A p300 and SIRT1 Regulated Acetylation Switch of C/EBPα Controls Mitochondrial Function

Graphical Abstract

Highlights
- p300 acetylates C/EBPα on several lysines
- SIRT1 deacetylates C/EBPα
- Hypoacetylated C/EBPα increases mitochondrial function
- C/EBPα is a mediator of SIRT1-controlled energy homeostasis

Authors
Mohamad A. Zaini, Christine Müller, Tristan V. de Jong, ..., Oliver H. Krämer, Victor Guryev, Cornelis F. Calkhoven

Correspondence
c.f.calkhoven@umcg.nl

In Brief
Zaini et al. show that the transcription factor C/EBPα is acetylated by p300 and deacetylated by the lysine deacetylase SIRT1. Hypoacetylated C/EBPα induces the transcription of mitochondrial genes and results in increased mitochondrial respiration. C/EBPα is a key mediator of SIRT1-controlled adaption of energy homeostasis to changes in nutrient supply.

Data and Software Availability
E-MTAB-6323

Zaini et al., 2018, Cell Reports 22, 497–511
January 9, 2018 © 2017 The Author(s).
https://doi.org/10.1016/j.celrep.2017.12.061
A p300 and SIRT1 Regulated Acetylation Switch of C/EBPα Controls Mitochondrial Function

Mohamad A. Zaini,1,2 Christine Müller,1 Tristan V. de Jong,1 Tobias Ackermann,1 Götz Hartlieben,1 Gertrud Kortman,1 Karl-Heinz Gührs,1 Fabrizia Fusetti,3 Oliver H. Krämer,3 Victor Guryev,1 and Cornelis F. Calkhoven1,4,*

1European Research Institute for the Biology of Ageing (ERIBA), University Medical Center Groningen, University of Groningen, 9700 AD Groningen, the Netherlands
2Leibniz Institute on Aging, Fritz Lipmann Institute, 07745 Jena, Germany
3Department of Biochemistry, Netherlands Proteomics Centre, Groningen Biological Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, the Netherlands
4Institute of Toxicology, University Medical Center Mainz, 55131 Mainz, Germany
5Lead Contact
*Correspondence: c.f.calkhoven@umcg.nl

https://doi.org/10.1016/j.celrep.2017.12.061

SUMMARY

Cellular metabolism is a tightly controlled process in which the cell adapts fluxes through metabolic pathways in response to changes in nutrient supply. Among the transcription factors that regulate gene expression and thereby cause changes in cellular metabolism is the basic leucine-zipper (bZIP) transcription factor CCAAT/enhancer-binding protein alpha (C/EBPα). Protein lysine acetylation is a key post-translational modification (PTM) that integrates cellular metabolic cues with other physiological processes. Here, we show that C/EBPα is acetylated by the lysine acetyl transferase (KAT) p300 and deacetylated by the lysine deacetylase (KDAC) sirtuin1 (SIRT1). SIRT1 is activated in times of energy demand by high levels of nicotinamide adenine dinucleotide (NAD⁺) and controls mitochondrial biogenesis and function. A hypoaecylated mutant of C/EBPα induces the transcription of mitochondrial genes and results in increased mitochondrial respiration. Our study identifies C/EBPα as a key mediator of SIRT1-controlled adaption of energy homeostasis to changes in nutrient supply.

INTRODUCTION

Studies in cell culture and with mouse models have demonstrated a key role for CCAAT/enhancer-binding protein alpha (C/EBPα) in regulating the transcription of metabolic genes. C/EBPα deficiency in mice results in severe metabolic phenotypes, particularly affecting the liver tissue structure and its functions in gluconeogenesis, glycogen synthesis, and bilirubin clearance, and its deficiency affects fat storage in white adipose tissue (WAT) (Wang et al., 1995; Darlington et al., 1995; Croniger et al., 1997; Inoue et al., 2004; Lee et al., 1997; Yang et al., 2005). In addition, C/EBPα and peroxisome proliferator-activated receptor gamma (PPARγ) are key factors in the transcriptional network controlling adipocyte differentiation (Lefterova et al., 2008; Rosen et al., 2002; Siersbaek and Mandrup, 2011), and mutations of phosphorylation sites in regulatory domains of C/EBPα result in dysregulated transcription of genes involved in glucose and lipid metabolism in vivo (Pedersen et al., 2007; Lefterova et al., 2008). Hence, C/EBPα is a key factor for the differentiation and function of hepatocytes and adipocytes and plays an essential role in the regulation of energy homeostasis.

