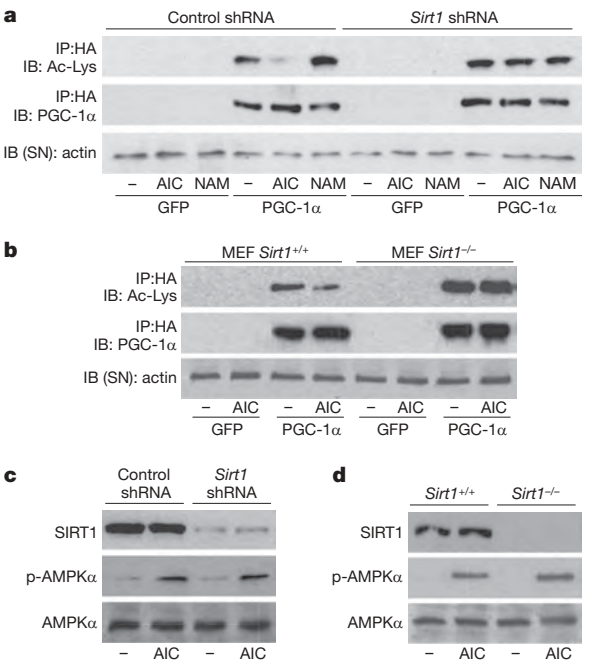

Figure 2 | SIRT1 mediates AMPK-induced PGC-1α deacetylation. **a**, **c**, C2C12 myocytes were infected with adenoviruses encoding GFP, PGC-1α and either control or *Sirt1* shRNAs. After 8 h (**a**) or 1 h (**c**) of AICAR treatment, PGC-1α acetylation and AMPK phosphorylation were analysed. **b**, **d**, *Sirt1*$^{+/+}$ and *Sirt1*$^{-/-}$ MEFs were infected with GFP and Flag–HA–PGC-1α and treated for 8 h (**b**) or 1 h (**d**) with AICAR to test PGC-1α acetylation and AMPK phosphorylation.

reduced over 60% when SIRT1 was knocked down in these cells (Fig. 3a, b). Conversely, AICAR only mildly activated PGC-1α in *Sirt1*$^{-/-}$ MEFs, but full AICAR action was recovered when SIRT1 was reintroduced (Fig. 3c). The lack of SIRT1 also compromised AICAR-induced PGC-1α-dependent transcriptional activity on promoters of other target genes, such as *Pdk4*[20] and *Mcad*[21] (also called *Acadm*; Supplementary Fig. 6a, b). The overexpression of the constitutively active PGC-1α R13 mutant, where the 13 acetylation sites are mutated, activated the *Pgc-1α* promoter, which could not be further enhanced by AICAR or inhibited by a *Sirt1* shRNA (Supplementary Fig. 6c). This indicates that SIRT1 and the deacetylation of PGC-1α are necessary for AMPK to increase PGC-1α activity.

We then tested whether the lack of SIRT1 affects AICAR-induced expression of genes related to mitochondrial metabolism and fatty acid utilization, which are under the control of PGC-1α. AICAR-induced expression of genes involved in mitochondrial gene expression (such as oestrogen-related receptor-α (*Esrra*) and *Pgc-1α*), mitochondrial architecture and electron transport (such as cytochrome *c* (*Cyc*), cytochrome *c* oxidase IV (*Cox4*) and mitofusin-2 (*Mfn2*)), and in fatty acid utilization (such as *Cpt1b* and *Pdk4*), was either prevented or robustly attenuated by knocking down SIRT1 expression in C2C12 myotubes (Fig. 3d and Supplementary Fig. 7) or deletion of the *Sirt1* gene in MEFs (Supplementary Fig. 8). The lack of SIRT1 also impaired the action of metformin on the expression of this gene set (Supplementary Fig. 9). In contrast, AICAR was unable to increase further the expression of PGC-1α targets in C2C12 myotubes overexpressing the PGC-1α R13 mutant (Supplementary Fig. 10). Together, these data indicate that, to a large extent, AMPK regulates the expression of mitochondrial and lipid metabolism genes through the modulation of PGC-1α activity by SIRT1.

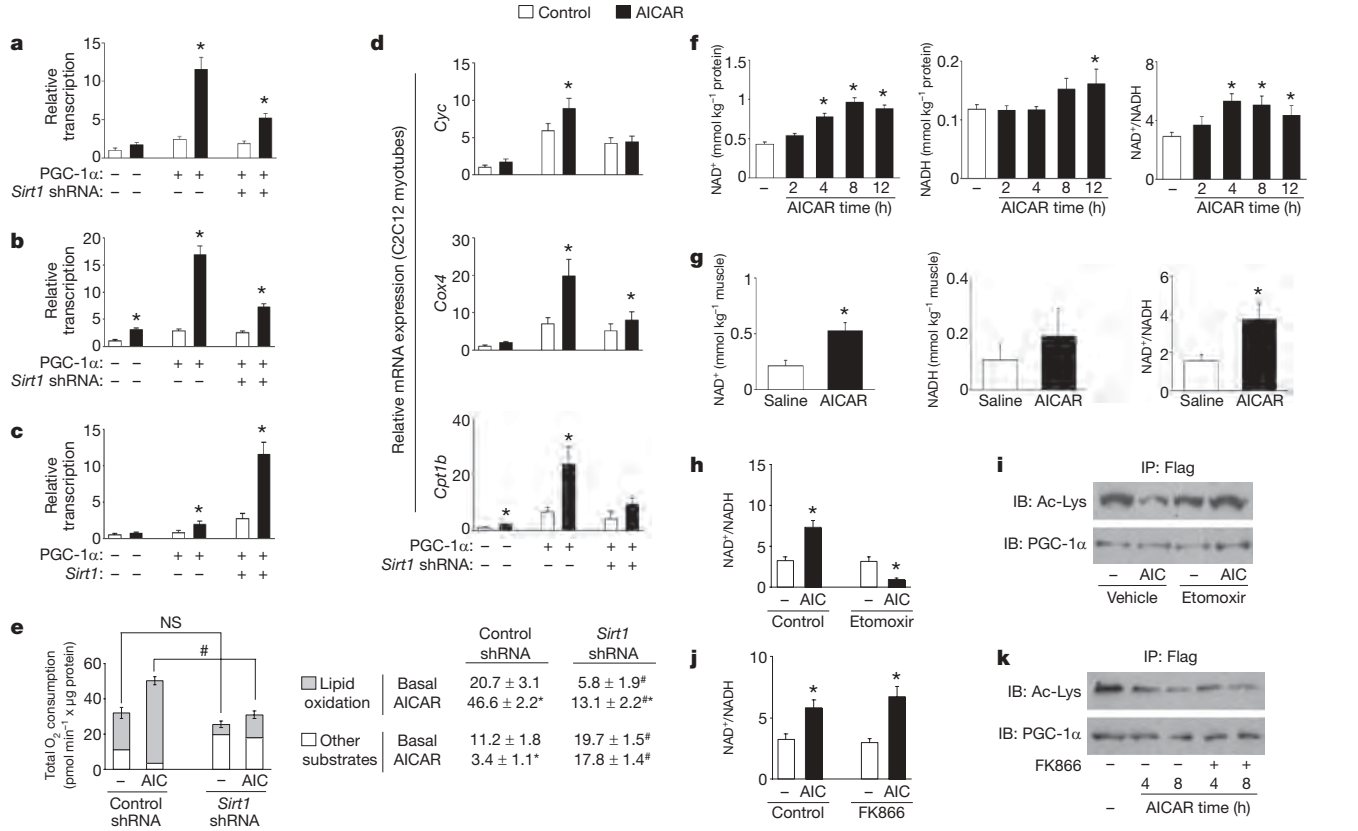

Figure 3 | AICAR modulates PGC-1α-dependent transcriptional activity, mitochondrial gene expression and oxygen consumption through SIRT1 and NAD^+ metabolism. **a**, C2C12 myocytes were transfected with a 2-kb mouse PGC-1α (mPGC-1α) promoter luciferase reporter, a plasmid for mPGC-1α and simultaneously infected with adenovirus encoding control or *Sirt1* shRNA. Thirty-six hours later, cells were treated with AICAR (12 h) and reporter activity was determined. **b**, $Sirt1^{+/+}$ MEFs were analysed as in **a**. **c**, $Sirt1^{-/-}$ MEFs were transfected with the 2-kb mPGC-1α reporter and expression plasmids for PGC-1α, SIRT1 or the corresponding empty vectors. Then, cells were treated and analysed as in **a**. **d**, C2C12 myotubes were infected with adenoviruses for GFP, PGC-1α and either control or *Sirt1* shRNAs. After AICAR treatment, target mRNAs were analysed by qRT–PCR. **e**, O_2 consumption in C2C12 myotubes infected with PGC-1α, and either control or *Sirt1* shRNAs. Total length of the bar equals total O_2 consumption. The white part of the bar is the O_2 consumption in each group when treated with etomoxir (1 mM). Therefore, the grey part represents lipid oxidation-derived O_2 consumption. Values for O_2 consumption due to the oxidation of lipids and other substrates are indicated on the right. **f**, NAD^+ and NADH content in C2C12 myotubes treated with AICAR for the times indicated. **g**, Whole tibialis anterior muscles from mice treated with saline or AICAR were used for the measurement of NAD^+ and NADH. **h**, **i**, C2C12 myotubes pre-incubated with vehicle or etomoxir (50 μM) for 1 h were treated with either vehicle (−) or AICAR (AIC). Then, NAD^+ and NADH (**h**) or PGC-1α acetylation levels (**i**) were measured. **j**, **k**, As in **h**, **i**, but using FK866 (10 nM) instead of etomoxir. All values are expressed as mean ± s.e.m. Asterisks indicate statistical difference versus vehicle/saline group at $P < 0.05$. Hash symbols indicate statistical difference versus respective control shRNA group at $P < 0.05$.

SIRT1 also had a major role in the ability of AMPK to increase mitochondrial respiration, as the long-term effects of AICAR on cellular O_2 consumption, a readout of oxidative metabolism, were severely blunted by knocking down SIRT1 (Fig. 3e). Thus, we determined the contribution of lipid oxidation to total O_2 consumption by blocking mitochondrial fatty acid uptake with etomoxir. Confirming previous observations[10], knocking down SIRT1 severely decreased lipid oxidation and increased the oxidation of alternative substrates (Fig. 3e). In control cells, AICAR markedly increased lipid oxidation-driven O_2 consumption, whereas it almost completely blocked the oxidation of other substrates, such as glucose. When SIRT1 expression was knocked down, AICAR action on lipid oxidation was blunted and, additionally, AICAR was unable to decrease O_2 consumption derived from the other substrates (Fig. 3e). In line with this, direct measurement of oleate oxidation confirmed that the chronic effects of AICAR on lipid oxidation were blunted in myotubes where SIRT1 expression was knocked down (Supplementary Fig. 11). The lack of SIRT1 hence alters the long-term actions of AICAR on lipid oxidation and global cellular O_2 consumption.

As AICAR or metformin cannot directly activate SIRT1 (Supplementary Fig. 12), our data suggest that AMPK signalling modulates SIRT1 activity indirectly. No changes in SIRT1 protein levels were detected after 8 h of AICAR (Supplementary Fig. 13a), when PGC-1α is already maximally deacetylated, indicating that changes in activity where not due to increased SIRT1 abundance. We could not observe interaction of AMPK with SIRT1 up to 8 h after AICAR, either in the presence or absence of PGC-1α (Supplementary Fig. 13b). Finally, AMPK could not phosphorylate SIRT1 *in vitro* either on the full-length protein (Supplementary Fig. 13c) or on different GST fragments (Supplementary Fig. 13d). These results suggest that AMPK regulates SIRT1 action by indirect mechanisms.

Because SIRT1 deacetylase activity is driven by NAD^+ levels[22], we examined whether AMPK indirectly activates SIRT1 by altering the intracellular NAD^+/NADH ratio. Supporting this hypothesis, AICAR increased the NAD^+/NADH ratio in C2C12 myotubes and in skeletal muscle (Fig. 3f, g, respectively). The increase in NAD^+/NADH ratio was evident 4 h after AICAR in C2C12 myotubes, and remained elevated after 12 h (Fig. 3f), a timing that perfectly correlates with PGC-1α deacetylation (Fig. 1a). Activation of AMPK by metformin, DNP, or overexpression of a constitutively active form of $AMPK\alpha_1$ also increased the NAD^+/NADH ratio (Supplementary Fig. 14a, b). A significant increase in NAD^+ was also evident 3 h after exercise in tibialis anterior muscle, further supporting the hypothesis that changes in NAD^+ levels translate AMPK effects onto SIRT1 activity (Supplementary Fig. 14c).

To determine how AMPK acutely increases the NAD^+/NADH ratio, we pharmacologically targeted different possible sources of cellular NAD^+ production. Inhibition of the glycolytic enzyme lactate dehydrogenase with oxamic acid did not affect the ability of AICAR to increase NAD^+ levels and the NAD^+/NADH ratio (Supplementary Fig. 15a). In contrast, inhibition of mitochondrial fatty acid oxidation with etomoxir was enough to hamper the increase in NAD^+/NADH induced by AMPK (Fig. 3h and Supplementary Fig. 15b), indicating that an increase in mitochondrial β-oxidation is required for AMPK to increase the NAD^+/NADH ratio. Supporting the role of NAD^+/NADH ratio on SIRT1 activity, etomoxir also abolished AMPK-induced PGC-1α deacetylation (Fig. 3i),

SIRT1 activity is inhibited by NAM, a product of the deacetylation reaction catalysed by the sirtuins[17]. NAM can be cleared and enzymatically reconverted into NAD^+ through the NAD^+ salvage pathway, the initial rate-limiting step of which is catalysed in mammals by nicotinamide phosphoribosyltransferase (NAMPT)[23]. Acute blockage of NAMPT activity with the specific inhibitor FK866[24], however, did not affect AICAR's capacity to modulate PGC-1α acetylation or NAD^+/NADH ratio up to 8 h after treatment (Fig. 3j, k and Supplementary Fig. 15c). These results apparently conflict with observations indicating that NAMPT can link AMPK and SIRT1 activities[25]. These differences may be explained by the fact that a chronic knock down of NAMPT may constitutively inhibit SIRT1 due to reduced intracellular NAM clearance[17]. Supporting this speculation, knock down of NAMPT for 48 h promotes PGC-1α hyperacetylation (Supplementary Fig. 16). The use of FK866 dissociates the direct effects of acute NAMPT inhibition from those of indirect SIRT1 inhibition and indicates that AMPK initially regulates the NAD^+/NADH ratio and SIRT1 activity independently of NAMPT. These results, however, do not rule out that NAMPT could participate to sustain the actions of AMPK on SIRT1.

Whereas it has been reported that AMPK activates PGC-1α through direct phosphorylation[8], our data show that deacetylation of PGC-1α on AMPK activation is also required to activate PGC-1α. To understand how both post-translational modifications intertwine, we used the PGC-1α 2A mutant lacking the two AMPK phosphorylation sites[8]. Mutation of these sites markedly reduced deacetylation of PGC-1α by AICAR (Fig. 4a), and, consistently, blunted AICAR action on the expression of mitochondrial genes (Fig. 4b). The expression of the PGC-1α 2A mutant, however, did not alter AICAR action on the NAD^+/NADH ratio, which increased to a similar extent as in myocytes expressing wild-type PGC-1α (Fig. 4c and Supplementary Fig. 17). This suggested that the activation of SIRT1 by AMPK should theoretically be unaffected by the PGC-1α 2A mutant and, consequently, still have an impact on other SIRT1 substrates beyond PGC-1α, like the FOXO transcription factors[26]. AICAR treatment triggered the deacetylation of endogenous FOXO1 (Fig. 4d), as well as other members of the FOXO family, such as FOXO3a (Supplementary Fig. 18), in C2C12 myotubes. The deacetylation of FOXO1 in response to AICAR was similar in myocytes expressing wild-type PGC-1α or the 2A mutant (Fig. 4e), demonstrating that the PGC-1α 2A mutation only alters PGC-1α deacetylation but not general SIRT1 activation in response to AICAR. Hence, the data suggest a scenario where phosphorylation of PGC-1α constitutes a priming signal for subsequent deacetylation by SIRT1 (Fig. 4f). Notably, AMPK can also phosphorylate the FOXO transcription factors[27], which are also targets for SIRT1 deacetylation[26]. It is therefore tempting to speculate that the coordinated sequential actions of AMPK and SIRT1 could be a conserved mechanism for AMPK to modulate the specificity among SIRT1 targets, with phosphorylation discriminating which substrates should be deacetylated and preventing random deacetylation.

This work demonstrates that deacetylation of PGC-1α is a key mechanism by which AMPK triggers PGC-1α activity in cultured myotubes and in skeletal muscle. We also unveil SIRT1 as a key, albeit not the sole, mediator of AMPK action on PGC-1α transcriptional activity, the expression of mitochondrial and lipid metabolism genes, and O_2 consumption (Fig. 3). The acute actions of AMPK on lipid oxidation alter the balance between cellular NAD^+ and NADH,

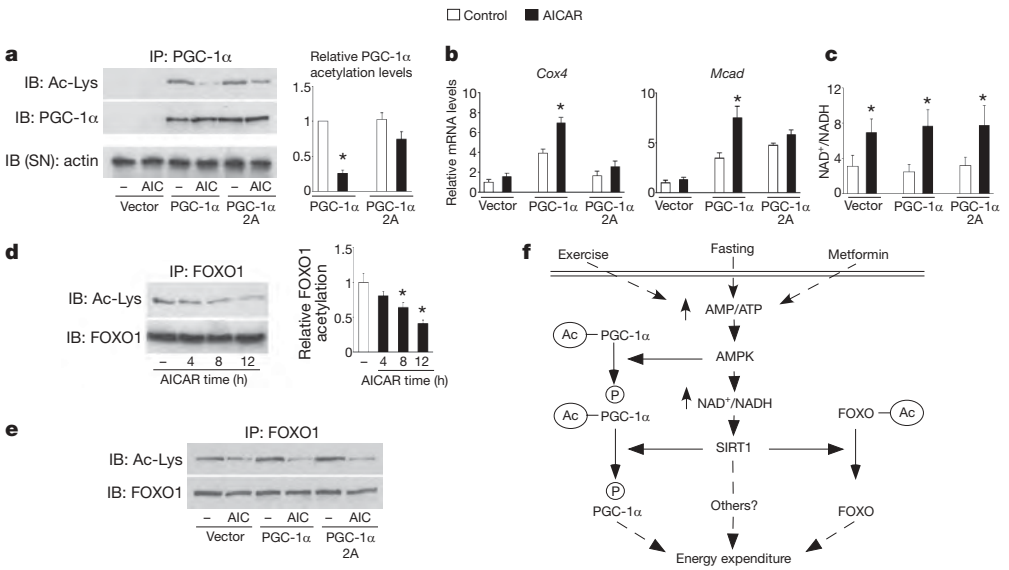

Figure 4 | The PGC-1α phosphorylation mutant is resistant to deacetylation. **a**, C2C12 myocytes were transfected with the wild-type or the 2A mutant form of PGC-1α, using empty vector as control. After 36 h, cells were treated with AICAR and total lysates were used to test PGC-1α acetylation. Relative acetylation levels of PGC-1α are shown on the right. **b**, Cells were treated as in **a**, and, after AICAR treatment, target mRNA levels were analysed by RT–qPCR. **c**, Cells were treated as in **a**, and acidic or alkali lysates were obtained to measure NAD^+ and NADH. **d**, C2C12 myotubes were treated with AICAR for the times indicated. Then, total protein lysates were used for immunoprecipitation of FOXO1. Relative FOXO1 acetylation is shown on the right. **e**, As in **a**, but immunoprecipitations were performed against FOXO1. **f**, Scheme illustrating the convergent actions of AMPK and SIRT1 on PGC-1α. Pharmacological (metformin) and physiological (fasting or exercise) activation of AMPK in muscle triggers an increase in the NAD^+/NADH ratio, which activates SIRT1. AMPK also induces the phosphorylation of PGC-1α and primes it for subsequent deacetylation by SIRT1. The impact of AMPK and SIRT1 on the acetylation status of PGC-1α and other transcriptional regulators, such as the FOXO family of transcription factors, will then modulate mitochondrial function and lipid metabolism. All values are presented as mean ± standard error. Asterisks indicate statistical difference versus corresponding vehicle group at $P < 0.05$.

which acts as a messenger to activate SIRT1. This study constitutes a step forward in the understanding of the mechanisms by which AMPK transcriptionally regulates energy expenditure. The implication of SIRT1 in the transcriptional actions of AMPK provides a possible explanation for the overlapping metabolic effects of SIRT1 and AMPK activators[7,12,16,28,29]. Furthermore, the interplay between SIRT1 and AMPK might be reciprocal, as specific SIRT1 agonists promote fatty acid oxidation and indirectly activate AMPK through metabolic adaptations[29]. Hence, the interdependent regulation of SIRT1 and AMPK provides a finely tuned amplification mechanism for energy homeostasis under low nutrient availability. Together, these findings constitute a conciliatory view of the possible implications of AMPK and SIRT1 on the pleiotropic beneficial effects of calorie restriction on metabolic homeostasis and lifespan, where both enzymes were known to participate, but never linked.

METHODS SUMMARY

Reagents and materials. The origin of all chemicals, antibodies, plasmids and adenoviruses is listed in Methods.

Cell culture. C2C12 skeletal muscle cells were grown and differentiated as described[12]. C2C12 cells were considered as myotubes after 96 h of differentiation. Differentiation medium was supplemented with 0.1 mM oleic acid. Unless otherwise stated, AICAR treatments lasted 8 h. Additional cell culture procedures can be found in Methods.

Animal experiments. All animals were purchased from Charles River. Eight-week-old C57BL/6J male mice were injected subcutaneously with AICAR ($1.0\,mg\,g^{-1}$ body weight) or saline. AICAR was dissolved in $50\,mg\,ml^{-1}$ saline. Injection time was at the start of the dark cycle, and animals had free access to food and water. AICAR injection did not alter food intake during the 12 h period (data not shown). Twelve hours after injection, hindlimb muscles were collected and immediately frozen in liquid nitrogen. The exercise protocol used can be found in Methods.

Statistics. Statistical analyses were performed with the Student's *t*-test for independent samples (nonparametric), and data are expressed as mean ± standard error unless otherwise specified. P value <0.05 was considered as statistically significant. In cell-based experiments the n ranged from 4 to 12 independent experiments. In animal-based studies, $n = 8$–10 animals per group.

Additional experimental procedures can be found in Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions C.C. designed and executed experiments, interpreted data and wrote the manuscript. Z.G.-H., J.C.M., J.N.F., M.L. and L.N. performed experiments and J.N.F. helped with writing. P.J.E. and P.P. provided crucial reagents and helped with data interpretation. J.A. supervised the design and interpretation of the experiments and participated in the writing of the manuscript.

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METHODS

Exercise protocol. 8-week-old non-fasted C57BL6/J male mice were subjected to a resistance running test, using a variable speed belt treadmill enclosed in a plexiglass chamber with a stimulus device consisting of a shock grid attached to the rear of the belt (Panlab). Animals were acclimatized to the chamber the day preceding the running test. For the habituation, mice run at 21 cm s^{-1} for 10 min with a 5° incline. For the actual test, we used a protocol at 5° incline where, beginning at 18 cm s^{-1}, speed increased gradually by 3 cm s^{-1} every 5 min. The distance run and the number of shocks were monitored during the test, and exhaustion was assumed when mice received more than 50 shocks in a 2.5 min interval. Mice were removed from the treadmill on exhaustion.

Preceding the running test, we randomly subdivided mice into three different groups (8 mice per group): one group that would be killed immediately after the exercise test, another which would be killed 3 h after the exercise test, and, finally, a group that would be killed 6 h after the cessation of exercise. The time and distance run before exhaustion was similar in the three groups (data not shown), around 600 m after 40 min of exercise. Mice killed 3 and 6 h after exercise had free access to food and water once the running protocol was finished.

Reagents and materials. AICAR was purchased from Toronto Research Chemicals. Anti-PGC-1α (H300) and anti-actin goat antibodies were purchased from Santa Cruz Biotechnology Inc. Anti-PGC-1α, anti-acetyl-lysine, anti-AMPKα, anti-phospho-AMPKα (Thr 172) and anti-FOXO1 polyclonal antibodies were purchased from Cell Signaling. Anti-Sir2 and anti-phospho AcetylCoA carboxylase (ACC) (Ser 79) were purchased from Upstate Biotechnology Inc. Anti-NAMPT antibody was purchased from Bethyl laboratories. Anti-Flag (M2) and anti-HA monoclonal antibodies as well as most commonly used chemicals were purchased from Sigma Aldrich. *Nampt* siRNAs were purchased from Dharmacon Inc. The A-769662 compound was a gift from G. Hardie. C. Ammann provided the FK866 compound.

Plasmids and adenoviral vectors. Adenoviruses encoding for GFP, Flag–HA–PGC-1α, Flag–R13-PGC-1α, control and *Sirt1* shRNAs were described previously[9]. Adenoviruses encoding for the different forms of AMPKα_1 subunit were a gift from P. Ferré and F. Foufelle[30]. The plasmids encoding for the mouse *Pdk4* gene promoter[20], mouse *Mcad* gene promoter[21], and the 2A-PGC-1α mutant[8] have all been described. The plasmid encoding for Flag-tagged FOXO3a was purchased from Addgene.

Cell culture, adenoviral infection and treatments. C2C12 skeletal muscle cells were grown and differentiated as described[12]. Unless otherwise stated, C2C12 were considered as myotubes after 96 h of differentiation. Differentiation medium was supplemented with 0.1 mM oleic acid. Adenoviral infections of C2C12 myocytes were performed after 48 h of differentiation. Cells were washed with PBS and left for 1 h in serum-free DMEM 4.5 g l^{-1} glucose containing the appropriate amount of viral particles (MOI = 100 per each virus used, using GFP as control to make even the final viral amount). Then, the media was replaced with fresh differentiation media for an additional 47 h before any treatment took place. MEFs were cultured in DMEM 4.5 g l^{-1} glucose supplemented with 10% fetal calf serum (FCS). Adenoviral infection of MEFs was performed when cells reached 70% of confluency, and processed as with C2C12 myocytes but using 10% FCS DMEM medium instead of differentiation medium at the end. DMSO was used as vehicle for the different treatments. Plasmids and siRNAs were transfected using Lipofectamine 2000 (Invitrogen) and following the manufacturer's instructions. *Sirt1*$^{+/+}$ and *Sirt1*$^{-/-}$ cells were a gift of F. Alt.

Total protein extraction. To obtain total protein extracts from cellular samples, cells were rapidly washed with ice-cold PBS before adding cold lysis buffer (25 mM Tris HCl pH = 7.9, 5 mM $MgCl_2$, 10% glycerol, 100 mM KCl; 1% NP40; 0.3 mM dithiothreitol, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, containing freshly added protease inhibitor cocktail (Calbiochem)). For acetylation studies, 5 mM nicotinamide and 1 mM sodium butyrate were added to the buffer. After 1 min, cells were scraped, transferred into an Eppendorf tube and left on ice for 5 more minutes. Then cells were homogenized with a 25-gauge needle, left for 5 more minutes on ice and centrifuged at 13,000 r.p.m. for 10 min. The supernatant was collected and kept at −80 °C.

Nuclear extracts. Nuclear extracts from gastrocnemius muscles were obtained as described previously[12].

Immunoprecipitation and western blot. Routinely, 500 μg of protein from cultured cells or 2 mg of protein from muscle samples (total lysates or nuclear extracts) were used for immunoprecipitation. Forty microlitres of protein G-sepharose re-suspended in lysis buffer were used for pre-clearing the sample and immunoprecipitation after conjugating the beads with 3–5 mg of antibody. The resulting immunoprecipitate was boiled with 50 μl of Laemmli sample buffer (LSB) and used for Western blot applications. For immunoprecipitation using rabbit polyclonal antibodies, protein-A-sepharose beads were used instead of Protein-G-conjugated beads. Western blot and protein detection was performed as described previously[12].

Gene expression analysis. RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA was generated using Superscript II enzyme (Invitrogen) and quantitative real-time PCR was performed as described previously[12] using acidic ribosomal protein (ARP) to normalize the expression. The oligonucleotides primers used for PCR analysis are provided at the end of the section.

Oxygen consumption. C2C12 myotubes or MEF cells were incubated for 5 h per day with AICAR during 2 days, in the presence of 0.1 mM oleic acid. Then oxygen consumption was measured using Seahorse Biotechnology XF24 equipment (Seahorse Bioscience Inc.) as described[31].

Oleate oxidation. The estimation of oleate oxidation rates was performed as described previously[32].

Reporter gene assays. C2C12 myocytes were transfected in 48-well plates at 90% of confluence with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells were left for 5 h with the DNA-Lipofectamine mix, and the corresponding adenoviruses were added (each adenovirus at MOI 100) for the last hour of transfection. Then, the medium was removed and replaced by differentiation medium supplemented with 0.1 mM oleic acid for 36 h before treatment with AICAR or vehicle for 12 h. For MEFs, the protocol was similar, but replacing the transfection medium by DMEM 4.5 g l^{-1} glucose 10% FCS supplemented with 0.1 mM oleic acid. Firefly luciferase activity was measured and normalized to β-gal activity (always transfected simultaneously). Empty pGL3basic reporter gene vector and pCDNA.3.1 vector were used as control vectors. pGL3-PGC-1α 2-kb promoter was purchased from Addgene. The pcDNA.3.1 HA–PGC-1α plasmid was generated in P.P.'s laboratory. pcDNA–Flag–SIRT1 plasmid was developed in our own laboratory. Reporter constructs for *Pdk4* and *Mcad* promoters were produced by D. P. Kelly's laboratory.

Measurement of SIRT1 activity. Experiments testing the direct effects of AICAR and metformin on SIRT1 activity were performed as described[28].

NAD^+/NADH measurements. NAD^+ and NADH nucleotides were directly measured as described before[33]. In brief, whole tibialis anterior muscles or two 10-cm dishes of C2C12 myotubes were homogenized in 200 μl of acid extraction buffer to measure the NAD^+ concentration, or 200 μl of alkali extraction to obtain NADH concentration. Then, homogenates were neutralized and the concentration of nucleotides was measured fluorimetrically after an enzymatic cycling reaction using 5 μl of sample. Values for both nucleotides were detected within the linear range. NAD^+/NADH ratios were calculated by comparing the ratios obtained from each animal (randomly, one tibialis was used for NAD^+ measurements and the other for NADH) or from parallel cell dishes in each experiment. The ratios obtained from different animals or individual cell culture experiment ratios were then used as individual values to calculate the mean and standard error.

Glycogen measurement. Muscle pieces (15–20 mg) were hydrolysed in 250 μl of 2 M HCl at 95 °C for 2 h. The solution was then neutralized with 250 μl 2 M NaOH, and the resulting free glycosyl units were assayed spectrophotometrically using a hexokinase-dependant assay kit (Amresco).

Protein kinase assays. For protein kinase assays on full-length SIRT1 and NAMPT, Flag-tagged proteins were produced using a coupled *in vitro* transduction and translation system (TNT, Promega Corporation). Active AMPK and SAMS peptide, as positive control substrate for AMPK, were purchased from Upstate Biotechnology. AMPK was mixed with either Flag–SIRT1, Flag–NAMPT, control vector or SAMS peptide (200 mM) in a solution containing 30 mM HEPES pH 7.4, 0.65 mM dithiothreitol, 0.02% Brij-35, 10 mM MgAc and 0.2 mM AMP. The reaction started by the addition of 0.1 mM ATP (containing [γ^{32}P]ATP at 1,000 c.p.m. pmol^{-1}), and was stopped after 20 min by adding 5 μl of 3% phosphoric acid, and 15 μl of the reaction mix were transferred to a piece of P81 phosphocellulose Whatmann paper and washed extensively with phosphoric acid solution. Then, the paper was dried with acetone and radioactivity was counted by Cherenkov counting. The rest of the reaction mix was diluted in LSB and boiled for 5 min. Western blots were performed to ensure the correct presence of the proteins in the mix (data not shown). For kinase assays on different GST–SIRT1 fragments and GST–PGC-1α 1–400 (as positive control), proteins were expressed in bacteria (BL21 strain; Invitrogen) and purified by using glutathione–Sepharose 4B beads (GE Healthcare, 17-0756-01). *In vitro* kinase assays were carried out according to the manufacturer's specifications (Millipore, 14-305). Briefly, recombinant protein was incubated with 32 mM HEPES pH 7.4, 0.01% Brij-35, 18.75 mM $MgCl_2$, 0.15 mM AMP, 0.125 mM ATP, 2.5 mCi [γ^{32}P]ATP and 0.65 mM dithiothreitol in the presence or absence of 200 ng activated AMPK for 30 min at 30 °C. The glutathione beads were then washed twice and eluted protein was analysed by SDS–PAGE and radiolabelled phosphate incorporation was assessed by autoradiography. Protein levels were determined by Coomassie blue staining.

Oligonucleotide primer list. Acidic ribosomal protein: reverse AAAGCCTGGAAGAAGGAGGTC, forward AGATTCGGGATATGCTGTTGG; *Pgc-1α*: reverse GGGTTATCTTGGTTFFCTTTATG, forward AAGTGTFFAAC TCTCTGGAACTG; *Pgc-1β*: reverse TGGAGACTGCTCTGGAAGGT, forward TGCTGCTGTCCTCAAATACG; *Nrf1*: reverse GATGACCACCTCGACCGTTT, forward CGGAGTGACCCAAACTGAAC; *Esrra*: reverse CACAGCCTCAGCAT CTTCAA, forward ACTGCCACTGCAGGATGAG; *Ppara*: reverse TTGAAGGA GCTTTGGGAAGA, forward AGGAAGCCGTTCTGTGACAT; *Pparb/d*: reverse ACTGGCTGTCAGGGTGGTTG, forward AATGCGCTGGAGCTCGATGAC; *Tfam*: reverse ATGTCTCCGGATCGTTTCAC, forward CCAAAAAGACCTCGT TCAGC; *Cyc*: reverse TCCATCAGGGTATCCTCTCC, GGAGGCAAGCATAAGA CTGG; *Cox4*: reverse GCTCGGCTTCCAGTATTGAG, forward AGAAGGAA ATGGCTGCAGAA; *Atp5g1*: reverse AFTTGGTGTGGCTGGATCA, forward GCTGCTTGAGAGATGGGTTC; mitofusin-2: reverse CAATCCCAGATGGCAG AACTT, forward ACGTCAAAGGGTACCTGTCCA; *Cpt1b*: reverse GCACCCAG ATGATTGGGATACTGT, forward TTGCCCTACAGCTGGCTCATTTCC; *Pdk4*: reverse GGAACGTACACAATGTGGATTG, forward ATCTAACATCGCCAGAA TTAAACC; *Mcad*: reverse AGCTGATTGGCAATGTCTCCAGCAAA, forward GATCGCAATGGGTGCTTTTGATAGAA; *Glut4*: reverse AGGTGAAGATGAAG AAGCCAAGC, forward CTTCTTTGAGATTGGCCCTGG; *Sod1*: reverse TTGTTTCTCATGGACCACCA, forward AGGCTGTACCAGTGCAGGAC; *Nampt*: reverse AGTGGCCACAAATTCCAGAGA, forward CCGCCACAGTA TCTGTTCCTT.

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GCN5-mediated Transcriptional Control of the Metabolic Coactivator PGC-1β through Lysine Acetylation*[S]

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Timothy J. Kelly[‡§¶1], **Carles Lerin**[‡§1], **Wilhelm Haas**[§], **Steven P. Gygi**[§], **and Pere Puigserver**[‡§2]

From the [‡]*Department of Cancer Biology, Dana-Farber Cancer Institute and* [§]*Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115 and the* [¶]*Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

Changes in expression levels of genes encoding for proteins that control metabolic pathways are essential to maintain nutrient and energy homeostasis in individual cells as well as in organisms. An important regulated step in this process is accomplished through covalent chemical modifications of proteins that form complexes with the chromatin of gene promoters. The peroxisome proliferators γ co-activator 1 (PGC-1) family of transcriptional co-activators comprises important components of a number of these complexes and participates in a large array of glucose and lipid metabolic adaptations. Here, we show that PGC-1β is acetylated on at least 10 lysine residues distributed along the length of the protein by the acetyl transferase general control of amino-acid synthesis (GCN5) and that this acetylation reaction is reversed by the deacetylase sirtuin 1 (SIRT1). GCN5 strongly interacts with PGC-1β and represses its transcriptional activity associated with transcription factors such as ERRα, NRF-1, and HNF4α, however acetylation and transcriptional repression do not occur when a catalytically inactive GCN5 is co-expressed. Transcriptional repression coincides with PGC-1β redistribution to nuclear foci where it co-localizes with GCN5. Furthermore, knockdown of GCN5 ablates PGC-1β acetylation and increases transcriptional activity. In primary skeletal muscle cells, PGC-1β induction of endogenous target genes, including MCAD and GLUT4, is largely repressed by GCN5. Functionally, this translates to a blunted response to PGC-1β-induced insulin-mediated glucose transport. These results suggest that PGC-1β acetylation by GCN5 might be an important step in the control of glucose and lipid pathways and its dysregulation could contribute to metabolic diseases.

Transcriptional control of gene expression is a very dynamic and regulated process that involves assembly of protein complexes organized in space and time. Often this assembly is directed by covalent modification of transcriptional proteins dictating novel physical interactions, resulting in tightly controlled expression of genes. Among these modifications, protein acetylation at lysine residues has been implicated in control of gene expression (1, 2).

Cells, either individually or organized in tissues, respond to environmental cues through multiple adaptive responses to maintain homeostasis. An important part of this cellular response involves rapid changes in expression of genes encoding proteins that will functionally adapt the cell or organism to the new condition. In mammalian cells, the PGC-1[3] family of transcriptional co-activators has emerged as important regulators of gene expression in response to nutrient and hormonal fluctuations. The PGC-1 family is composed of three members, PGC-1α, PGC-1β, and PRC, which play important metabolic roles in various tissues. These proteins contain a similar architecture with an activation domain at the N terminus, a middle region associated with repression, and a C terminus with two domains involved in RNA processing. A main mechanism of control of the PGC-1s is through regulation of their own gene expression. For example, PGC-1α is rapidly induced in response to low temperatures in brown adipose tissue, fasting in liver or exercise in skeletal muscle (3–5). An increase of cAMP levels is one of the main signals involved in this transcriptional response, which leads to activation of PGC-1α function (6, 7). Among the targets, *OXPHOS* genes are strongly induced by the PGC-1s through interaction with transcription factors such as ERRα, NRFs, and ying yang 1 (8–11). Multiple covalent chemical modifications play a large role in controlling PGC-1α function as well. For example, PGC-1α is phosphorylated by p38 MAPK (12), glycogen synthase kinase 3β (13, 14), and Akt (15), it is methylated by PRMT1 (16), ubiquitinated by SCF(Cdc4) (14), *O*-GlcNAcylated by *O*-GlcNAc transferase (17), and, more related to this work, acetylated and de-acetylated by GCN5 (18) and SIRT1 (19, 20), respectively.

Our group has previously shown that PGC-1α acetylation is regulated through nutrient pathways controlled by changes in glucose concentrations (19, 21, 22). In high glucose concentrations PGC-1α is largely acetylated on at least 13 lysine residues, which in turn are deacetylated by SIRT1 in response to low glucose. In addition, SIRT1 activators are sufficient to deacety-

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dana-Farber Cancer Institute, One Jimmy Fund Way, Smith 932C, Boston, MA 02115. Tel.: 617-582-7977; Fax: 617-632-4770; E-mail: pere_puigserver@dfci.harvard.edu.

[3] The abbreviations used are: PGC-1, peroxisome proliferators γ co-activator 1; PRC, PGC-1-related co-activator; ERRα, estrogen-related receptor alpha; GCN5, general control of amino-acid synthesis; HNFα, hepatocyte nuclear factor alpha; GLUT4, glucose transporter 4; SIRT1, sirtuin 1; MCAD, medium chain acyl CoA-dehydrogenase; NRF1, nuclear respiratory factor 1; PRMT1, protein arginine methyltransferase 1; SCF, Skp1/Cullin/F-box; MAPK, mitogen-activated protein kinase; shRNA, short hairpin RNA; HA, hemagglutinin; MS, mass spectrometry; MS/MS, tandem MS; LC, liquid chromatography; FT, Fourier transform; ICR, ion cyclotron resonance.

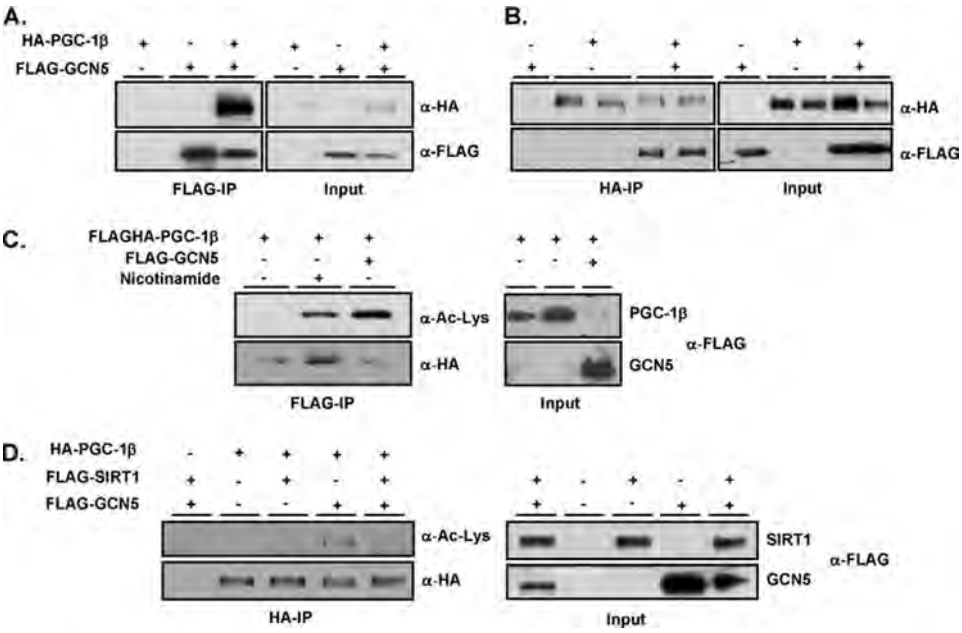

FIGURE 1. **GCN5 interacts with and acetylates the transcriptional co-activator PGC-1β.** Primary skeletal muscle myotubes were infected with the indicated adenoviruses encoding for FLAG-GCN5 and HA-PGC-1β proteins. *A* and *B*, immunoprecipitation with FLAG or HA antibodies linked to agarose was performed followed by Western blot analysis using the indicated antibodies. *C*, HEK 293 cells were transfected with the indicated plasmids encoding for either FLAG or HA-tagged proteins. PGC-1β was immunoprecipitated from whole cell extracts, and lysine-acetylation was detected using Western blot analysis with the indicated antibodies. *D*, similar experiments as in *C* were performed but using the SIRT1 plasmid.

late and activate PGC-1α leading to increases in energy expenditure (23, 24). The mechanism by which acetylated PGC-1α is less active is largely unknown but may involve altered subnuclear protein localization and reduced occupancy at gene promoters (18). Less is known about PGC-1β function, but like PGC-1α, it induces oxidative mitochondrial function, particularly in skeletal muscle (25, 26), and has been implicated in the hepatic feeding response, host innate response to bacterial pathogenesis and iron metabolism (25–29). Structurally, PGC-1β contains similar domains to PGC-1α along the protein, but with an extended middle region (30, 31).

Given the similarities between PGC-1α and PGC-1β and the regulatory role of PGC-1α acetylation, we investigated whether PGC-1β is acetylated by GCN5 and determined the functional consequences as it relates to gene expression and glucose transport. Here, we report that PGC-1β is a substrate for GCN5 and is acetylated on at least 10 lysine residues that are distributed along multiple domains of the protein. SIRT1 overexpression is sufficient to completely deacetylate PGC-1β. shRNA knockdown of GCN5 resulted in loss of basal acetylation and an induction of transcriptional activity. Moreover, wild-type GCN5 but not mutant, catalytically inactive GCN5 strongly repressed PGC-1β transcriptional activity. In primary skeletal muscle cells, GCN5 repressed expression of target genes involved in glucose and fatty acid utilization. Functionally, the effects of GCN5 on PGC-1β target gene expression correlated with a blockage of insulin-stimulated glucose uptake. These results indicate that acetylation of PGC-1β is sufficient to control its transcriptional co-activation activity and might have important consequences in metabolic diseases if dysregulated.

EXPERIMENTAL PROCEDURES

Constructs—pcDNA-PGC-1β construct was a gift from Bruce Spiegelman (Dana Farber Cancer Institute, Boston, MA). To generate GAL4-DBD and pAdTrack plasmids, PGC-1β was subcloned into the backbone of these plasmids. Constructs used in these studies for different transcription factors, wild-type and mutant GCN5, have already been described (19, 18). Plasmid maps and sequences of constructs used are available upon request.

Cell Culture and Treatments—HEK 293 cells were routinely cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Primary skeletal muscle cells were isolated and cultured as previously described (32). 80% confluent myoblasts were switched to the differentiation medium, Dulbecco's modified Eagle's medium containing 5% horse serum. Myotubes were transduced with adenoviruses encoding for PGC-1β or GCN5 for a period of 48 h.

Immunofluorescence Microscopy—Immunofluorescence experiments were performed 48 h after transfection with plasmids encoding for HA-PGC-1β and FLAG-GCN5. Immunofluorescence was performed using mouse anti-HA antibody to detect PGC-1β and rabbit anti-GCN5 antibody as previously described (18).

Analysis of Protein Acetylation—FLAG- or HA-tagged PGC-1β were expressed in HEK 293 cells via transfection using PolyFect (Qiagen) or in primary skeletal muscle cells by adenoviral infection. Whole cell extracts were used to immunoprecipitate PGC-1β or GCN5 with anti-FLAG M2 or HA antibody linked to agarose beads. After extensive washing, immunoprecipitates were separated by SDS-PAGE and immunoblotted using the acetyllysine antibody (Cell Signaling) and the M2 FLAG antibody (Sigma) or HA (BabCO) to detect lysine acetylation and total protein levels, respectively.

Identification of Acetylated Lysine Residues by MS Analysis—Mapping of acetylation on lysine residues of PGC-1β was performed by nanoscale microcapillary reverse phase liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) (33, 34). HEK 293 cells were infected with FLAG-HA-PGC-1β and FLAG-GCN5 and treated with 20 mM nicotinamide for a period of 12 h. Cells were harvested, and nuclear extracts were prepared as previously described (19). Immunoprecipitation with anti-FLAG M2 antibodies linked to agarose was performed in 300 mM NaCl, 1% Triton X-100. After extensive washes, proteins were eluted with 0.5 mg/ml FLAG peptide, and proteins were precipitated with trichloroacetic acid. Protein from Coomassie-stained gel bands was in-gel

A.

Acetylation Site	Peptide Sequence
K202	K.KTPTLR.A
K218	R.AQSRPCTELHKHLTSVLPCPR.V
K230	R.VKACSPTPHPSPR.L
K455	W.GRKRPGRGLPW.T
K469	R.KMDSSVCPVR.R
K645	K.LSPGQDTAPSLPSPEALPLTATPGASHKLPK.R
K674	V.SQAGQKRPF.S
K726	R.SWEPIGVHLEDLAQQGAPLPTETKAPR.R
K933	R.GQKHGFITFR.C
K994	R.YTDYDPTSEESLPSSGKSK.Y

B.

MAGNDCGALLDEELSSFFLNYLSDTQGGDSGEEQLCADLPELDLSQLDASDFDSATCFGELQWCPETSETEPSQYSP
DDSELFQIDSENEALLAALTKTLDDIPEDDVGLAAFPELDEGDTPSCTPASPAPLSAPPSPTLERLLSPASDVDELSLLQK
LLLATSSPTASSDALKDGATWSQTSLSSRSQRPCVKVDGTQDKKTPTLRAQSRPCTELHKHLTSVLPCPRVKACSPTP
HPSPRLLSKEEEEEVGEDCPSPWPTPASPQDSLAQDTASPDSAQPPEEDVRAMVQLIRYMHTYCLPQRKLPQRAPEPI
PQACSSLSRQVQPRSRHPPKAFWTEFSILRELLAQDILCDVSKPYRLAIPVYASLTPQSRPRPPKDSQASPAHSAMAEE
VRITASPKSTGPRPSLRPLRLEVKRDVNKPTRQKREEDEEEEEEEEEEEEKEEEEEWGRKRPGRGLPWTKLGRKM
DSSVCPVRRSRRLNPELGPWLTFTDEPLGALPSMCLDTETHNLEEDLGSLTDSSQGRQLPQGSQIPALESPCESGCGD
TDEDPSCPQPTSRDSSRCLMLALSQSDSLGKKSFEESLTVELCGTAGLTPPTTPPYKPMEEDPFKPDTKLSPGQDTAP
SLPSPEALPLTATPGASHKLPKRHPERSELLSHLQHATTQPVSQAGQKRPFSCSFGDHDYCQVLRPEAALQRKVLRS
WEPIGVHLEDLAQQGAPLPTETKAPRREANQNCDPTHKDSMQLRDHEIRASLTKHFGLLETALEGEDLASCKSPEYDT
VFEDSSSSSGESSFLLEEEEEEEEGGEEDDEGEDSGVSPPCSDHCPYQSPPSKASRQLCSRSRSSSGSSSCSSWSP
ATRKNFRRESRGPCSDGTPSVRHARKRREKAIGEGRVVYIRNLSSDMSSRELKKRFEVFGEIVECQVLTRSKRGQKHG
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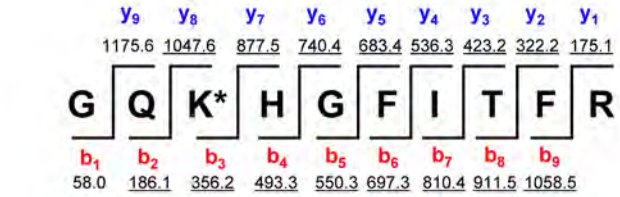
C.