Protein lysine acetylation is a key post-translational modification (PTM) that integrates cellular metabolic cues with other physiological processes, including cell growth and proliferation, circadian rhythm, and energy homeostasis (Menzies et al., 2016; Choudhary et al., 2014; Xiong and Guan, 2012). Acetylation may regulate various functions of the acetylated proteins, including changes in DNA binding, protein stability, enzymatic activity, protein-protein interactions, and subcellular localization. Protein acetylation is a reversible process in which an acetyl group is transferred from an acetyl coenzyme A (acetyl-CoA) to the target lysine residue by lysine acetyl transferases (KATs) and is removed by lysine deacetylases (KDACs). The KATs and KDACs consist of a large group of enzymes originally identified to acetylate histones as part of epigenetic mechanisms. Later also non-histone proteins were identified as KAT targets (Menzies et al., 2016). Sirtuins (class III KDACs) are KDACs that require nicotinamide adenine dinucleotide (NAD⁺) as co-factor for their enzymatic activity and therefore are activated in times of energy demand when NAD⁺ levels are high (high NAD⁺/NADH ratio) (Houtkooper et al., 2012).

Involvement of KATs in C/EBPα-mediated transcription has been reported in the past (Bararia et al., 2008; Erickson et al., 2001; Jurado et al., 2002; Yoshida et al., 2006), but the role C/EBPα protein lysine acetylation in the transcriptional regulation of metabolic genes has not been addressed. Because C/EBPα is a key regulator of metabolism, we hypothesized that reversible acetylation of C/EBPα is decisively involved in regulating metabolic homeostasis. Here we show that C/EBPα is acetylated on lysines K159 and K298 by the KAT p300, which modulates the transcriptional activity of C/EBPα. We show that acetylation of C/EBPα is dependent on glucose availability, and we identify sirtuin1 (SIRT1) as the sole sirtuin that mediates NAD⁺-dependent deacetylation of C/EBPα. A hypoaecylated mutant of C/EBPα induces the expression of genes involved in
Figure 1. Acetylation of C/EBPα by p300 Enhances Its Transactivation Activity
(A) Immunoblot analysis of immunoprecipitated (IP) C/EBPα and total lysates (Input) of Fao cells cultured overnight in either high-glucose (25 mM) or low-glucose (5 mM) medium. Antibody staining as indicated.
(B) Immunoblot analysis of immunoprecipitated (IP) C/EBPα and total lysates (Input) of HEK293T cells ectopically expressing C/EBPα or empty vector (E.V.) control. Antibody staining as indicated.
(C) HEK293T cells were transiently transfected with C/EBP-responsive firefly reporter vector, a Renilla expression vector for normalization, C/EBPα, and/or one of the lysines acetyl transferases (KATs) expressing vector as indicated. Luciferase activity was measured 48 hr later (n = 4).
(D) HEK293T cells were transiently transfected with luciferase C/EBP-responsive firefly reporter vector, Renilla expression vector for normalization, C/EBPα, and increased amounts of either WT p300-HA or ΔKAT:p300-HA (p300 with its lysine acetyl transferase domain deleted) expression vectors. Luciferase activity was measured 48 hr later (n = 4).
(E) Immunoblot analysis of HA-immunoprecipitated (IP) p300-HA and total lysates (Input) of HEK293T cells ectopically expressing C/EBPα and p300-HA or empty vector (E.V.) control. Antibody staining as indicated.

(legend continued on next page)
the function of the mitochondrion and oxidation-reduction processes, which is accompanied by an increase in mitochondrial mass and cellular oxygen consumption rates. Our study shows that reversible acetylation of C/EBPα in response to changed metabolic conditions alters its transcriptional function to adapt metabolic gene expression and plays an important role in SIRT1-controlled cellular metabolic homeostasis.

RESULTS

Acetylation of C/EBPα by p300 Enhances Its Transactivation Activity

The presence of 15 conserved lysines in sequences of vertebrate C/EBPα orthologs suggests that C/EBPα is a potential target for lysine acetylation (Figure S1). Glucose-rich cell culture conditions are known to increase protein-acetylation through increased availability of acetyl-CoA as substrate for KATs to donate an acetyl group to the target lysine (Shi and Tu, 2015). Acetylation of endogenous C/EBPα in lysates from the Fao rat hepatoma cell line was detected using an anti-acetylated lysine (anti-Ac-K) antibody following immunoprecipitation (IP) of C/EBPα under high-glucose (25 mM) conditions, which was reduced under low-glucose (5 mM) conditions (Figure 1A). Acetylation of immunoprecipitated C/EBPα was also detected in HEK293T cells lacking endogenous C/EBPα that were transfected with a C/EBPα expression vector (Figure 1B).