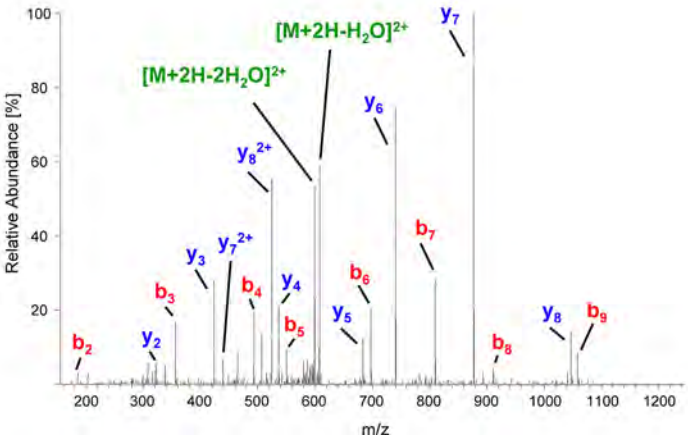


FIGURE 2. **Analysis and identification of PGC-1β lysine acetylation sites.** HEK 293 cells were infected with adenoviruses encoding for FLAG-HA-PGC-1β and FLAG-GCN5 and treated with nicotinamide (20 mM) for 12 h. After immunoprecipitation, PGC-1β was separated by SDS-PAGE and analyzed by tandem mass spectrometry. Acetylation was determined by subjecting a tryptic and a chymotryptic digest of the protein to microcapillary LC-MS/MS on a hybrid linear ion trap/FT-ICR mass spectrometer and assigning the acquired MS/MS data using the SEQUEST algorithm as described under "Experimental Procedures." *A*, sequences of identified acetylated peptides, including flanking amino acid residues. Acetylated lysine residues are shown in *red*. *B*, 87% of the amino acid sequence (74% of lysine residues) of PGC-1β was covered in the acetylation mapping experiment (covered residues are shown in *green*, acetylated lysine are residues in *red*). *C*, determination of acetylation on Lys-933. The *lower panel* shows the MS/MS of the doubly charged tryptic peptide Gly-931 to Arg-940 (*m/z* 616.82976, mass accuracy, −1.0 ppm) carrying an acetyl residue on Lyss-933 (*red*). The sequence of the peptide including *m/z* values for predicted fragment ions is shown above the spectrum. Detected fragment ions are *underlined*.

reduced with dithiothreitol, and cysteine residues were derivatized with iodoacetamide. In-gel digestion of the protein was performed using either trypsin or chymotrypsin (33), and the generated peptide mixtures were subjected to nanoscale microcapillary LC-MS/MS on a hybrid linear ion trap/FT-ICR mass spectrometer (LTQ FT, Thermo Electron) essentially as described previously (34). Briefly, peptides were separated on a 125-μm inner diameter microcapillary C_{18} column, and MS and MS/MS data were collected in an automated fashion. A high mass accuracy and high mass resolution FT-ICR MS survey scan was followed by ten linear ion trap MS/MS experiments on the ten most abundant ions detected in the survey scan before a consecutive experimental cycle was initiated with another FT-survey scan. MS/MS data were automatically assigned by searching them against the PGC-1β sequence using the SEQUEST (35) algorithm and allowing lysine residues to be acetylated. The precursor ion mass tolerance in the data base search was set to ±2 Da, and no enzyme specificity constraints were used. Search results were filtered for peptide assignments with high mass accuracy (−7 to 3 ppm), and both termini were consistent with the specificity of the proteases used for the digest of the protein. Additionally, a Δ*Cn* filter of 0.1 and XCorr filters of 1.5 for doubly, 2 for triply, and 3 for quadruply charged peptides were applied and MS/MS spectra of acetylated peptides were validated manually.

Luciferase-based Transcriptional Co-activation Assays—HEK 293 cells were transfected using a ratio of DNA:PolyFect (Qiagen) 1:2. After transfection, cells were lysed and luciferase assays were performed. We normalized transfection efficiency using the *Renilla* system (Promega).

Gene Expression Analysis—mRNA expression levels were analyzed by quantitative real-time PCR. Total RNA was prepared from primary skeletal muscle cells via TRIzol

extraction (Invitrogen). cDNA was generated by Superscript II enzyme (Invitrogen) and analyzed by quantitative reverse-transcriptase-mediated PCR using an iQ SYBR Green Supermix (Bio-Rad). All data were normalized to tubulin expression. The oligonucleotide primers can be provided upon request.

Glucose Uptake Assays—Primary muscle myotubes were infected with adenoviruses encoding for green fluorescent protein, PGC-1β, and GCN5 and incubated with 5% horse serum for 48 h. Cells were then incubated with Dulbecco's modified Eagle's medium supplemented with 0.5%bovine serum albumin for 6 h and 100 nM insulin was added during the last 20 min. Glucose uptake was measured by incubating with [^{3}H]2-deoxyglucose for 6 min and corrected by protein content.

RESULTS

GCN5 Interacts with and Acetylates PGC-1β—Based on our previous studies that PGC-1α is a substrate of the acetyl transferase GCN5 (18), we tested whether GCN5 might also interact with and acetylate PGC-1β. To this end, we infected primary skeletal myotubes with adenoviruses encoding for FLAG-GCN5 and HA-PGC-1β. Fig. 1*A* shows that immunoprecipitation of FLAG-GCN5 resulted in strong co-immunoprecipitation of HA-PGC-1β protein. Conversely, immunoprecipitated HA-PGC-1β was associated with FLAG-GCN5 suggesting physical interaction between both proteins (Fig. 1*B*). Similar to PGC-1α, ectopic expression of PGC-1β in cultured cells results in protein that is predominantly deacetylated, however, when co-expressed with GCN5, PGC-1β became strongly acetylated at lysine residues (Fig. 1*C*). In addition, two different types of experiments indicate that one of the PGC-1β deacetylases is the class III histone deacetylase sirtuin, SIRT1. First, PGC-1β acetylation is largely increased after treatment with nicotinamide (Fig. 1*C*) (a selective inhibitor of sirtuins). Second, expression of SIRT1 decreased GCN5-induced acetylation of PGC-1β (Fig. 1*D*). Taken together, these results indicate that PGC-1β is an acetylated protein and that its acetylation status is oppositely regulated by the enzymes GCN5 and SIRT1.

Identification of PGC-1β Lysine Residues Acetylated by GCN5—To map the PGC-1β lysines acetylated by GCN5, we initiated a large scale purification of PGC-1β expressed in HEK 293 cells double infected with adenoviruses encoding for FLAG-HA-PGC-1β and FLAG-GCN5 and treated with nicotinamide. Immunoprecipitated PGC-1β was analyzed by tandem mass spectrometry. We identified 10 acetylated lysines with 87% coverage of the full-length PGC-1β protein (Fig. 2). Interestingly, only one lysine site at the C terminus of PGC-1α and PGC-1β seems to be conserved (PGC-1α K778 and PGC-1β K994), although most of the lysine acetylation sites are in regions of homology between both co-activators (supplemental Fig. S1). These results indicate that, similar to PGC-1α, PGC-1β is acetylated in multiple lysine residues that are distributed across the major domains of the protein.

GCN5 Represses PGC-1β-mediated Transcriptional Co-activation—To determine the effects of GCN5 on PGC-1β transcriptional activity, we performed cell-based luciferase reporter assays using various transcription factors known to be co-activated by PGC-1β (30, 31). Fig. 3 shows that, as predicted,

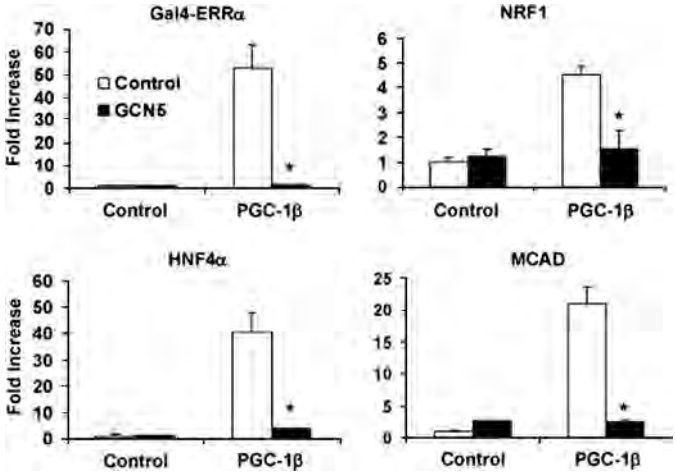


FIGURE 3. **Repression of PGC-1β transcriptional activity through GCN5.** HEK 293 cells were transfected with the indicated plasmids. Luciferase activities were measured as described under "Experimental Procedures." The luciferase reporters were either 5XUAS (for GAL4-ERRα), NRF-1 DNA-binding sites (NRF-1) (8), gAF1 of phosphoenolpyruvate carboxykinase promoter (HNF4α) (45), or the −375 MCAD promoter linked to luciferase as previously described (ERRα) (46). Values represent means ± S.E. of at least three independent experiments performed in duplicate. Statistical significance was determined by two-tailed unpaired Student's *t* test. *, $p < 0.05$ control *versus* GCN5.

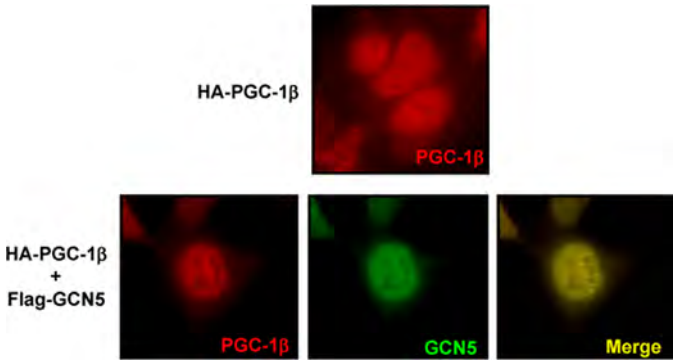


FIGURE 4. **PGC-1β nuclear redistribution induced by GCN5.** HEK 293 cells were transfected with HA-PGC-1β and FLAG-GCN5. Cells were fixed 48 h after transfection and immunofluorescence was performed using mouse anti-HA antibodies (shown in "*red*") and rabbit anti-GCN5 antibodies (shown in "*green*").

PGC-1β co-activated ERRα, NRF-1, as well as HNF4α transcription factors. Consistently, in all assays GCN5 potently repressed the ability of PGC-1β to activate these transcription factors. Similar repression effects were also observed in PGC-1β/ERRα-targeted promoters such as MCAD, an enzyme involved in mitochondrial fatty acid oxidation (Fig. 3). Together, these results indicate that GCN5 down-regulates the transcriptional activity of PGC-1β.

GCN5 Translocates PGC-1β to Nuclear Foci—To determine possible mechanisms by which GCN5 might control PGC-1β we performed cellular immunolocalization experiments. Expression of PGC-1β alone resulted in diffuse protein nuclear localization. However, co-expression of GCN5 resulted in re-localization of PGC-1β to nuclear foci also containing GCN5 protein (Fig. 4). These experiments suggest that localization of PGC-1β to nuclear foci by GCN5-mediated acetylation correlates with repression of its transcriptional activity.

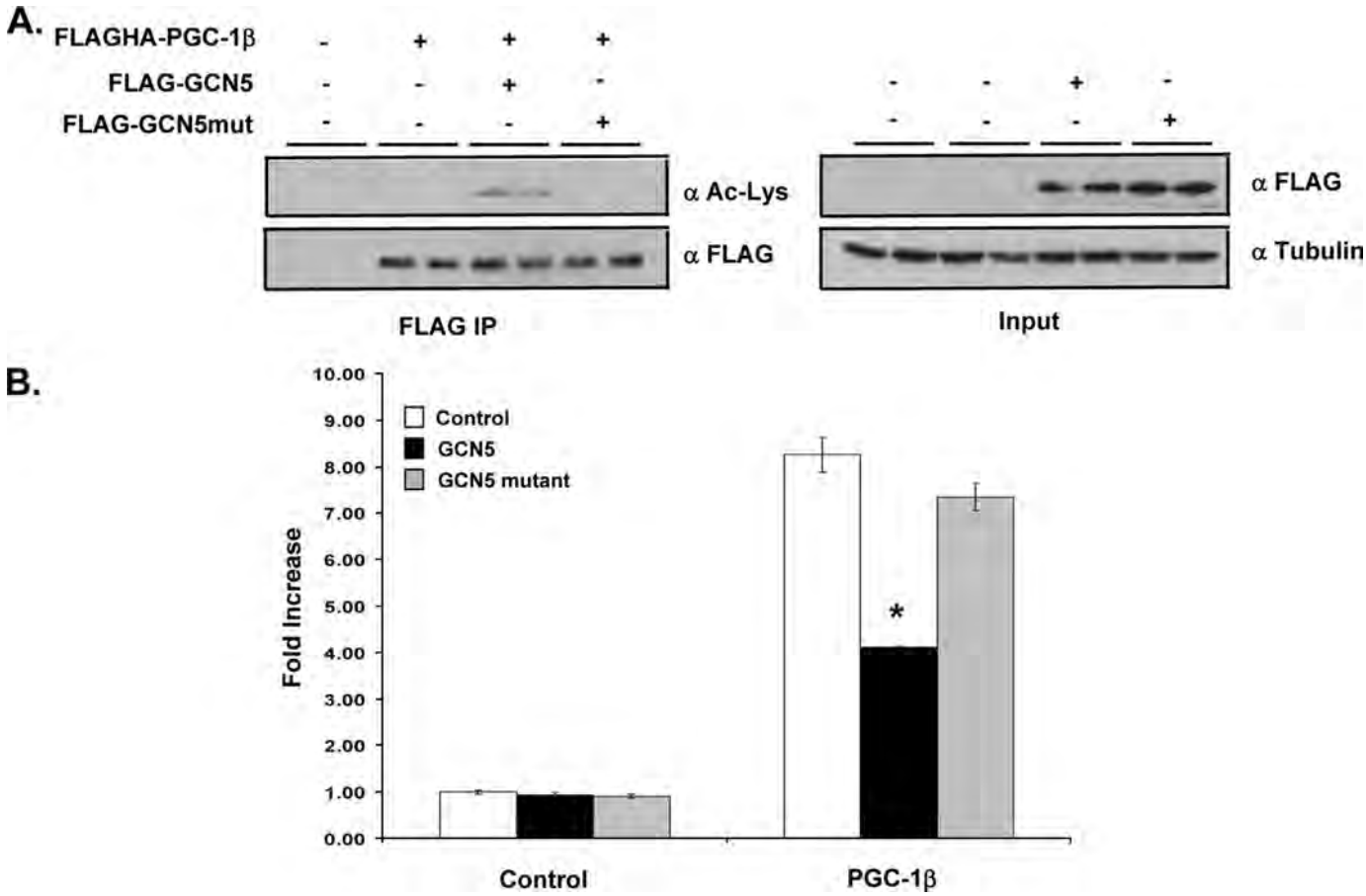


FIGURE 5. **Mutant GCN5 fails to acetylate PGC-1β and shows little impairment of transcriptional activity.** *A*, HEK 293 cells were transfected with the indicated plasmids encoding either wild-type GCN5 or mutant GCN5. FLAGHA-PGC-1β was overexpressed and immunoprecipitated using FLAG antibody linked to agarose beads. Detection of protein and acetylation levels was completed with Western blot as previous. *B*, HEK 293 cells were transfected with 5XUAS as well as GAL4-Errα and other indicated plasmids. Luciferase activities were measured as described under "Experimental Procedures." Values are representative of mean ± S.E. of two experiments each in triplicate Statistical significance was determined by two-tailed unpaired students *t* test. *, $p < 0.05$ PGC-1β *versus* PGC-1β plus GCN5.

GCN5 Repression of PGC-1β-mediated Transcription Requires Acetyltransferase Activity—To further establish that PGC-1β transcriptional activity is altered by GCN5 mediated acetylation, a mutant GCN5 lacking acetyltransferase activity was tested in both cell-based acetylation and luciferase reporter assays. As shown previously with PGC-1α, overexpressed PGC-1β is not acetylated when co-expressed with inactive GCN5 (Fig. 5*A*). Furthermore, transcriptional co-activation of PGC-1β on GAL4-Errα was not significantly repressed by overexpressed catalytically inactive GCN5 mutant, unlike wild-type GCN5 (Fig. 5*B*). These data suggest that regulation of PGC-1β by GCN5 is due to increased lysine acetylation.

Knockdown of GCN5 Results in Decreased Acetylation and an Increase in Transcriptional Activity—Knockdown of endogenous GCN5 by shRNA was used to probe the acetylation status and associated transcriptional activity of PGC-1β. 293HEK cells were transfected with either a control or GCN5-targeted shRNA and treated with nicotinamide to inhibit endogenous SIRT1 deacetylase activity. Overexpression and immunoprecipitation of PGC-1β revealed a reduction in acetylation to an undetectable level by Western blot, suggesting that endogenous GCN5 is a major PGC-1β acetyltransferase (Fig. 6*A*). Indeed, knockdown of GCN5 also resulted in a small but reproducible increase in transcriptional co-activation of PGC-1β on Errα at the MCAD promoter, as determined by luciferase activity (Fig. 6*B*). Taken together with the acetylation status of PGC-1β, these results suggest that GCN5 may regulate PGC-1β transcriptional activity by altering the acetylation status of the protein.

GCN5 Down-regulates Endogenous PGC-1β Target Genes—To further demonstrate the repressive effects of GCN5 on PGC-1β, endogenous expression of PGC-1β target genes were analyzed in primary skeletal muscle myotubes infected with adenoviruses encoding both proteins. PGC-1β expression alone was sufficient to induce mRNAs encoding for MCAD and GLUT4, two key enzymes involved in fatty acid and glucose utilization, respectively, ~5-fold. Consistent with the effects on acetylation status and luciferase reporter assays, expression of GCN5 largely repressed the induction of these PGC-1β target genes (Fig. 7). Taken together, these results further suggest that GCN5 is a transcriptional repressor of PGC-1β.

GCN5 Decreases Insulin-stimulated Glucose Transport Mediated by PGC-1β—The fact that PGC-1β strongly increased expression of the insulin-sensitive glucose transporter GLUT4, led us to test whether the effects of gene expression translated into glucose uptake. We therefore analyzed

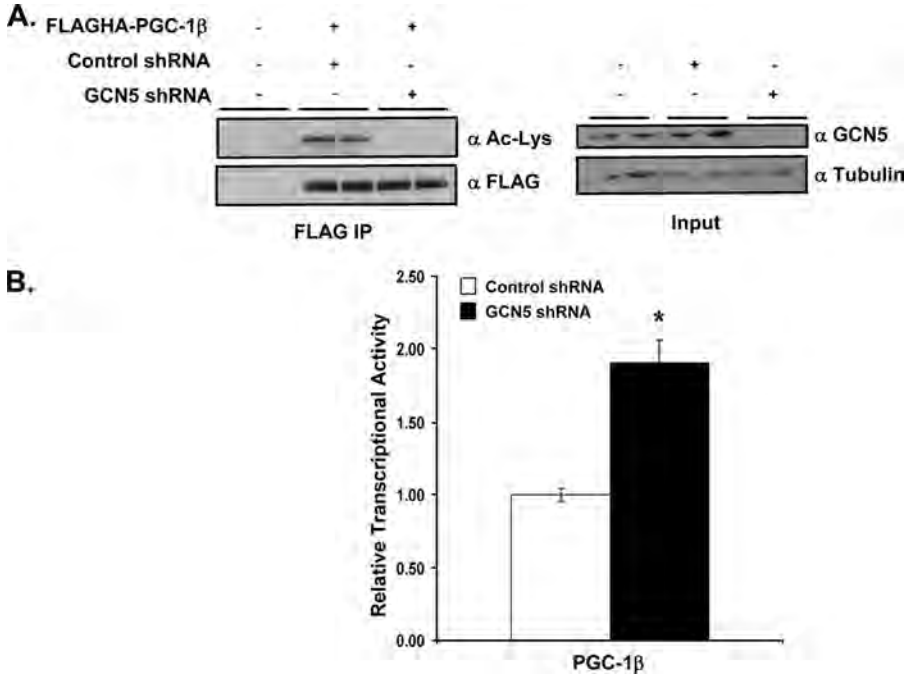


FIGURE 6. **Knockdown of GCN5 results in decreased acetylation and increased transcriptional activity of PGC-1β.** *A*, HEK 293 cells were transfected with control or GCN5 shRNA plasmid for 72 h and treated with 10 mM nicotinamide for the final 12 h. Overexpressed FLAGHA-PGC-1β was immunoprecipitated using FLAG antibody linked agarose beads and acetylation status was determined by Western blot. *B*, HEK 293 cells were transfected with control or GCN5 shRNA plasmid for 72 h along with PGC-1β, Errα, and the −375 MCAD promoter linked to luciferase. Luciferase activities were measured as described under "Experimental Procedures." Values represent relative means ± S.E. of three experiments each in triplicate. Statistical significance was determined by two-tailed unpaired Student's *t* test. *, $p < 0.005$ PGC-1β plus control shRNA *versus* PGC-1β plus GCN5 shRNA.

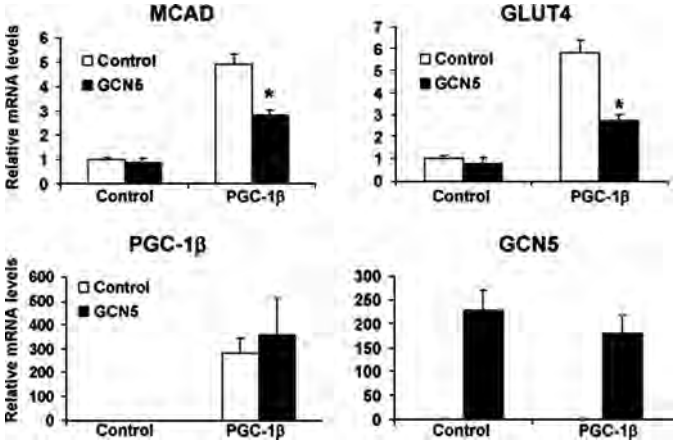


FIGURE 7. **GCN5 inhibition of PGC-1β-induced endogenous gene expression in skeletal muscle cells.** Primary skeletal muscle myotubes were infected with adenoviruses encoding the indicated proteins. Total RNA was analyzed by RT-PCR 2 days after infection. Values represent means ± S.E. of at least two independent experiments performed in duplicate. Statistical significance determined by two-tailed unpaired Student's *t* test. *, $p < 0.05$ PGC-1β *versus* PGC-1β plus GCN5.

insulin-mediated 2-deoxyglucose transport in primary skeletal myotubes. Interestingly, in this system expression of PGC-1β did not affect basal glucose uptake (data not shown). However, the transcriptional co-activator significantly increased the induction of glucose uptake in response to insulin (Fig. 8). Again, and consistent with our previous results in these studies, expression of GCN5 largely repressed the effects of PGC-1β on insulin-induced glucose uptake. These results indicate that the effects of this transcriptional co-activator on insulin-stimulated glucose transport are inhibited by expression of GCN5.

DISCUSSION

Fluxes of nutrients through the different metabolic pathways are largely determined by activities of enzymes and transporters. Although acute control of these proteins is via modulation of the catalytic or transport activity (*e.g.* post-translational modification, allosteric regulation, or translocation), in most metabolic pathways regulation of genes encoding for enzymes and transporters directly impact the rates and dynamics at which these pathways function (36, 37). Transcriptional regulation in response to fluctuation of nutrients or hormones is accomplished by changes in activities of transcriptional complexes that are bound to the promoters of genes as well as by alteration of promoter occupancy. In this metabolic regulatory context, we have described that the transcriptional co-activator PGC-1β, similarly to PGC-1α, is regulated by lysine acetylation through the acetyl transferase GCN5. Acetylation of PGC-1β coincides with a modified spatial subnuclear localization and repression of its transcriptional co-activation activity, while knockdown of GCN5 induces transcriptional co-activation activity. Functionally, the ability of PGC-1β to increase insulin-mediated glucose transport in skeletal muscle cells is blunted by GCN5.

PGC-1β-acetylated lysine residues are located in multiple domains suggesting that they might impact various binding partners and therefore activities of PGC-1β. For example, some of these lysines are close to the activation domain, whereas others are near nuclear localization signal sequences or in the proximity of the RNA processing motifs. Comparison of the identified acetylated lysines between PGC-1α and PGC-1β results in only one lysine at the C terminus that is conserved between both co-activators by BLAST analysis. However, most of the acetylated lysines are in regions of homology between PGC-1α and PGC-1β suggesting similar functions. It is possible that modification of specific lysines by acetylation could play specialized roles by defining interaction with particular sets of proteins. As a consequence, these interactions might lead to repression and translocation to nuclear foci. Moreover, binding affinities with specific transcription factors or other nuclear proteins that interact with different domains in PGC-1α or PGC-1β might change depending on the acetylation status. The specific function of these acetylation sites individually or in

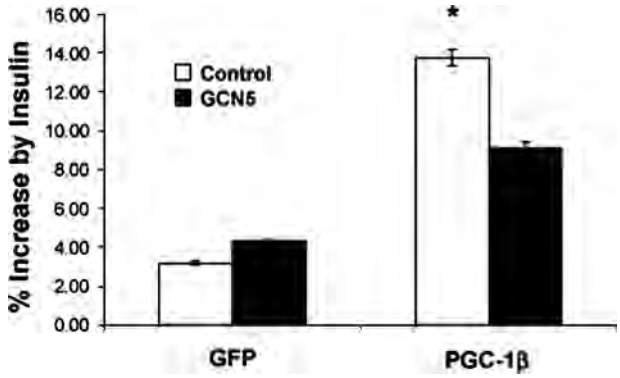


FIGURE 8. **GCN5 blocks PGC-1β-induced insulin-mediated glucose transport.** Primary skeletal muscle myotubes were infected with the indicated adenoviruses. Cells were incubated in Dulbecco's modified Eagle's medium with 0.5% bovine serum albumin, treated with 100 nM insulin for 20 min and incubated with [2-^{3}H]deoxyglucose as described under "Experimental Procedures." Values of % [2-^{3}H]deoxyglucose uptake increased by insulin represent means ± S.E. of six independent experiments performed in triplicate. Statistical significance determined by two-tailed unpaired Student's *t* test. *, $p <$ 0.05 control *versus* PGC-1β.

combination is currently under investigation. Another intriguing aspect of our studies is the fact that GCN5 acetylates histone 3 at lysine 9, which coincides with activation of gene expression (38). However, in our experiments GCN5 blocks induction of PGC-1β target genes. It is conceivable that GCN5 might initially acetylate histone H3 to promote gene expression, but then act in a negative feedback loop to acetylate and down-regulate PGC-1β transcriptional activity. In fact, similar molecular mechanisms have been proposed for nuclear hormone receptor co-activator ACTR (39). It is also possible be that other histone acetyltransferases such as CREB-binding protein or p300 play a role in acetylation of histones in PGC-1β target genes, similar to PGC-1α targets (40). Although we cannot completely rule out this possibility, initial chromatin immunoprecipitation studies expressing PGC-1β or co-expressing PGC-1β and GCN5 results in little change of histone H3 acetylation status at the MCAD promoter (data not shown), suggesting that acetylation of histone H3 plays little if any role in driving transcription in response to PGC-1β overexpression.

In these studies we have uncovered a new PGC-1β function associated with insulin-induced glucose uptake in skeletal muscle. The PGC-1β KO and hypomorphic allele transgenic mice present several metabolic abnormalities that include deficient adaptive thermogenesis, hepatic steatosis, and liver insulin resistance and impaired response to bacterial infection (26, 41, 27). Conversely, transgenic PGC-1β are resistant to obesity in response to high fat diet and display increased insulin sensitivity associated with an increase in oxidative metabolism (42, 43). In this context, we provide evidence in primary skeletal muscle cells that PGC-1β is sufficient to increase the response to insulin-mediated glucose transport and this correlates with increases in GLUT4 mRNA. Importantly, these effects were suppressed by GCN5. It is not clear how GCN5 might be modulated in skeletal muscle in response to insulin and in what metabolic context PGC-1β facilitates insulin action, at least in relation to glucose transport. In this regard, one of the acetylated PGC-1β lysine residues, Lys-202, precedes the A203P polymorphism that correlates with enhanced insulin-stimulated glucose metabolism in humans (44). It would be interesting to determine whether this amino acid substitution to proline can affect the Lys-202 acetylation and whether it might interfere with glucose transport.

In summary, the studies presented here illustrate that PGC-1β acetylation, regulated by the enzymatic activities of GCN5 and SIRT1, controls expression of metabolic genes involved in fatty acid oxidation and glucose transport. The fact that small molecules can regulate the catalytic activity of these two enzymes suggests that PGC-1β acetylation might be targeted in metabolic diseases to modulate its transcriptional activity.

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GCN5-mediated Transcriptional Control of the Metabolic Coactivator PGC-1β through Lysine Acetylation*[S]

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Timothy J. Kelly[‡§¶1], **Carles Lerin**[‡§1], **Wilhelm Haas**[§], **Steven P. Gygi**[§], **and Pere Puigserver**[‡§2]

From the [‡]*Department of Cancer Biology, Dana-Farber Cancer Institute and* [§]*Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115 and the* [¶]*Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

Changes in expression levels of genes encoding for proteins that control metabolic pathways are essential to maintain nutrient and energy homeostasis in individual cells as well as in organisms. An important regulated step in this process is accomplished through covalent chemical modifications of proteins that form complexes with the chromatin of gene promoters. The peroxisome proliferators γ co-activator 1 (PGC-1) family of transcriptional co-activators comprises important components of a number of these complexes and participates in a large array of glucose and lipid metabolic adaptations. Here, we show that PGC-1β is acetylated on at least 10 lysine residues distributed along the length of the protein by the acetyl transferase general control of amino-acid synthesis (GCN5) and that this acetylation reaction is reversed by the deacetylase sirtuin 1 (SIRT1). GCN5 strongly interacts with PGC-1β and represses its transcriptional activity associated with transcription factors such as ERRα, NRF-1, and HNF4α, however acetylation and transcriptional repression do not occur when a catalytically inactive GCN5 is co-expressed. Transcriptional repression coincides with PGC-1β redistribution to nuclear foci where it co-localizes with GCN5. Furthermore, knockdown of GCN5 ablates PGC-1β acetylation and increases transcriptional activity. In primary skeletal muscle cells, PGC-1β induction of endogenous target genes, including MCAD and GLUT4, is largely repressed by GCN5. Functionally, this translates to a blunted response to PGC-1β-induced insulin-mediated glucose transport. These results suggest that PGC-1β acetylation by GCN5 might be an important step in the control of glucose and lipid pathways and its dysregulation could contribute to metabolic diseases.

Transcriptional control of gene expression is a very dynamic and regulated process that involves assembly of protein complexes organized in space and time. Often this assembly is directed by covalent modification of transcriptional proteins dictating novel physical interactions, resulting in tightly controlled expression of genes. Among these modifications, protein acetylation at lysine residues has been implicated in control of gene expression (1, 2).

Cells, either individually or organized in tissues, respond to environmental cues through multiple adaptive responses to maintain homeostasis. An important part of this cellular response involves rapid changes in expression of genes encoding proteins that will functionally adapt the cell or organism to the new condition. In mammalian cells, the PGC-1[3] family of transcriptional co-activators has emerged as important regulators of gene expression in response to nutrient and hormonal fluctuations. The PGC-1 family is composed of three members, PGC-1α, PGC-1β, and PRC, which play important metabolic roles in various tissues. These proteins contain a similar architecture with an activation domain at the N terminus, a middle region associated with repression, and a C terminus with two domains involved in RNA processing. A main mechanism of control of the PGC-1s is through regulation of their own gene expression. For example, PGC-1α is rapidly induced in response to low temperatures in brown adipose tissue, fasting in liver or exercise in skeletal muscle (3–5). An increase of cAMP levels is one of the main signals involved in this transcriptional response, which leads to activation of PGC-1α function (6, 7). Among the targets, *OXPHOS* genes are strongly induced by the PGC-1s through interaction with transcription factors such as ERRα, NRFs, and ying yang 1 (8–11). Multiple covalent chemical modifications play a large role in controlling PGC-1α function as well. For example, PGC-1α is phosphorylated by p38 MAPK (12), glycogen synthase kinase 3β (13, 14), and Akt (15), it is methylated by PRMT1 (16), ubiquitinated by SCF(Cdc4) (14), *O*-GlcNAcylated by *O*-GlcNAc transferase (17), and, more related to this work, acetylated and de-acetylated by GCN5 (18) and SIRT1 (19, 20), respectively.

Our group has previously shown that PGC-1α acetylation is regulated through nutrient pathways controlled by changes in glucose concentrations (19, 21, 22). In high glucose concentrations PGC-1α is largely acetylated on at least 13 lysine residues, which in turn are deacetylated by SIRT1 in response to low glucose. In addition, SIRT1 activators are sufficient to deacety-

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dana-Farber Cancer Institute, One Jimmy Fund Way, Smith 932C, Boston, MA 02115. Tel.: 617-582-7977; Fax: 617-632-4770; E-mail: pere_puigserver@dfci.harvard.edu.

[3] The abbreviations used are: PGC-1, peroxisome proliferators γ co-activator 1; PRC, PGC-1-related co-activator; ERRα, estrogen-related receptor alpha; GCN5, general control of amino-acid synthesis; HNFα, hepatocyte nuclear factor alpha; GLUT4, glucose transporter 4; SIRT1, sirtuin 1; MCAD, medium chain acyl CoA-dehydrogenase; NRF1, nuclear respiratory factor 1; PRMT1, protein arginine methyltransferase 1; SCF, Skp1/Cullin/F-box; MAPK, mitogen-activated protein kinase; shRNA, short hairpin RNA; HA, hemagglutinin; MS, mass spectrometry; MS/MS, tandem MS; LC, liquid chromatography; FT, Fourier transform; ICR, ion cyclotron resonance.

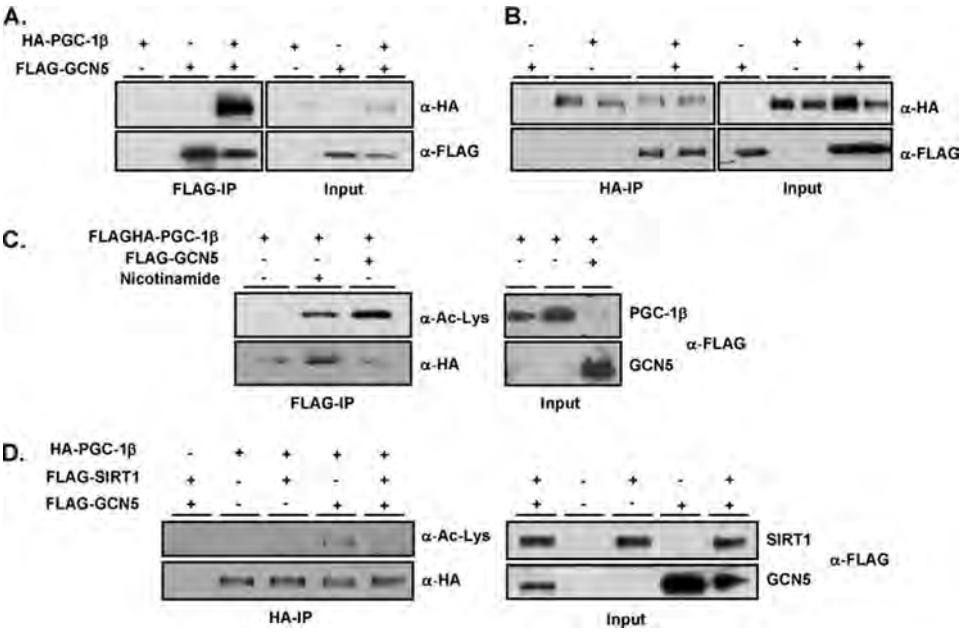

FIGURE 1. **GCN5 interacts with and acetylates the transcriptional co-activator PGC-1β.** Primary skeletal muscle myotubes were infected with the indicated adenoviruses encoding for FLAG-GCN5 and HA-PGC-1β proteins. *A* and *B*, immunoprecipitation with FLAG or HA antibodies linked to agarose was performed followed by Western blot analysis using the indicated antibodies. *C*, HEK 293 cells were transfected with the indicated plasmids encoding for either FLAG or HA-tagged proteins. PGC-1β was immunoprecipitated from whole cell extracts, and lysine-acetylation was detected using Western blot analysis with the indicated antibodies. *D*, similar experiments as in *C* were performed but using the SIRT1 plasmid.

late and activate PGC-1α leading to increases in energy expenditure (23, 24). The mechanism by which acetylated PGC-1α is less active is largely unknown but may involve altered subnuclear protein localization and reduced occupancy at gene promoters (18). Less is known about PGC-1β function, but like PGC-1α, it induces oxidative mitochondrial function, particularly in skeletal muscle (25, 26), and has been implicated in the hepatic feeding response, host innate response to bacterial pathogenesis and iron metabolism (25–29). Structurally, PGC-1β contains similar domains to PGC-1α along the protein, but with an extended middle region (30, 31).

Given the similarities between PGC-1α and PGC-1β and the regulatory role of PGC-1α acetylation, we investigated whether PGC-1β is acetylated by GCN5 and determined the functional consequences as it relates to gene expression and glucose transport. Here, we report that PGC-1β is a substrate for GCN5 and is acetylated on at least 10 lysine residues that are distributed along multiple domains of the protein. SIRT1 overexpression is sufficient to completely deacetylate PGC-1β. shRNA knockdown of GCN5 resulted in loss of basal acetylation and an induction of transcriptional activity. Moreover, wild-type GCN5 but not mutant, catalytically inactive GCN5 strongly repressed PGC-1β transcriptional activity. In primary skeletal muscle cells, GCN5 repressed expression of target genes involved in glucose and fatty acid utilization. Functionally, the effects of GCN5 on PGC-1β target gene expression correlated with a blockage of insulin-stimulated glucose uptake. These results indicate that acetylation of PGC-1β is sufficient to control its transcriptional co-activation activity and might have important consequences in metabolic diseases if dysregulated.

EXPERIMENTAL PROCEDURES

Constructs—pcDNA-PGC-1β construct was a gift from Bruce Spiegelman (Dana Farber Cancer Institute, Boston, MA). To generate GAL4-DBD and pAdTrack plasmids, PGC-1β was subcloned into the backbone of these plasmids. Constructs used in these studies for different transcription factors, wild-type and mutant GCN5, have already been described (19, 18). Plasmid maps and sequences of constructs used are available upon request.

Cell Culture and Treatments—HEK 293 cells were routinely cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Primary skeletal muscle cells were isolated and cultured as previously described (32). 80% confluent myoblasts were switched to the differentiation medium, Dulbecco's modified Eagle's medium containing 5% horse serum. Myotubes were transduced with adenoviruses encoding for PGC-1β or GCN5 for a period of 48 h.

Immunofluorescence Microscopy—Immunofluorescence experiments were performed 48 h after transfection with plasmids encoding for HA-PGC-1β and FLAG-GCN5. Immunofluorescence was performed using mouse anti-HA antibody to detect PGC-1β and rabbit anti-GCN5 antibody as previously described (18).

Analysis of Protein Acetylation—FLAG- or HA-tagged PGC-1β were expressed in HEK 293 cells via transfection using PolyFect (Qiagen) or in primary skeletal muscle cells by adenoviral infection. Whole cell extracts were used to immunoprecipitate PGC-1β or GCN5 with anti-FLAG M2 or HA antibody linked to agarose beads. After extensive washing, immunoprecipitates were separated by SDS-PAGE and immunoblotted using the acetyllysine antibody (Cell Signaling) and the M2 FLAG antibody (Sigma) or HA (BabCO) to detect lysine acetylation and total protein levels, respectively.

Identification of Acetylated Lysine Residues by MS Analysis—Mapping of acetylation on lysine residues of PGC-1β was performed by nanoscale microcapillary reverse phase liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) (33, 34). HEK 293 cells were infected with FLAG-HA-PGC-1β and FLAG-GCN5 and treated with 20 mM nicotinamide for a period of 12 h. Cells were harvested, and nuclear extracts were prepared as previously described (19). Immunoprecipitation with anti-FLAG M2 antibodies linked to agarose was performed in 300 mM NaCl, 1% Triton X-100. After extensive washes, proteins were eluted with 0.5 mg/ml FLAG peptide, and proteins were precipitated with trichloroacetic acid. Protein from Coomassie-stained gel bands was in-gel

A.

Acetylation Site	Peptide Sequence
K202	K.KTPTLR.A
K218	R.AQSRPCTELHKHLTSVLPCPR.V
K230	R.VKACSPTPHPSPR.L
K455	W.GRKRPGRGLPW.T
K469	R.KMDSSVCPVR.R
K645	K.LSPGQDTAPSLPSPEALPLTATPGASHKLPK.R
K674	V.SQAGQKRPF.S
K726	R.SWEPIGVHLEDLAQQGAPLPTETKAPR.R
K933	R.GQKHGFITFR.C
K994	R.YTDYDPTSEESLPSSGKSK.Y

B.

MAGNDCGALLDEELSSFFLNYLSDTQGGDSGEEQLCADLPELDLSQLDASDFDSATCFGELQWCPETSETEPSQYSP
DDSELFQIDSENEALLAALTKTLDDIPEDDVGLAAFPELDEGDTPSCTPASPAPLSAPPSPTLERLLSPASDVDELSLLQK
LLLATSSPTASSDALKDGATWSQTSLSSRSQRPCVKVDGTQDKKTPTLRAQSRPCTELHKHLTSVLPCPRVKACSPTP
HPSPRLLSKEEEEEVGEDCPSPWPTPASPQDSLAQDTASPDSAQPPEEDVRAMVQLIRYMHTYCLPQRKLPQRAPEPI
PQACSSLSRQVQPRSRHPPKAFWTEFSILRELLAQDILCDVSKPYRLAIPVYASLTPQSRPRPPKDSQASPAHSAMAEE
VRITASPKSTGPRPSLRPLRLEVKRDVNKPTRQKREEDEEEEEEEEEEEEKEEEEEWGRKRPGRGLPWTKLGRKM
DSSVCPVRRSRRLNPELGPWLTFTDEPLGALPSMCLDTETHNLEEDLGSLTDSSQGRQLPQGSQIPALESPCESGCGD
TDEDPSCPQPTSRDSSRCLMLALSQSDSLGKKSFEESLTVELCGTAGLTPPTTPPYKPMEEDPFKPDTKLSPGQDTAP
SLPSPEALPLTATPGASHKLPKRHPERSELLSHLQHATTQPVSQAGQKRPFSCSFGDHDYCQVLRPEAALQRKVLRS
WEPIGVHLEDLAQQGAPLPTETKAPRREANQNCDPTHKDSMQLRDHEIRASLTKHFGLLETALEGEDLASCKSPEYDT
VFEDSSSSSGESSFLLEEEEEEEEGGEEDDEGEDSGVSPPCSDHCPYQSPPSKASRQLCSRSRSSSGSSSCSSWSP
ATRKNFRRESRGPCSDGTPSVRHARKRREKAIGEGRVVYIRNLSSDMSSRELKKRFEVFGEIVECQVLTRSKRGQKHG
FITFRCSEHAALSVRNGATLRKRNEPSFHLSYGGLRHFRWPRYTDYDPTSEESLPSSGKSKYEAMDFDSLLKEAQQSL
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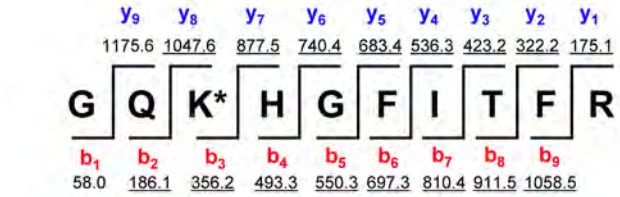
C.



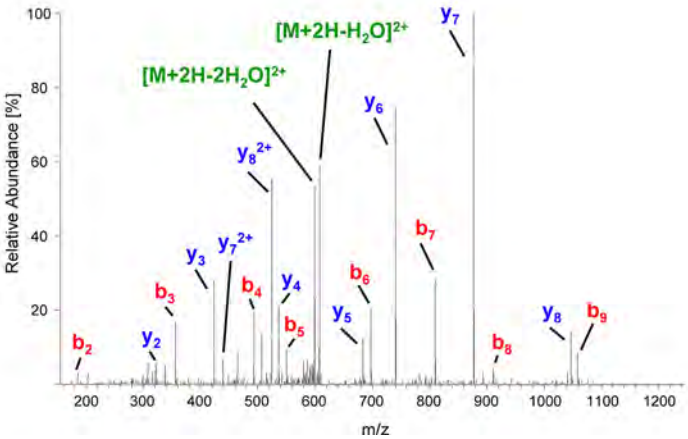


FIGURE 2. **Analysis and identification of PGC-1β lysine acetylation sites.** HEK 293 cells were infected with adenoviruses encoding for FLAG-HA-PGC-1β and FLAG-GCN5 and treated with nicotinamide (20 mM) for 12 h. After immunoprecipitation, PGC-1β was separated by SDS-PAGE and analyzed by tandem mass spectrometry. Acetylation was determined by subjecting a tryptic and a chymotryptic digest of the protein to microcapillary LC-MS/MS on a hybrid linear ion trap/FT-ICR mass spectrometer and assigning the acquired MS/MS data using the SEQUEST algorithm as described under "Experimental Procedures." *A*, sequences of identified acetylated peptides, including flanking amino acid residues. Acetylated lysine residues are shown in *red*. *B*, 87% of the amino acid sequence (74% of lysine residues) of PGC-1β was covered in the acetylation mapping experiment (covered residues are shown in *green*, acetylated lysine are residues in *red*). *C*, determination of acetylation on Lys-933. The *lower panel* shows the MS/MS of the doubly charged tryptic peptide Gly-931 to Arg-940 (*m/z* 616.82976, mass accuracy, −1.0 ppm) carrying an acetyl residue on Lyss-933 (*red*). The sequence of the peptide including *m/z* values for predicted fragment ions is shown above the spectrum. Detected fragment ions are *underlined*.

reduced with dithiothreitol, and cysteine residues were derivatized with iodoacetamide. In-gel digestion of the protein was performed using either trypsin or chymotrypsin (33), and the generated peptide mixtures were subjected to nanoscale microcapillary LC-MS/MS on a hybrid linear ion trap/FT-ICR mass spectrometer (LTQ FT, Thermo Electron) essentially as described previously (34). Briefly, peptides were separated on a 125-μm inner diameter microcapillary C_{18} column, and MS and MS/MS data were collected in an automated fashion. A high mass accuracy and high mass resolution FT-ICR MS survey scan was followed by ten linear ion trap MS/MS experiments on the ten most abundant ions detected in the survey scan before a consecutive experimental cycle was initiated with another FT-survey scan. MS/MS data were automatically assigned by searching them against the PGC-1β sequence using the SEQUEST (35) algorithm and allowing lysine residues to be acetylated. The precursor ion mass tolerance in the data base search was set to ±2 Da, and no enzyme specificity constraints were used. Search results were filtered for peptide assignments with high mass accuracy (−7 to 3 ppm), and both termini were consistent with the specificity of the proteases used for the digest of the protein. Additionally, a Δ*Cn* filter of 0.1 and XCorr filters of 1.5 for doubly, 2 for triply, and 3 for quadruply charged peptides were applied and MS/MS spectra of acetylated peptides were validated manually.

Luciferase-based Transcriptional Co-activation Assays—HEK 293 cells were transfected using a ratio of DNA:PolyFect (Qiagen) 1:2. After transfection, cells were lysed and luciferase assays were performed. We normalized transfection efficiency using the *Renilla* system (Promega).

Gene Expression Analysis—mRNA expression levels were analyzed by quantitative real-time PCR. Total RNA was prepared from primary skeletal muscle cells via TRIzol

extraction (Invitrogen). cDNA was generated by Superscript II enzyme (Invitrogen) and analyzed by quantitative reverse-transcriptase-mediated PCR using an iQ SYBR Green Supermix (Bio-Rad). All data were normalized to tubulin expression. The oligonucleotide primers can be provided upon request.

Glucose Uptake Assays—Primary muscle myotubes were infected with adenoviruses encoding for green fluorescent protein, PGC-1β, and GCN5 and incubated with 5% horse serum for 48 h. Cells were then incubated with Dulbecco's modified Eagle's medium supplemented with 0.5%bovine serum albumin for 6 h and 100 nM insulin was added during the last 20 min. Glucose uptake was measured by incubating with [^{3}H]2-deoxyglucose for 6 min and corrected by protein content.

RESULTS

GCN5 Interacts with and Acetylates PGC-1β—Based on our previous studies that PGC-1α is a substrate of the acetyl transferase GCN5 (18), we tested whether GCN5 might also interact with and acetylate PGC-1β. To this end, we infected primary skeletal myotubes with adenoviruses encoding for FLAG-GCN5 and HA-PGC-1β. Fig. 1*A* shows that immunoprecipitation of FLAG-GCN5 resulted in strong co-immunoprecipitation of HA-PGC-1β protein. Conversely, immunoprecipitated HA-PGC-1β was associated with FLAG-GCN5 suggesting physical interaction between both proteins (Fig. 1*B*). Similar to PGC-1α, ectopic expression of PGC-1β in cultured cells results in protein that is predominantly deacetylated, however, when co-expressed with GCN5, PGC-1β became strongly acetylated at lysine residues (Fig. 1*C*). In addition, two different types of experiments indicate that one of the PGC-1β deacetylases is the class III histone deacetylase sirtuin, SIRT1. First, PGC-1β acetylation is largely increased after treatment with nicotinamide (Fig. 1*C*) (a selective inhibitor of sirtuins). Second, expression of SIRT1 decreased GCN5-induced acetylation of PGC-1β (Fig. 1*D*). Taken together, these results indicate that PGC-1β is an acetylated protein and that its acetylation status is oppositely regulated by the enzymes GCN5 and SIRT1.

Identification of PGC-1β Lysine Residues Acetylated by GCN5—To map the PGC-1β lysines acetylated by GCN5, we initiated a large scale purification of PGC-1β expressed in HEK 293 cells double infected with adenoviruses encoding for FLAG-HA-PGC-1β and FLAG-GCN5 and treated with nicotinamide. Immunoprecipitated PGC-1β was analyzed by tandem mass spectrometry. We identified 10 acetylated lysines with 87% coverage of the full-length PGC-1β protein (Fig. 2). Interestingly, only one lysine site at the C terminus of PGC-1α and PGC-1β seems to be conserved (PGC-1α K778 and PGC-1β K994), although most of the lysine acetylation sites are in regions of homology between both co-activators (supplemental Fig. S1). These results indicate that, similar to PGC-1α, PGC-1β is acetylated in multiple lysine residues that are distributed across the major domains of the protein.

GCN5 Represses PGC-1β-mediated Transcriptional Co-activation—To determine the effects of GCN5 on PGC-1β transcriptional activity, we performed cell-based luciferase reporter assays using various transcription factors known to be co-activated by PGC-1β (30, 31). Fig. 3 shows that, as predicted,

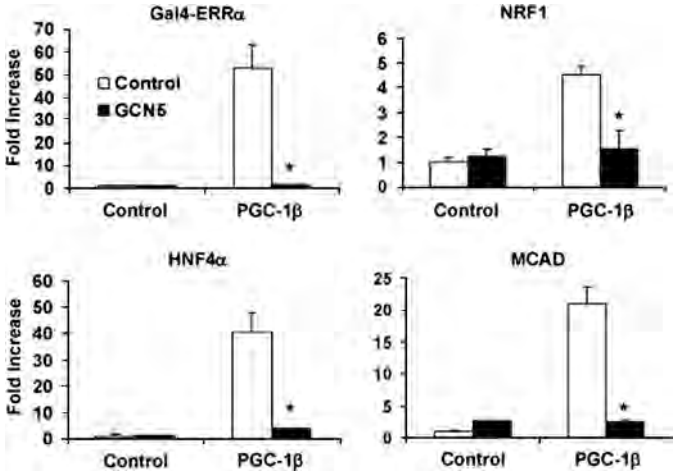


FIGURE 3. **Repression of PGC-1β transcriptional activity through GCN5.** HEK 293 cells were transfected with the indicated plasmids. Luciferase activities were measured as described under "Experimental Procedures." The luciferase reporters were either 5XUAS (for GAL4-ERRα), NRF-1 DNA-binding sites (NRF-1) (8), gAF1 of phosphoenolpyruvate carboxykinase promoter (HNF4α) (45), or the −375 MCAD promoter linked to luciferase as previously described (ERRα) (46). Values represent means ± S.E. of at least three independent experiments performed in duplicate. Statistical significance was determined by two-tailed unpaired Student's *t* test. *, $p < 0.05$ control *versus* GCN5.

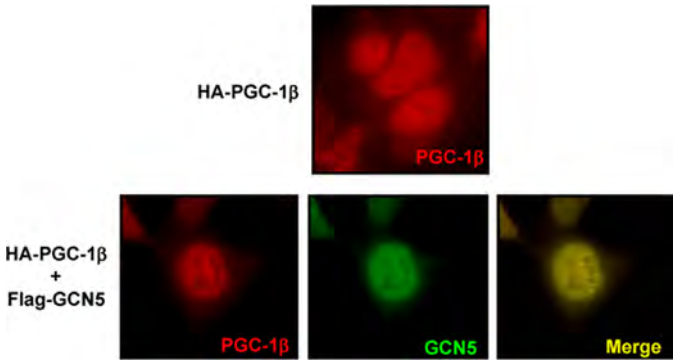


FIGURE 4. **PGC-1β nuclear redistribution induced by GCN5.** HEK 293 cells were transfected with HA-PGC-1β and FLAG-GCN5. Cells were fixed 48 h after transfection and immunofluorescence was performed using mouse anti-HA antibodies (shown in "*red*") and rabbit anti-GCN5 antibodies (shown in "*green*").

PGC-1β co-activated ERRα, NRF-1, as well as HNF4α transcription factors. Consistently, in all assays GCN5 potently repressed the ability of PGC-1β to activate these transcription factors. Similar repression effects were also observed in PGC-1β/ERRα-targeted promoters such as MCAD, an enzyme involved in mitochondrial fatty acid oxidation (Fig. 3). Together, these results indicate that GCN5 down-regulates the transcriptional activity of PGC-1β.

GCN5 Translocates PGC-1β to Nuclear Foci—To determine possible mechanisms by which GCN5 might control PGC-1β we performed cellular immunolocalization experiments. Expression of PGC-1β alone resulted in diffuse protein nuclear localization. However, co-expression of GCN5 resulted in re-localization of PGC-1β to nuclear foci also containing GCN5 protein (Fig. 4). These experiments suggest that localization of PGC-1β to nuclear foci by GCN5-mediated acetylation correlates with repression of its transcriptional activity.

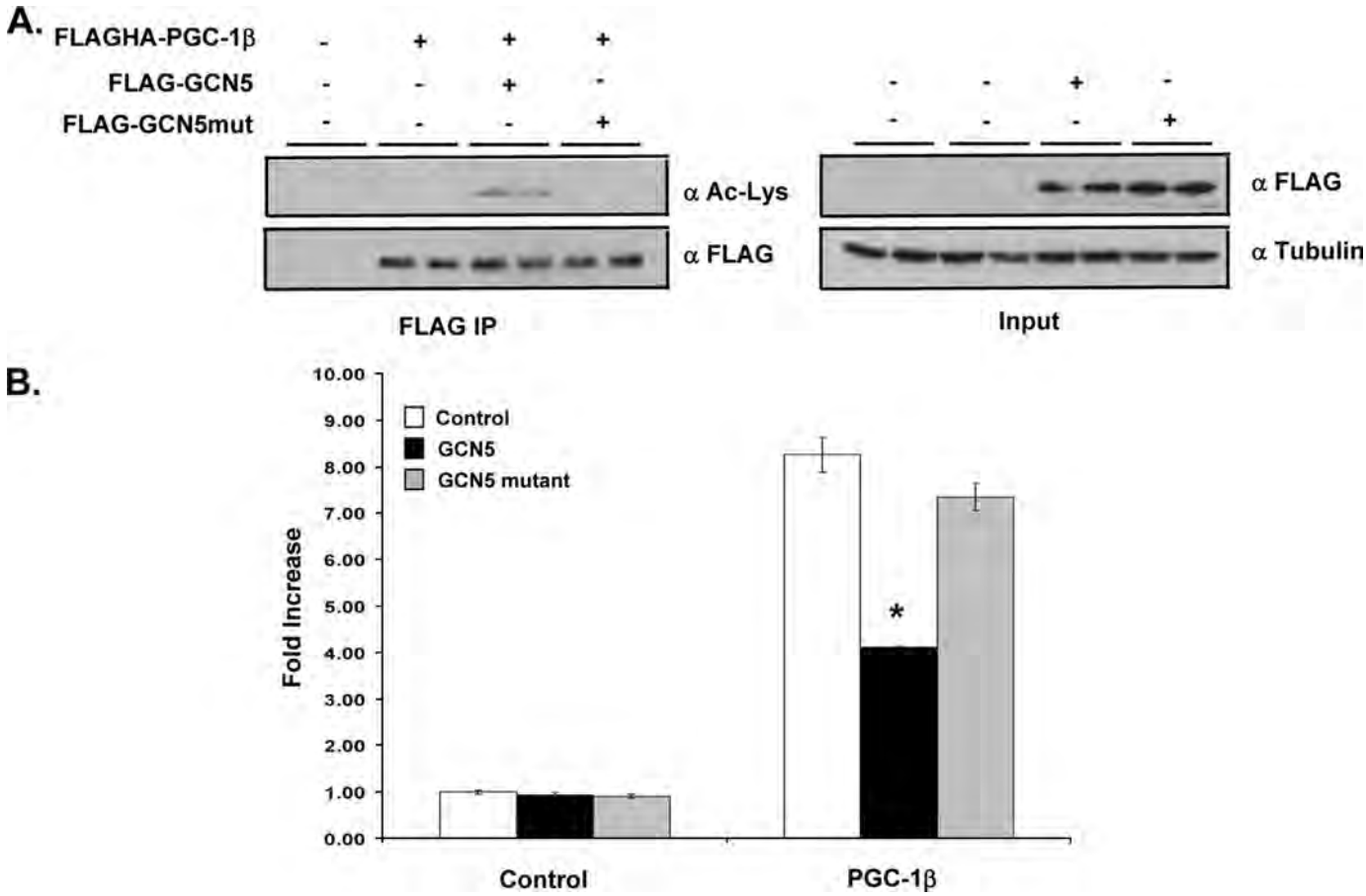


FIGURE 5. **Mutant GCN5 fails to acetylate PGC-1β and shows little impairment of transcriptional activity.** *A*, HEK 293 cells were transfected with the indicated plasmids encoding either wild-type GCN5 or mutant GCN5. FLAGHA-PGC-1β was overexpressed and immunoprecipitated using FLAG antibody linked to agarose beads. Detection of protein and acetylation levels was completed with Western blot as previous. *B*, HEK 293 cells were transfected with 5XUAS as well as GAL4-Errα and other indicated plasmids. Luciferase activities were measured as described under "Experimental Procedures." Values are representative of mean ± S.E. of two experiments each in triplicate Statistical significance was determined by two-tailed unpaired students *t* test. *, $p < 0.05$ PGC-1β *versus* PGC-1β plus GCN5.

GCN5 Repression of PGC-1β-mediated Transcription Requires Acetyltransferase Activity—To further establish that PGC-1β transcriptional activity is altered by GCN5 mediated acetylation, a mutant GCN5 lacking acetyltransferase activity was tested in both cell-based acetylation and luciferase reporter assays. As shown previously with PGC-1α, overexpressed PGC-1β is not acetylated when co-expressed with inactive GCN5 (Fig. 5*A*). Furthermore, transcriptional co-activation of PGC-1β on GAL4-Errα was not significantly repressed by overexpressed catalytically inactive GCN5 mutant, unlike wild-type GCN5 (Fig. 5*B*). These data suggest that regulation of PGC-1β by GCN5 is due to increased lysine acetylation.

Knockdown of GCN5 Results in Decreased Acetylation and an Increase in Transcriptional Activity—Knockdown of endogenous GCN5 by shRNA was used to probe the acetylation status and associated transcriptional activity of PGC-1β. 293HEK cells were transfected with either a control or GCN5-targeted shRNA and treated with nicotinamide to inhibit endogenous SIRT1 deacetylase activity. Overexpression and immunoprecipitation of PGC-1β revealed a reduction in acetylation to an undetectable level by Western blot, suggesting that endogenous GCN5 is a major PGC-1β acetyltransferase (Fig. 6*A*). Indeed, knockdown of GCN5 also resulted in a small but reproducible increase in transcriptional co-activation of PGC-1β on Errα at the MCAD promoter, as determined by luciferase activity (Fig. 6*B*). Taken together with the acetylation status of PGC-1β, these results suggest that GCN5 may regulate PGC-1β transcriptional activity by altering the acetylation status of the protein.

GCN5 Down-regulates Endogenous PGC-1β Target Genes—To further demonstrate the repressive effects of GCN5 on PGC-1β, endogenous expression of PGC-1β target genes were analyzed in primary skeletal muscle myotubes infected with adenoviruses encoding both proteins. PGC-1β expression alone was sufficient to induce mRNAs encoding for MCAD and GLUT4, two key enzymes involved in fatty acid and glucose utilization, respectively, ~5-fold. Consistent with the effects on acetylation status and luciferase reporter assays, expression of GCN5 largely repressed the induction of these PGC-1β target genes (Fig. 7). Taken together, these results further suggest that GCN5 is a transcriptional repressor of PGC-1β.

GCN5 Decreases Insulin-stimulated Glucose Transport Mediated by PGC-1β—The fact that PGC-1β strongly increased expression of the insulin-sensitive glucose transporter GLUT4, led us to test whether the effects of gene expression translated into glucose uptake. We therefore analyzed

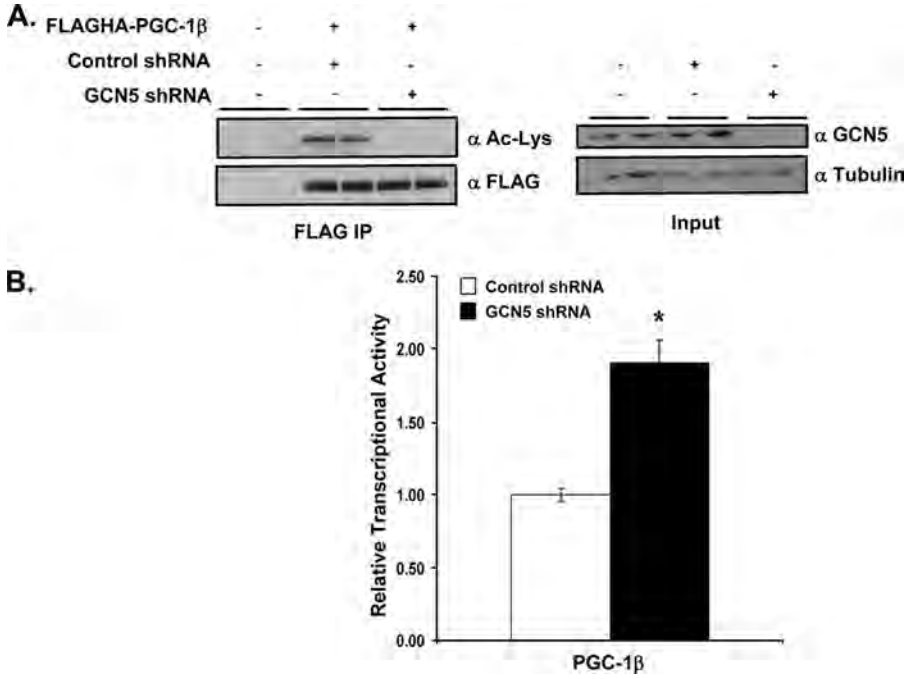


FIGURE 6. **Knockdown of GCN5 results in decreased acetylation and increased transcriptional activity of PGC-1β.** *A*, HEK 293 cells were transfected with control or GCN5 shRNA plasmid for 72 h and treated with 10 mM nicotinamide for the final 12 h. Overexpressed FLAGHA-PGC-1β was immunoprecipitated using FLAG antibody linked agarose beads and acetylation status was determined by Western blot. *B*, HEK 293 cells were transfected with control or GCN5 shRNA plasmid for 72 h along with PGC-1β, Errα, and the −375 MCAD promoter linked to luciferase. Luciferase activities were measured as described under "Experimental Procedures." Values represent relative means ± S.E. of three experiments each in triplicate. Statistical significance was determined by two-tailed unpaired Student's *t* test. *, $p < 0.005$ PGC-1β plus control shRNA *versus* PGC-1β plus GCN5 shRNA.

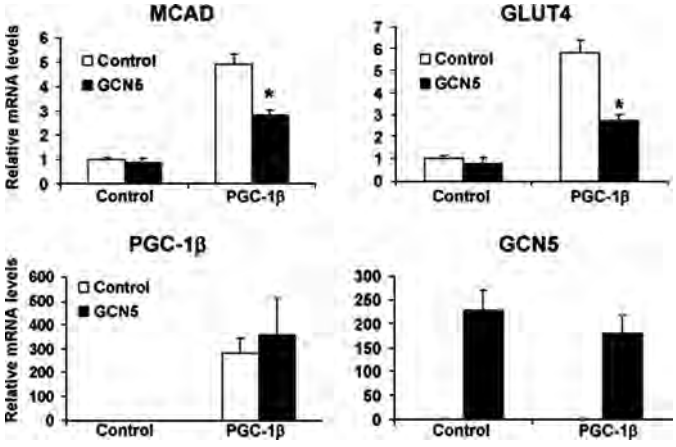


FIGURE 7. **GCN5 inhibition of PGC-1β-induced endogenous gene expression in skeletal muscle cells.** Primary skeletal muscle myotubes were infected with adenoviruses encoding the indicated proteins. Total RNA was analyzed by RT-PCR 2 days after infection. Values represent means ± S.E. of at least two independent experiments performed in duplicate. Statistical significance determined by two-tailed unpaired Student's *t* test. *, $p < 0.05$ PGC-1β *versus* PGC-1β plus GCN5.

insulin-mediated 2-deoxyglucose transport in primary skeletal myotubes. Interestingly, in this system expression of PGC-1β did not affect basal glucose uptake (data not shown). However, the transcriptional co-activator significantly increased the induction of glucose uptake in response to insulin (Fig. 8). Again, and consistent with our previous results in these studies, expression of GCN5 largely repressed the effects of PGC-1β on insulin-induced glucose uptake. These results indicate that the effects of this transcriptional co-activator on insulin-stimulated glucose transport are inhibited by expression of GCN5.

DISCUSSION

Fluxes of nutrients through the different metabolic pathways are largely determined by activities of enzymes and transporters. Although acute control of these proteins is via modulation of the catalytic or transport activity (*e.g.* post-translational modification, allosteric regulation, or translocation), in most metabolic pathways regulation of genes encoding for enzymes and transporters directly impact the rates and dynamics at which these pathways function (36, 37). Transcriptional regulation in response to fluctuation of nutrients or hormones is accomplished by changes in activities of transcriptional complexes that are bound to the promoters of genes as well as by alteration of promoter occupancy. In this metabolic regulatory context, we have described that the transcriptional co-activator PGC-1β, similarly to PGC-1α, is regulated by lysine acetylation through the acetyl transferase GCN5. Acetylation of PGC-1β coincides with a modified spatial subnuclear localization and repression of its transcriptional co-activation activity, while knockdown of GCN5 induces transcriptional co-activation activity. Functionally, the ability of PGC-1β to increase insulin-mediated glucose transport in skeletal muscle cells is blunted by GCN5.

PGC-1β-acetylated lysine residues are located in multiple domains suggesting that they might impact various binding partners and therefore activities of PGC-1β. For example, some of these lysines are close to the activation domain, whereas others are near nuclear localization signal sequences or in the proximity of the RNA processing motifs. Comparison of the identified acetylated lysines between PGC-1α and PGC-1β results in only one lysine at the C terminus that is conserved between both co-activators by BLAST analysis. However, most of the acetylated lysines are in regions of homology between PGC-1α and PGC-1β suggesting similar functions. It is possible that modification of specific lysines by acetylation could play specialized roles by defining interaction with particular sets of proteins. As a consequence, these interactions might lead to repression and translocation to nuclear foci. Moreover, binding affinities with specific transcription factors or other nuclear proteins that interact with different domains in PGC-1α or PGC-1β might change depending on the acetylation status. The specific function of these acetylation sites individually or in

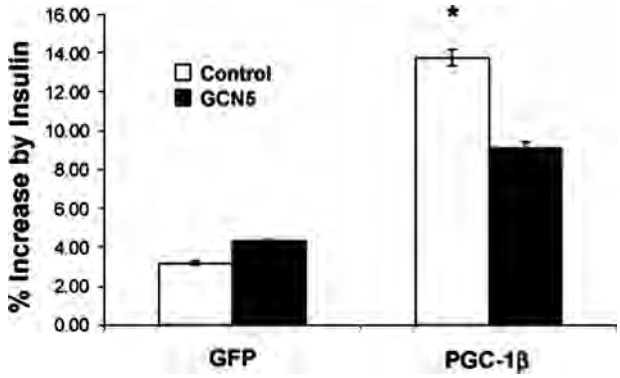


FIGURE 8. **GCN5 blocks PGC-1β-induced insulin-mediated glucose transport.** Primary skeletal muscle myotubes were infected with the indicated adenoviruses. Cells were incubated in Dulbecco's modified Eagle's medium with 0.5% bovine serum albumin, treated with 100 nM insulin for 20 min and incubated with [2-^{3}H]deoxyglucose as described under "Experimental Procedures." Values of % [2-^{3}H]deoxyglucose uptake increased by insulin represent means ± S.E. of six independent experiments performed in triplicate. Statistical significance determined by two-tailed unpaired Student's *t* test. *, $p <$ 0.05 control *versus* PGC-1β.

combination is currently under investigation. Another intriguing aspect of our studies is the fact that GCN5 acetylates histone 3 at lysine 9, which coincides with activation of gene expression (38). However, in our experiments GCN5 blocks induction of PGC-1β target genes. It is conceivable that GCN5 might initially acetylate histone H3 to promote gene expression, but then act in a negative feedback loop to acetylate and down-regulate PGC-1β transcriptional activity. In fact, similar molecular mechanisms have been proposed for nuclear hormone receptor co-activator ACTR (39). It is also possible be that other histone acetyltransferases such as CREB-binding protein or p300 play a role in acetylation of histones in PGC-1β target genes, similar to PGC-1α targets (40). Although we cannot completely rule out this possibility, initial chromatin immunoprecipitation studies expressing PGC-1β or co-expressing PGC-1β and GCN5 results in little change of histone H3 acetylation status at the MCAD promoter (data not shown), suggesting that acetylation of histone H3 plays little if any role in driving transcription in response to PGC-1β overexpression.

In these studies we have uncovered a new PGC-1β function associated with insulin-induced glucose uptake in skeletal muscle. The PGC-1β KO and hypomorphic allele transgenic mice present several metabolic abnormalities that include deficient adaptive thermogenesis, hepatic steatosis, and liver insulin resistance and impaired response to bacterial infection (26, 41, 27). Conversely, transgenic PGC-1β are resistant to obesity in response to high fat diet and display increased insulin sensitivity associated with an increase in oxidative metabolism (42, 43). In this context, we provide evidence in primary skeletal muscle cells that PGC-1β is sufficient to increase the response to insulin-mediated glucose transport and this correlates with increases in GLUT4 mRNA. Importantly, these effects were suppressed by GCN5. It is not clear how GCN5 might be modulated in skeletal muscle in response to insulin and in what metabolic context PGC-1β facilitates insulin action, at least in relation to glucose transport. In this regard, one of the acetylated PGC-1β lysine residues, Lys-202, precedes the A203P polymorphism that correlates with enhanced insulin-stimulated glucose metabolism in humans (44). It would be interesting to determine whether this amino acid substitution to proline can affect the Lys-202 acetylation and whether it might interfere with glucose transport.

In summary, the studies presented here illustrate that PGC-1β acetylation, regulated by the enzymatic activities of GCN5 and SIRT1, controls expression of metabolic genes involved in fatty acid oxidation and glucose transport. The fact that small molecules can regulate the catalytic activity of these two enzymes suggests that PGC-1β acetylation might be targeted in metabolic diseases to modulate its transcriptional activity.

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Minireview

Metabolic adaptations through the PGC-1α and SIRT1 pathways

Joseph T. Rodgers, Carles Lerin, Zachary Gerhart-Hines, Pere Puigserver*

Dana-Farber Cancer Institute and Department of Cell Biology, Harvard Medical School, One Jimmy Fund Way, Smith-936C, Boston, MA 02115, USA

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Abstract **Energy homeostasis in mammals is achieved through tight regulation of tissue-specific metabolic pathways that become dysregulated in metabolic diseases including diabetes and obesity. At the molecular level, main nutrient and hormonal signaling pathways impinge on expression of genes encoding for metabolic enzymes. Among the major components of this transcriptional circuitry are the PGC-1α transcriptional complexes. An important regulatory mechanism of this complex is through acetylation and SIRT1-mediated lysine de-acetylation under low nutrient conditions. Activation of SIRT1 can mimic several metabolic aspects of calorie restriction that target selective nutrient utilization and mitochondrial oxidative function to regulate energy balance. Thus, understanding the PGC-1α and SIRT1 pathways might have important implications for comprehending metabolic and age-associated diseases.**

Keywords: PGC-1α; SIRT1; Mitochondrial oxidation; Glucose metabolism; Lipid metabolism; Aging

1. Molecular mechanisms of PGC-1α and SIRT1 function

The initial studies in lower organisms found that the gene SIR2 (silent information regulator; ortholog of mammalian sirtuin SIRT1) potentially mediated the effects of calorie restriction on longevity [1], prompting different groups to investigate if SIRT1 had a role in metabolic function and longevity in mammals. Another active line of investigation was the search for genes that control mammalian energy and nutrient homeostasis. In this context, PGC-1α (peroxisome proliferator-activated receptor gamma-coactivator-1α) was identified as transcriptional coactivator that could define tissue-specific metabolic pathways in the adaptive response to environmental and nutritional stimuli [2]. Work by our group and Finkel's group first identified the functional interaction and deacetylation of PGC-1α and SIRT1. Furthermore, this interaction and deacetylation could be regulated by energy fluctuations and nutrient levels, thus leading to direct transcriptional control of metabolic enzymes and pathways [3,4]. We will discuss in this section how these two proteins function mechanistically in the context of controlling metabolic gene expression.

1.1. PGC-1α functions as a transcriptional coactivator

PGC-1α belongs to a small family of transcriptional coactivators, including PGC-1β and PRC which possess a common function in mitochondrial physiology, in addition to control over separate specific biological programs. PGC-1α was the first member identified as a cofactor for the nuclear hormone receptor PPARγ that is required for the adaptive thermogenic response to lower temperatures [2]. Until mid 1990s most of the regulation of gene expression and its biological implications were focused on transcription factors. During that time the discovery of the first transcriptional coactivators and corepressors for nuclear hormone receptors and other transcriptions factors initiated a novel concept centered around the control of specific genetic programs coordinated at the level of the transcriptional cofactor. PGC-1α as a transcriptional coactivator functions through direct physical interaction with transcription factors directly bound to DNA promoter regions. For example, LXXLL motifs in the PGC-1α N-terminus interact with different hormone nuclear receptors, including PPARs, HNF4α, GR and ERRα. In addition, other parts of the protein bind to other transcription factors such as NRF-1 in the 200–400 region, MEF2C in the 400–565 region or FoxO1 and YY1 in the RNA-processing C-terminal region (Fig. 1). Importantly, the ability of PGC-1α to interact with different transcription factors allows for the coordinated expression of gene sets in response to specific signals. However, another important and interesting implication is the possibility to activate gene expression in very specific contexts. As an example, PPARγ binding sites are present in UCP-1 and aP2 promoters, however PGC-1α only actives UCP-1, but not aP2 [2]. So far, the molecular basis of this specification is unknown, but it might involve DNA-binding affinities or facilitating interactions with other transcription factors or protein complexes to promote availability to access the promoter.

The mechanisms through which PGC-1α activates gene expression are poorly understood. Initial studies identified an extremely powerful autonomous transcriptional activity at the N-terminal region. This activation function correlates with the ability of PGC-1α to dock on this domain two other coactivators with acetyl transferase activity, SRC-1 and CBP/p300. In addition, the C-terminal region contains an SR motif and an RNA-binding domain. This region is required to induce expression of certain endogenous genes and interacts with proteins involved in RNA processing and the TRAP complex involved in transcriptional initiation [5]. Moreover, PGC-1α has been found in association with two additional different transcriptional complexes, the GCN5 and TIP60 acetyl transferases complexes and many of their associated protein

*Corresponding author. Fax: +1 617 632 4779.
E-mail address: pere_puigserver@dfci.harvard.edu (P. Puigserver).

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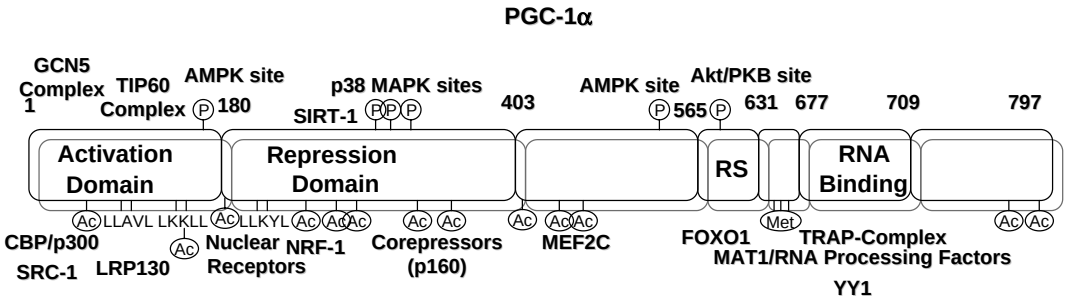

Fig. 1. Architecture of the PGC-1α transcriptional Coactivator. PGC-1α contains several functional domains that correlate with the interactions with different proteins and complexes. Additionally, PGC-1α contains specific phosphorylated, acetylated and methylated amino acids which can modulate its activity. See text for further details.

components [6]. GCN5 directly acetylates PGC-1α at multiple lysine residues and negatively regulates its transcriptional activity, at least in part, through nuclear sublocalization. How spatially and temporally all these complexes are assembled to PGC-1α to control expression of genes is unknown. A current model is that PGC-1α binds to specific transcription factors at promoters, then, additional recruitment of p300 and TRAP complexes would open the chromatin through histone acetylation thereby allowing initiation of transcription through RNA polII. Although GCN5 may acetylate histone H3 in this complex, it would initially be refractory to acetylate PGC-1α. The fact that the PGC-1α complex contains several proteins involved in RNA elongation and processing also suggests that it might move with the elongating phospho-RNA polII and participate in fully maturation of the mRNAs [6]. To terminate gene expression, GCN5 would acetylate PGC-1α resulting in relocalization to repressive subnuclear foci where PGC-1α has been shown to co-localize with the transcriptional repressor RIP140 [6]. Conversely, SIRT1 activation will maintain PGC-1α in a deacetylated active form bound to the chromatin and increasing rates of transcription (Fig. 2) [4,7].

However, this model is perhaps simplistic and does not take into account the other PGC-1α modifications such as phosphorylation and methylation, as well as interaction with other proteins including corepressors. Three kinases have been demonstrated that directly phosphorylate PGC-1α. The stress-activated p38 MAP kinase phosphorylates PGC-1α at three residues (Thr262; Ser265; Thr298) in the 200–400 repression domain and correlates with a more active and stable protein [8]. In addition to enhanced stability, the activity is further increased due to the inability of the phospho-PGC-1α to bind the p160 corepressor [9]. AMP kinase directly phosphorylates PGC-1α at residues Thr177 and Ser538 resulting in a more active protein [10]. Moreover, PGC-1α is phosphorylated by Akt/PKB at Ser570 in the SR domain and leads to a more unstable protein with lower activity [11]. Therefore, it seems that a major mechanism by which phosphorylation functions is through control of PGC-1α protein degradation and/or interaction

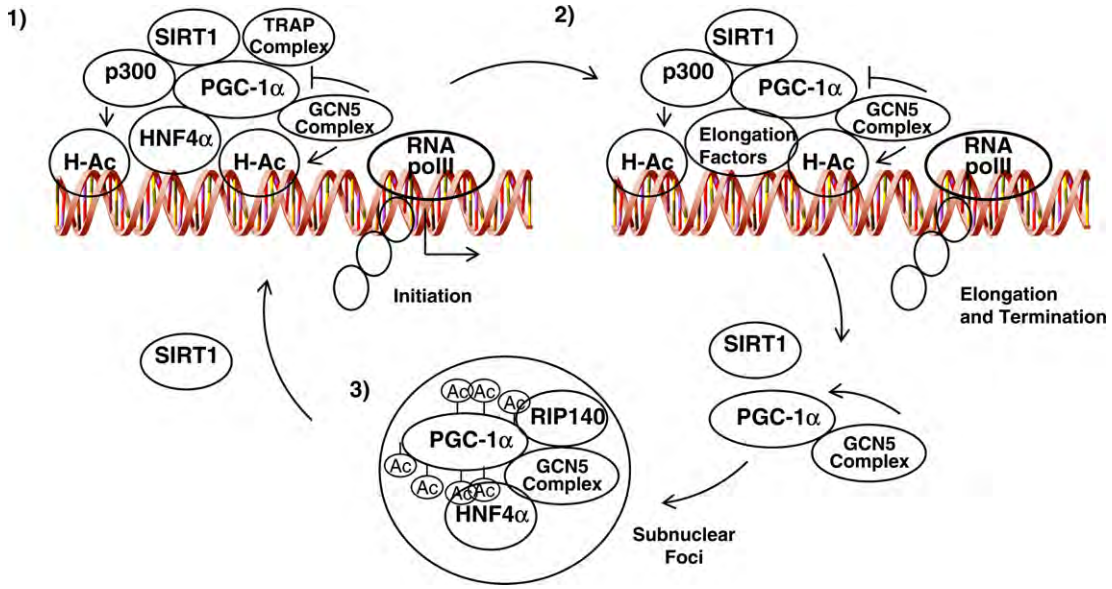

Fig. 2. Model for PGC-1α transcriptional gene expression activity. (1) PGC-1α is part of multiprotein complexes that contain histone acetyl transferase activity that open and remodels chromatin to allow the transcription factor to bind DNA. In the transcriptional initiation complex SIRT1 would maintain deacetylated PGC-1α. (2) PGC-1α and its associated proteins will move with RNA processing, elongation factors and RNA polII to transcribe the mRNA. (3) After the mRNA processing, GCN5 would acetylate PGC-1α localizing the whole complex to a RIP140 containing subnuclear repressive foci. To initiate another cycle, SIRT1 would deacetylate PGC-1α freeing it from the repressive foci, allowing it to become incorporated into protein complexes at promoter regions.

with corepressor proteins. It is not known how these specific phosphates control PGC-1α stability and the proteins involved in this regulation. Furthermore, PGC-1α is a target of the protein arginine methyltransferase 1 (PRMT1) that also coactivates nuclear hormone receptors. PRMT1 activates PGC-1α transcriptional activity through methylation at three arginine residues (Arg665, Arg667 and Arg669) at the C-terminus of the protein [12]. It is conceivable that PGC-1α functions in multiple protein complexes whose composition might depend on the specific target gene as well as the combination of different signals that are on and/or off. For example, LRP130 (leucine rich protein 130), a gene mutated in Leigh Syndrome French Canadian, is part of the PGC-1α complex that regulates a specific set of PGC-1α target genes in liver including gluconeogenic genes and certain mitochondrial genes (Fig. 1) [13]. Further identification of the signals, specific protein components and chemical modifications controlling these proteins will certainly provide novel mechanistic information as to how the whole PGC-1α metabolic biochemical machinery operates.

1.2. SIRT1 functions as a protein deacetylase

Sir2 was first identified as a factor which regulated silencing at the yeast mating type loci. Sir2 physically associates with chromatin in regions of the genome that were transcriptionally silent (mating type loci, telomere and centromeres) and also functions in preventing recombination of rDNA repeats [1]. Sir2, as well as SIRT1, are both NAD^+-dependent deacetylase but neither used NAD^+ as a redox acceptor. The NAD^+ molecule is instead cleaved in the deacetylation reaction, transferring the acetyl group to the ribose sugar, thereby producing *O*-acetyl-ADP-ribose and nicotinamide (Fig. 3). The released nicotinamide ring can also function as an inhibitor of this reaction [14,15]. As it relates to metabolism, the initial studies searching for yeast aging genes in the caloric restriction nutrient pathway identified Sir2. In worms and flies it appears that Sir2 is also involved in certain aspects of connecting calorie restriction to lifespan [16,17].

Most of the biological functions attributed to Sir2 or SIRT1 depend on the enzymatic activity. So far, the SIRT1 molecular mechanisms of function have been related to regulation of gene expression. In some cases, SIRT1 represses transcription and it is present in histone deacetylases and polycomb protein complexes [18]. SIRT1 specifically represses transcription factors such as PPARγ [19] and p53 [20]. The mechanism of repression is not completely understood but involves physical interaction with NCoR for PPARγ and direct deacetylation of p53 that decreases DNA-binding. In the case of FoxO proteins, SIRT1 can be either positive or negative depending on the type of target genes, but the mechanistic basis for this is not completely understood [21]. SIRT1 acts positively on activation of other genes through direct deacetylation of PGC-1α [4,7] and HIV Tat [22]. SIRT1 activates Tat-mediated transcription of the HIV long terminal repeat. SIRT1 interacts with PGC-1α in the 200–400 region and deacetylates PGC-1α in at least 13 lysines in different domains of the proteins (Fig. 1). Mutation of these residues to arginine leads to a more active PGC-1α allele [23].

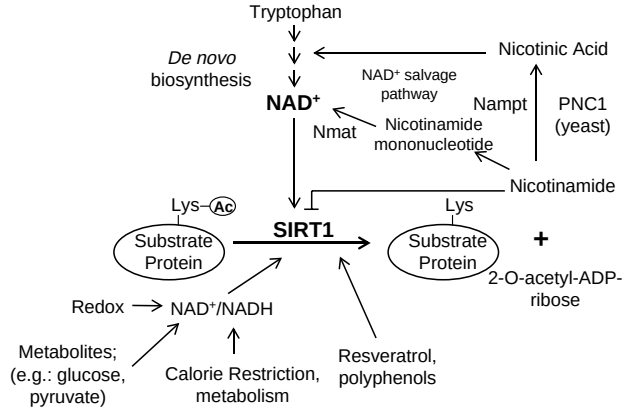

Fig. 3. Regulation of SIRT1 enzymatic activity. SIRT1 catalyzes a NAD^+-dependent protein deacetylase activity. This biochemical reaction is regulated by different metabolic inputs as well as several polyphenols. Moreover, several metabolic pathways including NAD^+ *de novo* biosynthesis from tryptophan and increasing the NAD^+ salvage pathway influence SIRT1 enzymatic activity.

Interestingly, it is also possible that SIRT1 functions outside transcriptional control of gene expression. For example, AcetylCoA synthetases are specifically deacetylated by SIRT1 increasing its enzymatic activity [24], these enzymes seem to be very ancient substrates of Sir2 proteins that were originally identified bacteria [25].

Several mechanisms of regulation for SIRT1 have been described. First, nutrient deprivation (fasting or calorie restriction) upregulates SIRT1 protein levels and seems to be independent of its own mRNA induction [4]. In cultured hepatocytes, pyruvate, a metabolite increased in fasting conditions, induces SIRT1 protein levels affecting translation of the protein. In skeletal muscle cells, resveratrol also increases SIRT1 protein levels [26]. How SIRT1 protein levels are regulated and to what extent this is only at the translational level without degradation control is currently unknown. Second, fluctuations in NAD^+ or ratios of NAD^+/NADH as well as nicotinamide concentrations will directly affect SIRT1 enzymatic activity. Thus, redox state of the cell controlled by the activities of different metabolic pathways will change the ratio of NAD^+/NADH. In addition, changes in activities of enzymes that control levels of these metabolites can affect SIRT1 activity. For example, NAD^+ biosynthesis from nicotinamide is catalyzed in two reactions, one of the steps is controlled by the enzyme Nicotinamide phosphoribosyltransferase (Nampt) also known as PBEF or visfatin. Changes in the activity of this enzyme that occur in fasting conditions positively regulate SIRT1 activity. Finally, aside from these direct catalytic regulators, it is possible that endogenous or natural compounds might act similarly to resveratrol to increase SIRT1 activity (Fig. 3).

2. Tissue-specific metabolic functions

2.1. Liver

Liver is a major buffering tissue that ensures nutrient homeostasis in fed and fasting conditions. Metabolic adaptation to fasting requires an important control at the transcriptional level. PGC-1α is induced in the fasted liver to activate gluconeogenic and fatty acid oxidation genes. PGC-1α deficient mice lack this response and display hypoglycemia and hepatic steatosis [27,28]. As part of the starvation response, signaling of the fasting hormones glucagon and glucocorticoids increases transcription of the PGC-1α gene. Glucagon signaling causes translocation and de-phosphorylation of the transcriptional

coactivator TORC2 to the nucleus, where it then binds to and coactivates the CREB transcription factor which is present on the PGC-1α and gluconeogenic promoters [29]. Once PGC-1α is induced it will coactivate FoxO1 and HNF4α on the gluconeogenic genes such as PEPCK and G-6-Pase, increasing transcription of these genes (Fig. 4). The entirety of PGC-1α gluconeogenic activity requires these transcription factors, genetic ablation of HNF4α and FoxO1 completely abolishes PGC-1α's ability to induce gluconeogenesis [30]. The counteregulatory effects of insulin impact this network through activation of Akt which phosphorylates at least three components of this signaling and transcriptional regulatory pathway. Akt directly phosphorylates FoxO1 at three residues and results in translocation to the cytoplasm forming a complex with 14-3-3 proteins [31]. Akt phosphorylates and activates the Ser/Thr kinase SIK2. Activated SIK2 induces TORC2 phosphorylation that is degraded by the 26S proteasome in association with COP1, a substrate receptor for an E3 ligase complex that promoted TORC2 ubiquitination and degradation [29]. PGC-1α is directly phosphorylated by Akt and increases PGC-1α protein degradation [11]. The effects of PGC-1α in hepatic lipid and mitochondrial metabolism are likely to function through the nuclear hormone receptors PPARα, ERRα and HNF4α. Moreover, lipin1a, a gene associated with lipodistrophy interacts with PGC-1α and positively controls fasted genes of fatty acid oxidation [32].

Recently, our laboratory found that in parallel to this hormonal regulation there is a nutrient signaling that involves SIRT1 and PGC-1α. SIRT1 is induced in the fasted liver and interacts and deacetylates PGC-1α to activate gluconeogenic and fatty acid oxidation genes. This nutrient signaling involves increases in pyruvate and NAD^+ levels resulting in elevated SIRT1 protein amounts as well as enzymatic activity. Expression of a PGC-1α acetylation mutant – in which 13 lysines have been mutated to arginines – maintains elevated expression of gluconeogenic genes in the fed state [4,23]. These data imply that PGC-1α undergoes cycles of acetylation and deacetylation during the fed and fasted states that are dependent on SIRT1 activity. Furthermore, it also seems that the functions of SIRT1 might go beyond the fed/fasted response. This is evidenced by the observation that independently of the fed status of mice, hepatic SIRT1 controls systemic and hepatic cholesterol levels likely dependent on increases in LXRα and PGC-1β expression [23].

A current hypothesis poses that increased intracellular lipids and inefficiency to couple β-oxidation of fatty acids to mitochondrial respiration in tissues such as liver and skeletal muscle is a possible cause of insulin resistance and type 2 diabetes. In the liver, this metabolic defect leads to non-alcoholic fatty liver disease. Another main problem in diabetic patients is, although they display hepatic insulin resistance, the constant activation of lipogenesis also contributes to lipid accumulation and metabolic dysfunction. Mice lacking PGC-1α develop fasting hepatic steatosis probably due to lower rates of fatty acid oxidation that might play a causative role to develop insulin resistance [27,28]. Intriguingly, PGC-1α KO mice are more insulin sensitive and resistant to high fat diet that could be related to hyperactivity. Alternatively, profound defects in fatty acid oxidation might be compensated with increases in glucose utilization to maintain energetic status. Moreover, lower rates of fatty acid oxidation and OXPHOS activity can also lead to decreases in rates of glucose synthesis that might improve glucose tolerance and insulin sensitivity [33,34]. Notably, SIRT1 knock-down in the liver causes an increase of hepatic free fatty acids which might also compromise insulin signaling [23]. Consistent with that, it has been recently shown that SIRT1, similar to FoxO1, increases insulin signaling. In the case of SIRT1 part of these effects seems through inhibition of PTB1B [35].

One of the main contributors of hyperglycemia in diabetic patients is increased hepatic glucose production in both fed and fasted states. Knock-down of PGC-1α and SIRT1 in liver lowers blood glucose in normal and db/db mice [36,23]. This reduction correlates with lower hepatic glucose production from pyruvate and increased glucose intolerance. Currently, due to this PGC-1α/SIRT1 dual effect in lipid and glucose metabolism is difficult to predict what it might be the final outcomes of the pharmacological activation of both proteins in the liver in diabetes. It is possible that facilitation of fatty acid

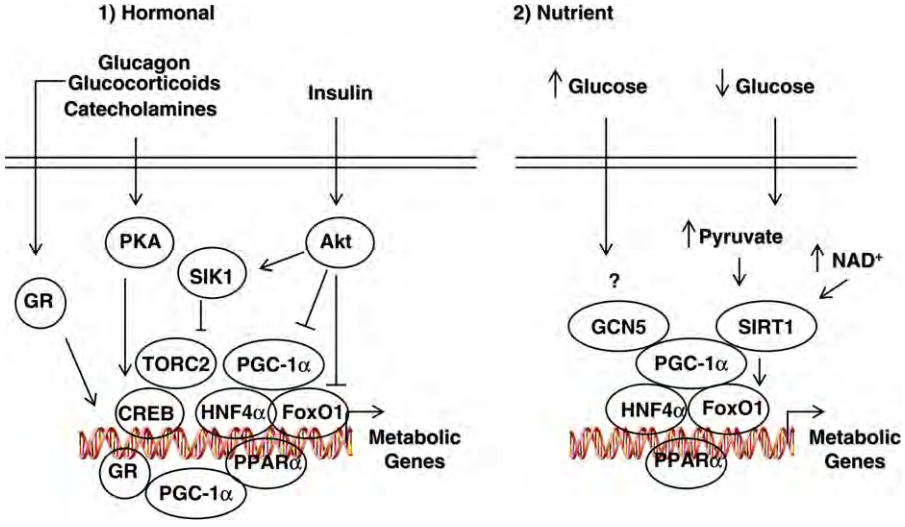

Fig. 4. Hormonal and nutrient regulation of hepatic PGC-1α function. (1) Hormones of the fed (insulin) and fasted (glucagon, glucocorticoids and catecholamines) through different signaling cascades that target transcriptional complexes control expression of metabolic genes such as gluconeogenic enzymes. (2) Nutrient Regulation of PGC-1α through GCN5 Acetyl transferase and SIRT1 deacetylase. See the text for further detail.

oxidation and improvement in insulin sensitivity might be predominant over glucose production. Thus, in normal insulin sensitivity states, this hormone can effectively suppress PGC-1α activation of gluconeogenic genes. This would also be consistent with the increases in insulin sensitivity and normal blood glucose levels observed in mice treated with resveratrol [26].

2.2. Skeletal and cardiac muscle

Skeletal muscle represents a significant percentage of the total body mass and is energetically very active, especially in conditions of increased physical activity induction of mitochondrial oxidative function become essential to adapt and maintain the whole body energy balance. Several signaling pathways are important to activate mitochondrial function in skeletal muscle, for example Ca^{2+}-regulated CAMKIV-calcineurin/NFAT and MEF2 axis, adrenergic and cholinergic signaling and AMPK activation. These signaling/transcription pathways can either induce and/or activate PGC-1α. Thus, skeletal muscle-tissue specific PGC-1α transgenic mice display higher mitochondrial content and a switch to oxidative type-1 fibers [37]. On the contrary, skeletal muscle-specific PGC-1α KO mice have deficient expression of mitochondrial proteins and develop myopathy that might be also related to its role to regulate neuromuscular gene expression [38]. In the heart, mitochondrial mass is also controlled by PGC-1α and is important to maintain cardiac function in response to a variety of stresses [39]. Similar to the liver, PGC-1α increases rates of oxidation of fatty acids that are important in fasting and neonatal conditions.

Little is known about the function of SIRT1 in skeletal muscle. In culture muscle cells SIRT1 seems to be involved in early steps of differentiation by interfering with myogenic factors [40]. Our group has found that in myotubes SIRT1 is required for the switch from glucose to fatty acid utilization that occurs in low nutrient conditions. Skeletal muscle cells trigger this switch in a cell autonomous manner depending on glucose concentrations. One of the mechanisms by which SIRT1 exerts these effects is through PGC-1α deacetylation. Consistent with SIRT1 activation, PGC-1α is deacetylated in fasting and in low glucose conditions to induce a whole battery of genes involved in mitochondrial fatty acid oxidation [7]. Intriguingly, resveratrol mimics the effects of low glucose by targeting SIRT1 and deacetylating PGC-1α. Mice treated with resveratrol have increased mitochondrial function in several tissues and display other metabolic changes that correlate with extension of life span [41]. In addition, resveratrol induces a genetic program in skeletal muscle that is consistent with higher energy expenditure that largely prevents increases in body weight under high fat diet [26]. It would be also of interest to investigate if the beneficial effects of PGC-1α in muscular degeneration are also improved with SIRT1 activation.

In the context of type 2 diabetes, the role of PGC-1α and SIRT1 in the liver seems to be more complex due to the positive effects on both fatty acid oxidation and hepatic glucose production (note that these two processes are activated in nutrient deprivation conditions). However, the effects in skeletal muscle of pharmacological activation of PGC-1α and SIRT1 would be consistent with beneficial results of both proteins. Thus, increases in insulin sensitivity and energy expenditure are observed in mice treated with resveratrol. It is also important to highlight the role that these two proteins might play in exercise and physical activity. These energy-demanding conditions activate AMPK that directly phosphorylates PGC-1α at two residues (Thr177 and Ser53). Notably, increases of target genes of AMPK such as GLUT4 and cytochrome *c* totally depend on PGC-1α [10]. Full activation of PGC-1α in skeletal muscle would allow efficient β-oxidation of fatty acids and coupling to mitochondrial oxidative phosphorylation. In addition, PGC-1α maintains higher number of active mitochondria and OXPHOS proteins that are decreased in type 2 diabetes. As it relates to SIRT1 in skeletal muscle, transgenic SIRT1 mice have increased physical activity and this also correlates with higher insulin sensitivity [42] and increase in physical activity during calorie restriction requires SIRT1 KO [43]. Furthermore, increases in mitochondrial function and fatigue resistance during exercise are also observed in mice treated with resveratrol [26].

The physiological role of SIRT1 in the heart is unclear. Transgenic mice with moderate expression of SIRT1 in heart are more resistant to oxidative stress under pressure overload. However, at high expression of SIRT1 mice develop cardiac hypertrophy. Similar phenotypes have also been observed with transgenic mice expressing PGC-1α in the heart [44]. Interestingly, mice treated with resveratrol under high fat diet have lower heart beat that could be related a decreases in locomotor spontaneous activities [26].

2.3. Brain

The brain constitutes perhaps the most vulnerable tissue to oxidative stress. One of the strong phenotypes observed in the PGC-1α KO are lesions in the striatal region of the brain that controls movement. These mice are hyperactive and display progressive loss of striatal neurons that is reminiscent of Huntington Disease [27]. Although is possible that some of this phenotype might be partially due to a defective mitochondrial gene expression and function, PGC-1α also activates genes that encode enzymes involved in ROS detoxyfication [45]. Thus, ROS induces PGC-1α expression and the lack of PGC-1α correlates with a loss of protection against oxidative stress damage. So far, the data of PGC-1α function in the brain is consistent with a major role in neuroprotection. Interestingly, SIRT1 plays a similar role in oxidative stress function in combination with FoxO transcription factors [21]. Moreover, studies with mice treated with resveratrol and ectopic expression of SIRT1 (that correlated with deacetylation of PGC-1α) in the hippocampus further support the neuroprotective role of SIRT1 in Alzheimer's disease and amyotrophic lateral sclerosis [46]. Together, these studies suggest that PGC-1α and SIRT1 might be potential targets to treat neurodegenerative diseases.

Another important physiological aspect related to the brain is the role in energy balance through hypothalamic control of food intake, energy expenditure and physical activity. Since the hypothalamus controls food intake in fed and fasted conditions through leptin, insulin and nutrient signaling pathways, it is likely that PGC-1α and SIRT1 might play a role in modulating this response. However, PGC-1α KO mice, SIRT1 transgenic and resveratrol treated mice do not present any difference in food intake compared to control animals. Whether other compensatory mechanisms are involved in keeping this function is currently unknown.

A key component in energy balance controlled at the CNS is energy expenditure. The observation that PGC-1α is induced in conditions of elevated energy expenditure, such as cold via adrenergic stimulation, suggests that it might control part of this response. The conclusions about metabolic phenotypes in the PGC-1α and SIRT1 KOs should be taken with caution and would be need to validate with tissue-specific and inducible models. The total PGC-1α KO mice are cold-intolerant indicating defective energy expenditure at lower temperatures [27,28]. However, it seems to become more complex when these mice are fed with high fat diet. The fact that these mice are hyperactive with high spontaneous locomotor activity seems to make them resistant to increases in body weight after high fat diet feeding. With regard to the role of SIRT1 in energy balance, the studies from the whole SIRT1 KO mice also provide little information; these mice are smaller and present several abnormalities. However, transgenic SIRT1 animals have higher physical activity [42] and mice treated with resveratrol display remarkably increase in energy expenditure [26]. In these two mouse models, whether the effects are due, at least in part, to the SIRT1 function in the brain is unknown. Although more data is needed from PGC-1α and SIRT1 tissue-specific KO or transgenic mice, it appears that functions of these two proteins correlate with increases in energy expenditure and physical activity.

2.4. Pancreas

Pancreatic β cells, together with a group of neurons in the hypothalamus, constitute very sensitive cellular sensors for systemic glucose levels. Small increases in glucose levels will stimulate β cells that will secrete insulin to the blood. Little is known about the role of PGC-1α in β cells, although initial studies indicated that overexpression of PGC-1α correlated with a decrease in insulin secretion [47]. However, further studies with PGC-1α KO mice should be performed to confirm this effect. This is important given the relevance of mitochondrial metabolism as well as ATP levels in insulin secretion. On the other hand, it appears that SIRT1 promotes insulin secretion through PPARγ-mediated repression of the uncoupling protein UCP2 and altering intracellular concentrations of ATP [48]. Similar to the skeletal muscle cells, β cells that lack SIRT1 do not fully sense fluctuations of glucose and release normal amounts of insulin. Given the protective effects of both SIRT1 and PGC-1α in other cell types it would be interesting to know whether these proteins might maintain β cell mass under oxidative stress conditions.

2.5. Adipose tissues

Among the first studies in mammalian cells that provided a metabolic functional role for PGC-1α as well as SIRT1 came from the two types of adipose tissues; PGC-1α in brown adipose tissue [2] and SIRT1 in white adipose tissue [19]. Cold is a potent activator of brown adipose tissue thermogenic function that maintains body's temperature. PGC-1α is strongly induced in this situation and lack of PGC-1α correlates with impaired cold tolerance. In addition, cultured brown adipocytes lacking PGC-1α possess a deficient thermogenic program but are still able to differentiate into brown adipocytes [49]. This later function seems to be dependent on a new transcriptional cofactor named PRDM16 [50].

The thermogenic, similar to the gluconeogenic, response largely depends on the cAMP pathway and PGC-1α controls a large set of the genes regulated by this pathway [49]. In addition, the lack of the three β-adrenoreceptors also produces cold intolerance, supporting the necessity of this pathway to maintain corporal temperature. Thermogenic capacity in brown adipose tissue depends on a high number of mitochondria with uncoupling respiration that leads to heat production. This process depends on a very abundant mitochondrial protein termed UCP-1 which is exclusively expressed in brown adipocytes. Expression of this protein entirely depends on β-adrenergic stimulation and mice deficient in UCP-1, similar to PGC-1α and β-adrenoreceptors, are also cold intolerant.

In white adipose tissue PGC-1α is expressed at much lower levels, however in situations of remodeling or activation by β-agonists PGC-1α as well as its mitochondrial target genes are increased. Ectopic expression of PGC-1α in white adipocytes induces a thermogenic program strongly resembling that of brown adipocytes. Another metabolic target of PGC-1α in white adipocytes is glycerol kinase that might promote a futile cycle of triglyceride hydrolysis and fatty acid reesterification [51], especially in combination with lipolytic activators such as catecholamines.

SIRT1 is activated by low nutrient conditions in white adipose tissue and induces systemic fatty acid mobilization. Similar to pancreatic β cells it appears that the mechanism by which SIRT1 induces lipolysis and prevent triglyceride accumulation in white adipocytes is through repression of PPARγ. The mechanism involves SIRT1-dependent recruitment of the transcriptional corepressor NCoR on PPARγ targets such as the abundant aP2 fat-selective gene. Furthermore, similar to skeletal muscle cells, SIRT1 also interferes with the ability of white adipocytes to fully differentiate, most likely by suppressing PPARγ function [19]. However, how SIRT1 controls the adipocyte lipolytic action and whether PPARγ is involved is unknown. Recent transgenic mice in which SIRT1 is overexpressed in different tissues including white adipose tissue display some of the metabolic features of caloric restricted mice such as lower body weight, reduction in glucose and insulin and increased glucose tolerance [42]. The role of SIRT1 in brown adipose tissue and thermogenic function has not been explored. Emerging genomic studies suggest a common cell precursor between brown adipocytes and myotubes that coincides with SIRT1 ability to block myogenesis and promote brown adipocyte differentiation with increased mitochondrial biogenesis [52]. In fact, other genomic studies have correlated expression of SIRT1 with increases in mitochondrial genes that is fully consistent with our own finding [7]. Along the same lines, mice treated with resveratrol show higher PGC-1α deacetylation in brown fat and expression of thermogenic genes including UCP-1 [26]. However, future studies will need to address SIRT1 function in this tissue and the possibility that SIRT1 activators might be used to treat metabolic diseases by increasing uncoupled respiration in brown adipose tissue. In light of some new findings, the possibility of therapeutically targeting brown fat thermogenic activities in humans might take a new twist. It has been thought for a long time that brown adipose tissue depots were only important in humans at birth but not in adults, however, recent observations with PET (positron emission tomography) analysis seems to provide evidence that adult humans have active brown fat depots localized in different areas of the upper body [53].

3. Concluding remarks and implications for metabolic diseases

The abundance of human metabolic diseases gives prime illustration of what occurs when energetic homeostatic pathways become dysregulated. An interesting approach to the problem of these diseases seems to be the study of the beneficial health effects of prolonged calorie restriction and a more detailed analysis of tissue-specific metabolic pathways including how they function and adapt in normal physiology. In this context, we have concentrated on how the PGC-1α and SIRT1 pathways seem to largely function together to promote tissue-specific metabolic adaptation to nutrient fluctuations. It may be of no coincidence that dysregulation of many PGC-1α/SIRT1 targets occurs in, or is even causative to metabolic diseases.

Nutrient signals impinge on the PGC-1α and SIRT1 to control expression of genes encoding for proteins that execute tissue-specific metabolic functions in response to this signal. In most cases, these proteins are metabolic enzymes or additional regulators that will change metabolite fluxes within the cell or tissue as well as impact functions of other tissues. Although the specific nutrient signals which the PGC-1α/SIRT1 pathways respond to remain elusive, it seems very likely that a nutrient signal that fluctuates in normal physiology would serve to synchronize the metabolic networks of the entire body. It is therefore very plausible that the metabolic dysregulation that occurs in disease might be due to the inability of the body to couple normal nutrient/hormonal fluctuation to the appropriate metabolic response. It is important also to mention that by investigating and identifying other genes and signals that are upstream or downstream of the PGC-1α and SIRT1 complex might provide novel insights into the pathology of these diseases.

Although we have concentrated in this review at the connection of the PGC-1α and SIRT1 pathways, other functions of these proteins could be independent of each other. In this regard, future studies of how PGC-1α acetylation functions and what sites are specific for different biological activities will also help to understand the implications of this protein complex in metabolic regulation. Furthermore, we also need to take into consideration other members of the PGC-1 family, for instance PGC-1β also targets mitochondrial genes and induces mitochondrial biogenesis. In fact, knock-down of both PGC-1α and PGC-1β results in a collapse in mitochondrial gene expression [49]. Another issue not covered in this review is the possible effects of PGC-1α and SIRT1 in other metabolic tissues or cells. For example, how it might these proteins contribute to energy balance its function in the thyroid or in macrophages that also have considerable metabolic importance.

A major defect in type 2 diabetes and obesity can be defined as a lack of metabolic stability in which the body, tissue and cells are not responsive to normal nutrient or hormonal fluctuations. A clear example is insulin resistance in type 2 diabetes. However, many pathways are also likely to be defective in these complex diseases. For example, old animals do not respond similarly to exercise as young, the blunted response of of AMPK and PGC-1α in old mice could exacerbate any metabolic complications. In addition, dysregulation of glucose and lipid metabolic pathways will completely change intracellular and systemic metabolite levels which will also further compromise the cells to respond to hormonal and nutrient signals appropriately. Related to this problem, it seems that at the "heart" of type 2 diabetes and obesity are inflammatory responses and chronic oxidative stress that leads to ROS production that might additionally complicate the pathology of these diseases. In this context, PGC-1α, SIRT1 and SIRT1 targets, such as FoxO proteins [21] and p53, control ROS formation and are important to maintain survival [20]. Notably, increases in free radical production have been causatively associated with insulin resistance.

As it relates to the human genetics, several SNPs have been found in PGC-1α that in certain populations are associated with the different aspects of the metabolic syndrome including diabetes, obesity and hypertension. Recently SNPs for SIRT1 have also been identified in humans that were associated with energy expenditure [26]. Finally, further genetic and metabolic studies in different human populations with different susceptibilities to metabolic diseases will provide more information of how these pathways might be therapeutically exploited to treat these diseases.

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Review

Nutrient-dependent regulation of PGC-1α's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5

John E. Dominy Jr., Yoonjin Lee, Zachary Gerhart-Hines, Pere Puigserver *

Dana-Farber Cancer Institute and Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Mammals possess an intricate regulatory system for controlling flux through fuel utilization pathways in response to the dietary availability of particular macronutrients. Under fasting conditions, for instance, mammals initiate a whole body metabolic response that limits glucose utilization and favors fatty acid oxidation. Understanding the underlying mechanisms by which this process occurs will facilitate the development of new treatments for metabolic disorders such as type II diabetes and obesity. One of the recently identified components of the signal transduction pathway involved in metabolic reprogramming is PGC-1α. This transcriptional coactivator is able to coordinate the expression of a wide array of genes involved in glucose and fatty acid metabolism. The nutrient-mediated control of PGC-1α activity is tightly correlated with its acetylation state. In this review, we evaluate how the nutrient regulation of PGC-1α activity squares with the regulation of its acetylation state by the deacetylase Sirt1 and the acetyltransferase GCN5. We also propose an outline of additional experimental directives that will help to shed additional light on this very powerful transcriptional coactivator.

1. Introduction

The availability of food is one of the most capricious environmental variables that mammals have been subjected to over the course of their natural history. At first glance, an unreliable supply of food would seem to pose a major dilemma for homeothermic organisms like mammals, which have an intrinsically high metabolic rate. Compounding this problem is the metabolic peculiarity that although most mammalian tissues are able to use glucose, fatty acids, or amino acids as fuel substrates there are some tissues, such as the central nervous system, retina, red blood cells, and renal medulla, that rely almost exclusively upon an uninterruptable supply of glucose as fuel. In spite of these existential problems, however, mammals have evolved a highly regulated and effective system of fuel utilization pathways that manages to accommodate the tissue-specific requirements of fuels over a wide range of food and macronutrient availability.

When a mammal is deprived of food, its body undergoes both quantitative and qualitative changes in the way in which stored macronutrients are mobilized and metabolized as fuel. These changes constitute a homeostatic response that ultimately aids in the preservation of a constant supply of circulating blood glucose for those tissues that exclusively require it and in the reduction of overall energy expenditure. Part of this response includes switching to the utilization of free fatty acids as the major macronutrient fuel in peripheral tissues such as muscle as well as increasing the rate of glucose synthesis in the liver and kidney. The coordinated initiation and subsequent maintenance of this remarkable change in fuel utilization is a highly complicated process that requires the integration of many hormonal, transcriptional, translational, and allosteric signals. Identification of the regulatory components in this response has been an important research endeavor for the purpose of aiding not just our understanding of basic biology but also in the development of treatments for pathophysiological conditions where glucose homeostasis has clearly gone awry, such as in obesity and type II diabetes. Elucidation of this regulatory pathway may also help to shed light on the mechanism by which caloric restriction is able to retard or prevent the age-related deterioration of mammalian tissues. One exciting branch of research into this problem over the past 10 years has indicated that a major participant in the physiological process of glucose maintenance and fuel utilization is the protein Peroxisome Proliferator-Activated Receptor Gamma-Coactivator-1 α (PGC-1α). In this review, we discuss the role of PGC-1α in regulating the central pathways of metabolism. We also discuss how the acetyl transferase GCN5 and the deacetylase Sirt1 are capable of changing the acetylation state of PGC-1α in response to nutrient state and reciprocally alter the transcriptional coactivating properties of PGC-1α.

2. PGC-1α and its effects on fuel metabolism in fasting

PGC-1α was first identified as a binding partner and co-activator of the transcriptional activity of PPARγ as part of a study to identify transcriptional components of the pathway responsible for the

* Corresponding author.
E-mail address: pere_puigserver@dfci.harvard.edu (P. Puigserver).

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metabolic changes accompanying adaptive thermogenesis [1]. Since that time, however, the list of transcription factors to which PGC-1α is known to bind to and consequently modulate the activity thereof has expanded considerably. This list includes PPARα, glucocorticoid receptor, hepatic nuclear factor-4α, members of the estrogen related receptor (ERR) family, myocyte enhancer factor 2, nuclear respiratory factors 1 and 2, FoxO1, and YY1 [2-6].

The precise mechanism by which PGC-1α is able to enhance the ability of these proteins to initiate gene transcription is not altogether clear, although it is clear that the enhancement of activity is contingent upon the physical interaction of PGC-1α and its cognate transcription factor [7]. Most transcriptional co-activators possess intrinsic enzymatic activity, such as histone acetyltransferase activity, that enables them to modify chromatin to make the genetic locus at which they are located more amenable to transcription. PGC-1α does not appear to possess any enzymatic activity. Its N-terminus, however, is an effective docking site for two well-established histone acetyltransferases, SRC-1 and CBP/p300, which have been shown to be strong enhancers of PGC-1α activity [7]. Although they enhance the activity of PGC-1α, SRC-1 and CBP/p300 do not acetylate PGC-1α. In fact, acetylation of PGC-1α is correlated with a decrease in PGC-1α activity (see below).

It should be noted that although PGC-1α is a co-activator it is not merely a blunt instrument for effecting a wholesale change in the transcriptional activity of its binding partners—its co-activational properties can apparently be selectively tuned for different promoters even when coupled to the same transcription factor. The UCP-1 and aP2 genes, for instance, contain conserved PPARγ binding sites within their promoters but it is only the gene expression of UCP-1 that changes in response to levels of PGC-1α [1]. The mechanism by which this specificity is mediated has not been fully explored.

From a physiological standpoint, the co-activation of a litany of cognate transcription factors by PGC-1α has important metabolic repercussions; collectively, the set of transcription factors to which PGC-1α binds controls the expression of genes involved in gluconeogenesis, glycolysis, lipogenesis, peroxisomal and mitochondrial fatty acid oxidation, and mitochondrial respiration efficiency. Thus, PGC-1α can single handedly coordinate the gene expression of multiple energy pathways. This point is underscored by the PGC-1α knock-out mouse, which shows a reduced respiratory capacity, diminished hepatic TCA cycle flux, reduced rates of hepatic gluconeogenesis and β-oxidation, hepatic steatosis under fasting conditions, and hypoglycemia [8-10].

2.1. PGC-1α function in the liver

In terms of its contribution to the problem of diet-dependent maintenance of energy homeostasis in mammals, there is a body of evidence to suggest that the co-transcriptional activity of PGC-1α is important for the compensatory metabolic responses that occur during food deprivation. The two organs for which this process is best understood are the liver and the skeletal muscle. In the liver during fasting, gluconeogenesis is profoundly upregulated primarily at the level of transcription. A compelling argument can be made for dissecting the upregulation of gluconeogenesis into two temporally distinct phases. In the first and more acute phase (<12–18 h), an increase in the transcription of gluconeogenic enzymes is initiated in part by the activation of cAMP response element binding protein (CREB) and its coactivator CRTC2 (Fig. 1). With prolonged fasting (>12-18 h), however, CRTC2 protein is degraded [11] and its contribution to the fasting transcriptional response is significantly diminished [12]. The initial signal for CRTC2 degradation appears to be deacetylation by Sirt1 [11]. The maintenance of the gluconeogenic response through prolonged fasting is thought to be mediated by PGC-1α and its cognate transcription factors. Indeed, hepatic levels of PGC-1α protein significantly increase under fasting conditions, driven in part by an increase in transcription caused by CRTC2 activation [13] and in part by an increase in the half-life of the protein induced by an attenuation of insulin signaling [14]. The elevated levels of PGC-1α facilitate increased hepatic glucose output by promoting the expression of gluconeogenic genes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase, most likely through the co-activation of HNF4α and FoxO1 [15]. Interestingly, whereas the increase in Sirt1 activity that accompanies prolonged fasting attenuates CRTC2 signaling, it actually improves PGC-1α's ability to increase hepatic gluconeogenesis (see below).

2.2. PGC-1α function in skeletal muscle

In skeletal muscle during fasting, induction of PGC-1α helps to orchestrate a series of metabolic changes that results in muscle tissue using less glucose and more fatty acids for oxidative phosphorylation (Fig. 2). PGC-1α has been shown to increase the expression of the glucose transporters GLUT1 and GLUT4 in muscle tissue [16-18]. In the case of GLUT4, this effect is dependent upon the transcription factor MEF-2c [18]. Although PGC-1α can induce glucose transporter expression, net utilization of glucose by skeletal myocytes is significantly mitigated by an increase in PGC-1α activity. The mechanism by which this occurs involves at least two different processes. The first mechanism is a PGC-1α-induced decrease in the expression of phosphofructokinase and an accompanying diminution in glycolytic flux [16].

The second proposed mechanism involves a PGC-1α-induced increase in the expression of pyruvate dehydrogenase kinase-4 [16,19,20], a negative regulator of pyruvate dehydrogenase, which abates the entry of glucose-derived pyruvate into the TCA cycle. PGC-1α's coactivation of ERRα is responsible for increased PDK4 expression. Higher levels of PGC-1α also permit muscle cells to oxidize more fatty acids. PGC-1α facilitates the delivery of free fatty acids across the cell membrane by increasing the expression of the integral membrane protein CD36, which is capable of binding to long-chain fatty acids and mediates their internalization into the cytosol [16,17]. Transport of free fatty acids from the cytoplasm across the outer mitochondrial membrane is also facilitated by an increase in the expression of the free fatty acid transporter carnitine palmitoyltransferase 1 (CPT-1) [16,20]. The complete oxidation of fatty acids and its coupling to ATP production within the mitochondria are facilitated by an increase in the levels of medium chain acyl CoA dehydrogenase (MCAD), cytochrome oxidases II and IV, isocitrate dehydrogenase 3A, β-ATP synthase, and cytochrome *c* [20].

3. Regulation of PGC-1α co-transcriptional activity: the contribution of acetylation

Given the preponderance of evidence supporting a role for PGC-1α in coordinating the compensatory metabolic responses that accompany food restriction, it is logical to inquire how the biological activity of PGC-1α changes under these circumstances. One conceivable mode of regulation is simply that the absolute concentration of PGC-1α protein is increased under low nutrient conditions and forces, by the principle of mass action, an increase in the activity of its transcription factor binding partners. This is definitely possible within the liver, where the concentration of PGC-1α protein significantly increases during periods of acute fasting [21]. Evaluations of skeletal muscle, on the other hand, have shown that there is no appreciable change in the level of PGC-1α during fasting despite very clear increases in the target genes of PGC-1α [20,22]. Such data strongly suggest the involvement of post-translational regulation of protein activity by covalent modification, allosteric modulation, or changes in intracellular localization/compartmentation.

One proposed mechanistic model for explaining changes in PGC-1α activity under fed and fasting conditions that does not require a

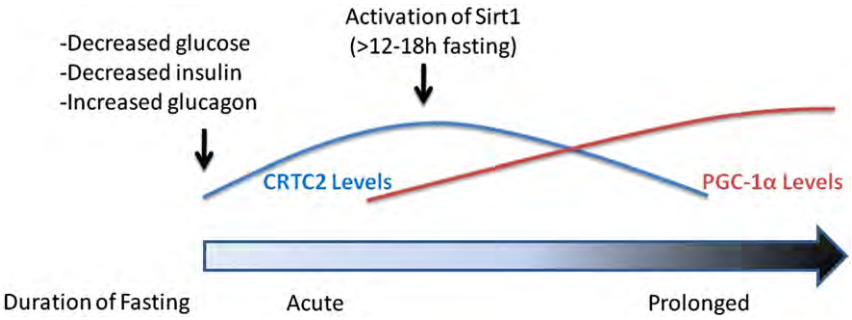

Fig. 1. A proposed theory for the temporal regulation of the two major transcriptional coactivators involved in hepatic gluconeogenesis during fasting. At the onset of fasting, circulating levels of glucose fall. This results in an increase in blood levels of glucagon and a decrease in insulin. This hormonal change produces an increase in both the levels and activity of the transcriptional coactivator CRTC2, which facilitates an increase in the expression of hepatic gluconeogenic genes. CRTC2 also increases the transcription of PGC-1α. With sustained fasting (>12–18 h), Sirt1 becomes activated and deacetylates CRTC2. This event permits the ubiquitination of the protein by the E3 ligase COP1 and targets the enzyme for destruction [70]. The activation of Sirt1 also results in the deacetylation of PGC-1α, which is associated with an increase in the activity of this transcriptional coactivator.

change in total protein is the alteration of activity by acetylation state [21,23]. At least 13 lysine acetylation sites have been identified on PGC-1α [21]. Under an acetylation-based control model, it follows that nutrient availability affects the steady-state level of PGC-1α by altering the relative rates at which the protein is either acetylated by GCN5 or deacetylated by Sirt1 and this is subsequently translated into a change in the co-transcriptional activity of the protein (Fig. 3). Under high nutrient conditions and low intracellular NAD^+ concentrations, PGC-1α is hyperacetylated by GCN5 and located within punctate nuclear bodies along with its transcription factor binding partners. In this state, the PGC-1α complex is effectively transcriptionally inactive. As cells are confronted with low nutrient availability, however, intracellular NAD^+ levels increase and lead to an increase in the rate at which PGC-1α is deacetylated by Sirt1. The change in PGC-1 α acetylation coincides with an increased occupancy of PGC-1α at the promoters of its target genes and an increase in transcriptional activation by remodeling of the local chromatin environment, by proteins such as p300, and greater interaction with general transcriptional machinery, facilitated by proteins such as the TRAP/Mediator complex [24].

Support for this model is readily found in correlative evidence from whole animal studies that have consistently shown, in both liver [21] and skeletal muscle [20,25], that steady-state levels of acetylated PGC-1α are high when animals are fed food and decline significantly when animals are deprived of food. Biochemical and genetic dissection of the association between nutrient supply and PGC-1α acetylation has revealed that the nutrient status of cells can directly alter both the rate at which PGC-1α is acetylated as well as the rate that it is deacetylated. What has been less well defined, however, is how acetylation is physically communicated into a change in the

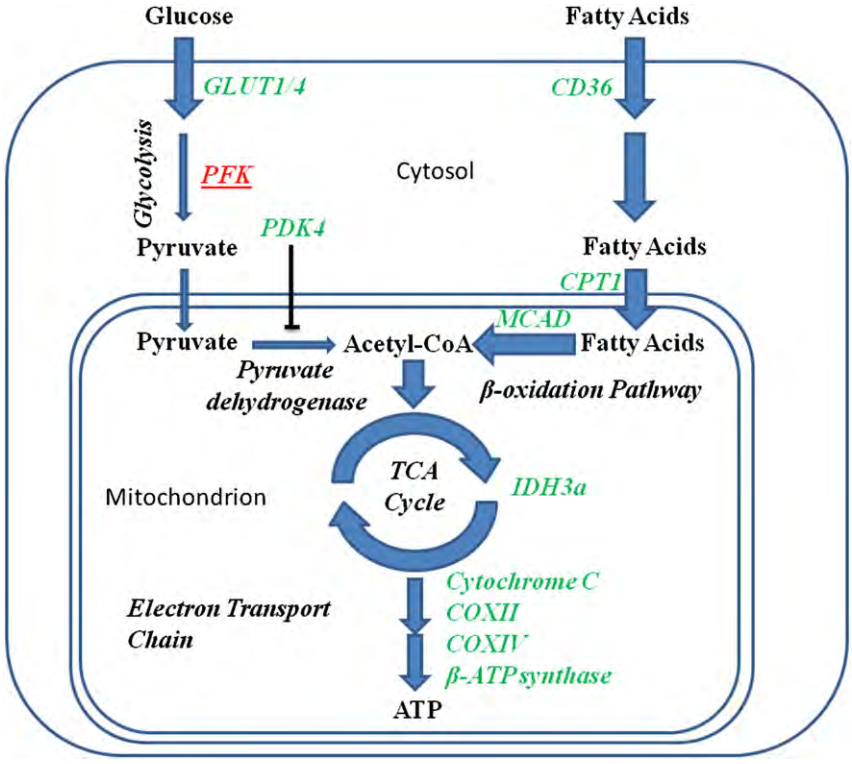

Fig. 2. Conditions that increase PGC-1α activity, such as fasting, increase the oxidation of fatty acids and decrease the oxidation of glucose in skeletal muscle. Genes that are upregulated by increases in PGC-1α activity are indicated in green whereas those that are downregulated are indicated in red and are underlined. PGC-1α increases the rates at which both glucose and fatty acids are transported into myocytes. Both the rate of glycolysis and the rate at which glucose-derived pyruvate is converted into acetyl-CoA, however, are significantly reduced by PGC-1α. Fatty acid transport into mitochondria is significantly enhanced, on the other hand, by PGC-1α. The rate at which fatty acids are oxidized in the mitochondrion is similarly increased, resulting in an increase in the acetyl-CoA pool derived from this macronutrient. Genes involved in TCA cycle flux, electron transport, and proton-coupled ATP synthesis are upregulated by PGC-1α, improving the efficiency with which ATP can be generated from fatty acids.

biological activity of PGC-1α. These aspects and how they pertain to the regulatory model as a whole are discussed below.

3.1. Nutrient supply and the regulation of GCN5: an endogenous acetyltransferase of PGC-1α.

Overexpression experiments with p300 suggested that this enzyme could be the acetyltransferase responsible for acetylating PGC-1α [26]. Subsequent experiments involving the purification of intact PGC-1α protein complexes from cells, however, identified the acetyltransferase General Control Non-repressed Protein 5 (GCN5) as being bound to PGC-1α and showed that this enzyme was a far more efficient acetyltransferase for PGC-1α than p300 *in vivo* [23].

The interaction of GCN5 and PGC-1α is dependent upon the C-terminal bromodomain of GCN5 and a stretch of amino acids located within the first 200 residues of PGC-1α. This region within PGC-1α, incidentally, is also an effective docking site for other acetyltransferases such as SRC-1 and CBP/p300 which strongly induce PGC-1α activity but do not result in significant increases in PGC-1α acetylation [7]. In contrast to these other acetyltransferases, the binding to and acetylation of PGC-1α by GCN5 is associated with a decrease in PGC-1α co-transcriptional activity. Overexpression of GCN5, for example, was able to effectively suppress the induction of PGC-1 α target genes caused by the overexpression of wild-type PGC-1α in cultured hepatoma cells [23]. Along these same lines, knockdown of GCN5 expression in these cells enhanced the effect of wild-type PGC-1α expression. When extended to whole animal models, the hepatic overexpression of GCN5 during fasting resulted in a suppression of the gluconeogenic genes PEPCK and glucose-6-phosphatase as well as in a reduction of *de novo* synthesis of glucose from pyruvate [23]. Although the metabolic effects of GCN5 overexpression *in vivo* are consistent with the PGC-1α acetylation model established in cells, additional experiments in which GCN5 is overexpressed in mice where PGC-1α expression has been knocked down or genetically extirpated are needed to prove that the effects of this acetyltransferase are actually contingent upon PGC-1α.

GCN5 also affects the subcellular localization of PGC-1α [23]. Assessment of ectopically expressed PGC-1α has revealed that most of the intracellular pool of PGC-1α is localized to the nucleus. Within this organelle, PGC-1α resides in a largely homogenously diffuse distribution. In the presence of ectopically overexpressed GCN5, however, the majority of nuclear PGC-1α becomes concentrated into numerous punctate speckles. The exact composition of these speckles has yet to be adequately resolved, although it is known that they co-localize with the nuclear receptor co-repressor RIP140 [23].

The relocalization of PGC-1α to nuclear speckles is dependent upon the acetyltransferase activity of GCN5: a catalytically incompetent mutant of GCN5 does not cause PGC-1α to relocalize to speckles [23]. Despite the fact that PGC-1α relocalization is dependent upon GCN5 activity, it has yet to be directly demonstrated that the PGC-1α in nuclear speckles is actually acetylated. The possibility thus remains that relocalization is a consequence of GCN5 activity that is independent of PGC-1α acetylation. Additional experiments with mutant isoforms of PGC-1α that cannot be acetylated by GCN5 are needed to clarify this point. It is known, however, that relocalization is strictly dependent upon an intact C-terminus of PGC-1α, a region that is not involved in GCN5 binding but does contain a RNA binding domain as well as an arginine/serine (RS)-enriched domain that has been identified in splicing complex proteins [27]. This domain also contains two known acetylation sites [21], raising the possibility that these acetylations are important in the relocalization signaling PGC-1α. From a functional standpoint, the GCN5-induced movement of PGC-1α to nuclear speckles correlates with a significant decrease in the binding of PGC-1α to the promoters of its target genes [23]. This could be interpreted to mean that the reduction in PGC-1α activity

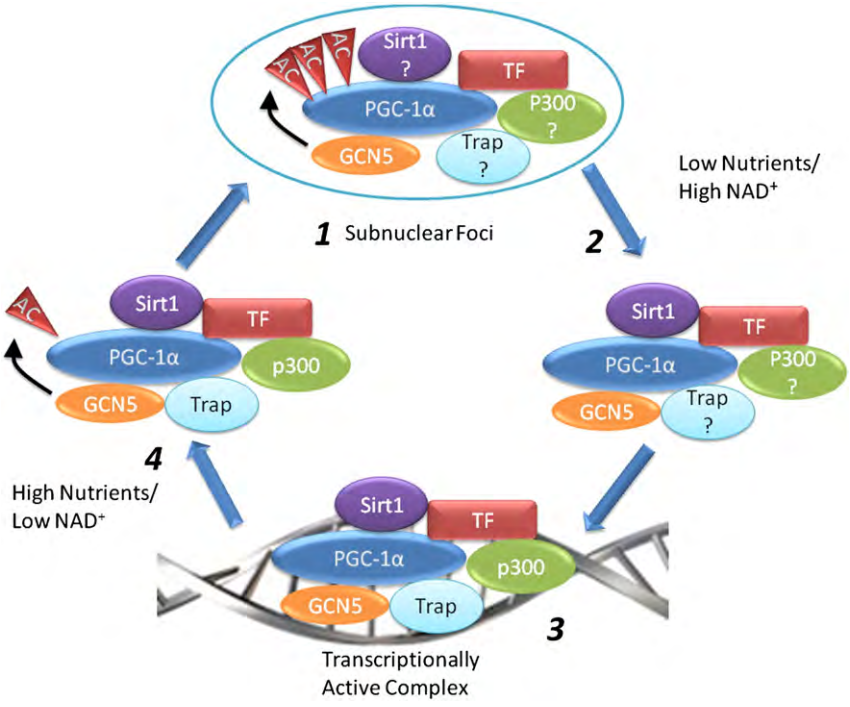

Fig. 3. A model for the regulation of PGC-1α acetylation in response to nutrient availability. When nutrient availability is high and intracellular NAD^+ is low, PGC-1α is acetylated and re-located within punctate nuclear bodies along with its transcription factor partners (TF) (1). As nutrients are exhausted and NAD^+ rises, Sirt1 activity is enhanced and results in the deacetylation of PGC-1α (2). This coincides with a greater occupancy of PGC-1α at its target promoters and an increase in the transcription of the target genes (3). When nutrient levels return to higher levels, the activity of Sirt1 relative to GCN5 declines and PGC-1α is acetylated (4) and sequestered within specific nuclear foci (1). See text for additional details. Abbreviations used: TF, transcription factor.

by GCN5 is operating via a spatial discrimination mechanism whereby PGC-1α is recompartmentalized away from its transcriptional targets and rendered functionally inactive.

There are some data to suggest that macronutrient availability could affect the ability of GCN5 to acetylate PGC-1α through changes in steroid receptor coactivator protein (SRC-3). SRC-3 positively regulates the activity of GCN5 in mammalian cells. SRC-3 has been shown to bind to GCN5 and this interaction is crucial to ability of SRC-3 to coactivate retinoic acid receptor [28]. Knockout of SRC-3 in mice has been shown to significantly reduce the expression of GCN5 in muscle and brown adipose tissue [22]. In SRC-3 knockout mice, PGC-1α acetylation levels were found to be similarly reduced in both muscle and brown adipose tissue. Correlating with the change in PGC-1α acetylation levels, many mitochondrial gene targets of PGC-1α, such as CPT-1, UCP-1 through 3, cytochrome C, and ATP synthase, were significantly elevated relative to wild-type controls [22]. In wild-type mice, both SRC-3 and GCN5 expression positively correlated with nutrient availability. Following a short-term fast (6 h), levels of SRC-3 in gastrocnemius in wild-type animals were found to decline along with the level of GCN5 [22]. Concomitant with the decrease in GCN5, the total acetylation state of PGC-1α fell. When wild-type animals were fed a high-fat diet for 4 weeks, SRC-3 protein in gastrocnemius was elevated as was GCN5 expression. According to the model proposed by the authors, diet composition/nutrient availability directly regulates the level of SRC-3 which subsequently influences the expression of GCN5 which, in turn, alters the acetylation state/co-transcriptional activity of PGC-1α. More detailed molecular analysis of this model, however, has raised additional questions over how SRC-3 is able to alter the expression and/or activity of GCN5. Knockdown of SRC-3 in C2C12 cells significantly reduced the expression of GCN5, reduced the acetylation of PGC-1α, and increased the expression of PGC-1α regulated mitochondrial genes [22]. Overexpression of SRC-3 by itself, however, increased endogenous GCN5 transcription but did not elevate the amount of PGC-1α acetylation in cultured C2C12 myotubes. Only when SRC-3 was overexpressed in conjunction with GCN5 overexpression was there a significant increase in PGC-1α acetylation. The disparity in results between the whole animal and cell culture experiments warrants further pursuit of the mechanism that connects changes in SRC-3 levels with changes in PGC-1α acetylation and target gene expression. Of particular interest would be an evaluation of the effects of SRC-3 on PGC-1α gene targets in cells which have had either GCN5 or PGC-1α knocked down.

Although abundant evidence suggests that GCN5 is able to acetylate PGC-1α and suppress its activity, evidence for the dietary control of this process is somewhat limited. Far more evidence exists, however, in support of the hypothesis that the changes in both acetylation state and co-transcriptional activity of PGC-1α accompanying dietary intake are principally due to fluctuations in the rate at which it is deacetylated by the protein Sirtuin 1.

3.2. Nutrient supply and the regulation of Sirt1: an endogenous deacetylase of PGC-1α

Sirtuin 1 (Sirt1) is a NAD^+-dependent protein deacetylase that has been implicated in a panoply of physiological processes in mammals, including control of lipolytic rates in white adipose tissue [29], modulation of insulin secretion from pancreatic β-cells [30], control of cytoplasmic and mitochondrial acetyl-CoA synthetase activity [31], regulation of the circadian clock [32-34], and regulation of the genetic response to various stressors such as heat shock [35], genotoxicity [36], and hypoxia [37]. To this long list of biological functions, one can also add the regulation of PGC-1α acetylation state. Sirt1 has thus far been the only identified protein capable of binding to PGC-1α and deacetylating it both *in vivo* and *in vitro* [21,26]. Sirt1 binds to a region of PGC-1α that is contained within amino acid residues 200–400 and deacetylates the protein in a NAD^+-dependent manner [21,26].

Alterations in the level of Sirt1 activity are closely associated with changes in the expression of PGC-1α targets in liver and muscle tissue. In hepatoma cultures, knockdown of Sirt1 significantly increased the acetylation of PGC-1α, and reduced both the induction of gluconeogenic genes and the corresponding rate at which glucose was produced following PGC-1α overexpression [21]. In myotube cultures, Sirt1 was found to be bound to the promoters of PGC-1α target genes and occupancy at these sites could be significantly enhanced by Sirt1 overexpression [20]. Overexpression of Sirt1 was also accompanied by a significant decrease in PGC-1α acetylation and an increase in the expression of PGC-1α targets; knockdown, on the other hand, produced a corresponding decrease in the expression of PGC-1α targets. Activation of endogenous Sirt1 activity by resveratrol in cultures of C2C12 myotubes was also able to induce the deacetylation of overexpressed PGC-1α protein and potentiate the effects of PGC-1α on MCAD, ERRα, and cytochrome *c* [38]. Resveratrol, however, was not able to enhance the activity of PGC-1α when all 13 of the known acetylated lysine residues were mutated to an arginine.

Whole animal studies have largely supported the mechanistic associations borne out by the cell culture experiments. In agreement with the cell culture studies, overexpression of Sirt1 in the liver significantly increased the expression of PEPCK and glucose-6-phosphatase in a PGC-1α-dependent manner [39]. Similarly, knockdown of Sirt1 attenuated hepatic glucose output induced by PGC-1α overexpression in a pyruvate tolerance test in mice [39] and reduced both fasting glucose levels and hepatic gluconeogenesis in rats [40]. Pharmacological activation of Sirt1 in animals has also provided corroborating evidence for the models of PGC-1α regulation that were developed in cell culture systems, with the caveat that there is currently controversy over whether these activators are actually directly modulating Sirt1 activity *in vivo* [41,42]. The Sirt1 activator SRT1720 was associated with a metabolic change in mouse skeletal muscle to an oxidative fiber type, a decrease in PGC-1α acetylation, and an increase in the expression of PGC-1α targets involved in fatty acid oxidation [43]. Treatment of animals with resveratrol increased the expression of a much larger number of PGC-1α targets in skeletal muscle [38]. Interestingly, resveratrol administration also appeared to be activating AMP kinase in these animals. Evidence that PGC-1α is phosphorylated by AMP kinase has been shown to increase the biological activity of PGC-1α therefore warrants consideration as an alternate explanation for these findings [25,44].

It should be noted that the findings of two whole animal studies have somewhat contradicted the activational effects of Sirt1 on PGC-1α activity observed in cell culture. In one of these studies [45], transgenic mice overexpressing a single copy of Sirt1 showed significantly reduced glucose output by the liver under fed conditions. Nevertheless, isolated hepatocytes from these mice did show increased PGC-1α activity in the form of elevated expression of gluconeogenic genes. This suggests that the whole animal metabolic phenotype could be due to counter regulatory non-cell autonomous factors such hormones. In the second study [46], electrotransfection of Sirt1 into rat hindlimb skeletal muscle increased total Sirt1 expression but decreased the level of PGC-1α protein. Concomitant with the decrease in PGC-1α protein was a decrease in proteins associated with mitochondrial biogenesis including cytochrome oxidase IV and mitochondrial transcription factor A.

One major issue for forwarding our understanding of the control of steady-state levels of PGC-1α acetylation by Sirt1 is identification of the potential mechanisms by which changes in nutrient availability are translated into changes in Sirt1 activity. Although there is evidence to suggest that post-translational modifications such as phosphorylation [47] and sumoylation [48] can modulate Sirt1 activity as well as some interacting proteins such as AROS [49] and DBC1 [50,51], most proposals for how Sirt1 activity is controlled by nutrient availability *in vivo* involve either changes in the total level of Sirt1 protein or with the availability of NAD^+.

Levels of Sirt1 have been reported to change in response to nutrients. Some investigators have found that Sirt1 transcription is inversely responsive to changes in nutrient load by a mechanism that is dependent upon either the transcription factor Foxo3a [52] or the transcriptional co-repressor CtBp and its binding partner Hypermethylated In Cancer [53]. Others, however, report that there are increases in Sirt1 protein caused by nutrient deprivation in both cultured cells and mice, but are not accompanied by changes in the rates of transcription or in steady-state mRNA levels [21,54]. Among these reports there is disagreement as to whether Sirt1 actually increases in the liver. From this body of work, it is clear that additional studies need to be done to establish the exact mechanism by which Sirt1 protein is controlled by nutriture and if this control is cell autonomous or tissue specific.

Levels of NAD^+ are also known to change in response to nutrient availability. NAD^+ is a necessary co-substrate for the deacetylase activity of sirtuin proteins. The strict requirement of this substrate is one of the two linchpins for the hypothesis that the major regulator of sirtuin proteins is the concentration of NAD^+. The second is that the concentration of NAD^+ is ordinarily at or below the sirtuins' K_m for NAD^+ (100-300 μM) [55,56]. Cell culture experiments in which NAD^+ biosynthesis from nicotinamide was artificially manipulated by varying the expression level of nicotinamide phosphoribosyl transferase (NAMPT)–an enzyme involved in the salvaging of NAD^+ from nicotinamide–have shown that it is possible to produce significant changes in Sirt1 activity by altering NAD^+ levels [57]. Interestingly, an ancillary effect of NAMPT activity is to reduce intracellular nicotinamide levels–a product of the sirtuin deacetylase reaction and an inhibitor of their activity–and raises the possibility that NAMPT increases sirtuin activity not only by increasing the concentration of the co-substrate but also by alleviating product inhibition. It has also been recently shown that nicotinamide monoadeninenucleotide transferase, the other enzyme involved in the NAD^+ salvage pathway positioned immediately downstream of NAMPT, is physically complexed with Sirt1 at the promoters of certain genes and plays a supporting role in allowing Sirt1 to activate gene transcription [58]. In light of these observations, physiological states that result in a perturbation of steady-state NAD^+ levels may very well alter Sirt1 activity and PGC-1α acetylation.

One of the outstanding mysteries in mammalian NAD^+ biology is how changes in dietary intake cause the intracellular NAD^+ concentration of tissues to change. Intracellular concentrations of liver and muscle NAD^+ have been shown to increase in rodents during fasting [21,25,59]. Nevertheless, under these circumstances, it is not clear if cells are responding to a change in a particular macronutrient such as glucose or fatty acids, one of their respective downstream metabolites, or perhaps a hormonal signal. Because cultured cells show an increase in intracellular NAD^+ following the withdrawal of glucose in culture medium [20,21], however, it is possible that tissues *in vivo* are also altering intracellular NAD^+ based on glucose availability. Research with cell culture models has led to the hypothesis that a reduction in glucose availability causes a decrease in the synthesis of ATP and an increase in AMP levels, which results in an activation of AMP-activated protein kinase (AMPK). The activation of AMPK, in turn, produces an increase in the rate of NAD^+ biosynthesis and the resulting elevation in NAD^+ stimulates sirtuin activity. Experimental dissection of the proposed pathway, however, has yielded some conflicting results about how changes in AMPK activity are eventually translated into an increase in NAD^+ biosynthesis. Some researchers have posited that an activation of AMPK causes an increase in the expression of NAMPT and increases the rate at which NAD^+ is synthesized from nicotinamide—indeed, knockdown of NAMPT was able to prevent NAD^+ levels from rising in C2C12 cells cultured in low glucose medium [60]. Another study employing C2C12 cultures, however, has show that low glucose increases both AMPK activity and NAD^+ levels but does so by a mechanism that is independent of changes in NAMPT expression [25]. The significant time lag between the onset of AMPK activation and when changes in intracellular NAD^+ levels were observed in the latter study, however, suggests that some as yet unidentified intermediary transcriptional response may be involved in initiating the change in NAD^+. Although these cell culture studies have formulated an intriguing connection between the activities of AMPK and Sirt1, any generalization of the involvement of AMPK as a proximate sensor for dietary glucose/energy availability and master regulator of Sirt1 activity *in vivo* is tempered by the fact that many tissues in rodents fail to show substantial increases in AMPK activity following short-term fast or long-term caloric restriction [61,62]—dietary conditions known to significantly increase Sirt1 activity. Empirical validation for the applicability of the hypothesis *in vivo* could be obtained by seeing what effect inhibition of AMPK and/or NAMPT has on the ability of fasting/caloric restriction to induce Sirt1 activity and PGC-1α deacetylation in living animals.

4. Overall regulatory model for PGC-1α acetylation

A synthesis of the current data for the regulation of PGC-1α acetylation in response to nutrient availability is presented in Fig. 3. Under conditions of high nutrients/low NAD^+ levels, PGC-1α is heavily acetylated and biologically inactive (***1***). It is sequestered within subnuclear foci and away from its target genes. PGC-1α is physically associated with GCN5 in these bodies and it is this acetyltransferase that maintains the overall acetylation state of PGC-1α. Unpublished data from our lab suggest that acetylated PGC-1α is able to bind to its cognate transcription factors with the same relative affinity as unacetylated PGC-1, and thus we have assumed in this model that they are also complexed with PGC-1α in the subnuclear foci (***1***). We have also presumed that Sirt1 is associated with PGC-1α in these bodies, but its catalytic activity relative to that of GCN5 is insufficient to result in a net deacetylation of PGC-1α (***1***). Additional experiments are needed to verify this as well as the binding state of other accessory proteins such as SRC-1, p300, and the TRAP complex.

An increase in intracellular NAD^+ levels, as would occur following fasting, causes an acute increase in Sirt1 activity and a net deacetylation of PGC-1α protein (***2***). Under conditions of high Sirt1 activity, such as that achieved by Sirt1 overexpression, it is known that the occupancy of PGC-1α at its target genes is enhanced [20]. Because of this, along with unpublished data showing that overexpression of Sirt1 is able to overcome GCN5-induced inhibition of PGC-1 activity, we have assumed that deacetylation somehow releases PGC-1α from the inactive nuclear foci and permits the protein to be directed to its target genes coupled with the aid of a bound transcription factor (***3***). Nevertheless, there have not been any studies to directly confirm that activation of Sirt1 causes a release of PGC-1α from GCN5-induced sequestration in nuclear foci. This is a salient experiment for establishing mechanistic causality in light of a report showing that overexpression of the transcription factor ERRα is able to result in a relocalization of PGC-1α from discrete foci within the nucleus to a more diffuse pattern of distribution [63]. Such a result suggests that there may be more than one mechanism of inducing a change in PGC-1α localization.

Once located at the promoter of a target gene, the PGC-1α-transcription factor complex is able to induce RNA polymerase II (***3***). This is accomplished by the recruitment of a coterie of histone remodeling proteins including p300, Src-1, and the TRAP complex. Again, the binding sequence of these proteins to PGC-1α is not known. It is equally unknown as to whether the binding of these protein factors to PGC-1α is sensitive to acetylation state. This latter point is important given the observation that the docking of PGC-1α to its cognate transcription factors is not strictly sufficient to induce co-transcriptional activity. An intact N-terminal domain is needed to induce transcriptional co-activation [7,64]. Interestingly, several of

the identified acetylation sites of PGC-1a fall within this region and could alter PGC-1α's affinity for specific factors needed for full co-activational activity.

The termination of PGC-1α activity occurs under nutrient replete conditions. When nutrient availability increases, there is an accompanying decrease in NAD^+ levels. As a consequence, the activity of Sirt1 relative to GCN5 falls and PGC-1α becomes acetylated (***4***). This results in the transcriptional inactivation of the PGC-1α protein complex and a relocalization of PGC-1α to punctuate nuclear bodies (***1***).

5. Additional avenues of research

Again, we acknowledge that the regulatory model for PGC-1α acetylation and its relationship to the biological activity of the protein may be far more baroque than the simple model presented here. It is our hope, however, that the model will serve as a useful platform for the design of additional experiments that may shed much needed light on this highly important transcriptional coactivator. Some other interesting issues related to the biology of PGC-1α that we believe are worth pursuing are:

(i). Why are there 13 acetylated residues in PGC-1α? Are these sites differentially regulated in response to various stimuli? Which of these residues are primarily responsible for altering the activity of PGC-1α?

(ii). Is it possible that it acetylated form of PGC-1α possesses an activity that is antagonistic to its deacetylated form? In our proposed model, we have assumed that the acetylated form has no biological activity. It is also possible that the acetylated form of PGC-1α may have a biological activity that is completely divorced from metabolic regulation.

(iii). PGC-1α undergoes other forms of post-translational modifications such as phosphorylation [14,65,66], ubiquitination [67], O-linked β-N-acetylglucosamination [68], and methylation [69]. How do these modifications interact with the acetylation state of PGC-1α and modify the protein's intrinsic activity?

(iv). Why is the C-terminal RNA binding domain of PGC-1α important for the GCN5-induced relocalization of PGC-1α? Does relocalization of PGC-1α affect its RNA-binding properties?

Addressing these issues will help to settle some of the key questions surrounding the role of PGC-1α acetylation in the control of this protein's biological activity. From a much broader perspective, however, the work will no doubt widen our understanding of how environmental cues, such as diet, are able to regulate the activity of mammalian transcriptional machinery. Thorough elucidation of the mechanistic underpinnings behind such external stimulus-transcriptional response pathways will improve our ability to design specific therapeutic interventions to alter the nature of the response for the betterment of disorders such as obesity, type II diabetes, and metabolic syndrome.

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