Next we investigated whether co-expression of the four major KATs, p300, P/CAF, GCN5, and Tip60, alters the transcriptional activity of C/EBPα using a luciferase-based reporter solely containing two natural C/EBP-binding sites of the cMGF promoter (Sternbeck et al., 1992). Co-transfection with p300 resulted in an increase in C/EBPα-induced promoter activity in a dose-dependent manner, whereas co-transfection with the other KATs had no significant effect (Figures 1C, 1D, and S2A). To investigate a direct interaction between C/EBPα and p300 as well as three additional major KATs, we co-expressed C/EBPα with p300-HA, P/CAF-FLAG, GCN5-FLAG, and Tip60 in HEK293T cells and performed coimmunoprecipitation (co-IP) experiments using anti-C/EBPα antibodies. C/EBPα co-precipitated with p300, P/CAF, and GCN5, but not Tip60 (Figure S2B), which was confirmed by reciprocal co-IP of the C/EBPα with the same KATs (Figures 1E and S2C). To examine whether the intrinsic KAT function of p300 is involved in C/EBPα acetylation and transactivation potential, we co-expressed C/EBPα with either p300 or p300 with its KAT domain deleted (p300ΔKAT-HA) and analyzed C/EBPα acetylation and p300 binding by C/EBPα coIP. C/EBPα acetylation was abolished by expression of p300ΔKAT-HA (Figure 1F). In addition, the p300-dependent C/EBPα transactivation activity is abrogated by deletion of the p300-KAT (Figure 1D). In addition, p300-mediated acetylation of C/EBPα in HEK293 cells is strongly reduced under low-glucose conditions (5 mM), confirming that protein acetylation is facilitated under conditions of high acetyl-CoA availability (Figure 1G). Moreover, in Fao cells, acetylation of endogenous C/EBPα was abolished by treatment with the p300 inhibitor C646 (Figure 1H). Therefore, we propose that p300 catalyzes the acetylation of C/EBPα and thereby alters its transcriptional function.

Lysine (K) 298 of C/EBPα was recently identified as an acetylation site using the anti-Ac-K298-C/EBPα antibody (Barania et al., 2016). Using this antibody, a co-expression experiment with p300 in HEK293T cells showed that K298 of C/EBPα is also acetylated by p300 (Figure S2D). In addition, both the endogenously expressed C/EBPα isoforms p42 and p30 (Calkhoven et al., 2000) in Fao cells are acetylated at K298, which is dependent on high-glucose conditions (Figure S2E). Changes in nutrient and calorie intake can influence acetylation of regulatory proteins through changes in cellular concentrations of acetyl-CoA and NAD+ (Houtkooper et al., 2012; Verdin and Ott, 2015). To examine C/EBPα acetylation under different metabolic conditions in vivo, we analyzed livers from mice that were subjected to either calorie restriction (CR; 4 weeks) or a high-fat diet (HFD; 20 weeks). By using anti-Ac-K298-C/EBPα, we found a decrease in C/EBPα K298-acetylation in livers of CR mice and an increase of its acetylation in livers of HFD mice (Figures S2F and S2G; shown is the p30-C/EBPα). Taken together, our data show that C/EBPα acetylation changes with nutritional status in vivo.

The IP experiments described above do not reveal to what extent or which of the lysines in C/EBPα are acetylated by p300 beyond K298. To examine the distribution of lysine acetylation, we purified acetylated C/EBPα protein derived from HEK293T cells co-expressing C/EBPα and p300 and examined protein acetylation by mass spectrometric analysis (Figure 2). Of the 15 lysines in C/EBPα, 11 were covered by the analyzed peptides, of which 5 (K159, K250, K273, K275, and K276) were found acetylated and 6 (K92, K169, K280, K304, K313, and K352) not acetylated (Figure 2). Taken together, our analyses suggest that C/EBPα is subject to extensive acetylation mediated by p300 and that acetylation enhances its transactivation activity.

C/EBPα Binds to and Is Deacetylated by SIRT1

Lysine acetylation is a reversible PTM, which implies that specific KDACs may be responsible for C/EBPα deacetylation. The dependence of C/EBPα acetylation on glucose (Figures 1A and 1G) and the fact that C/EBPα and sirtuins both regulate glucose and fatty acid metabolism suggested that the NAD+1-dependent sirtuin deacetylases (SIRTs) could be involved. We examined the potential involvement of the four cytoplasmic KDACs specifically by performing immunoblot analysis of lysates from HFD-fed and CR mice that were immunoprecipitated with the anti-Ac-K298-C/EBPα antibody (Bararia et al., 2016). We found that C/EBPα K298-acetylation in livers of CR mice and total lysates (Input) of Fao cells treated overnight with either DMSO or p300 inhibitor (C646, 10 µM). Antibody staining as indicated.

Statistical differences were analyzed using Student’s t tests. Error bars represent ± SD. ***p < 0.001; NS, not significant.
and nuclear sirtuins, SIRT1, SIRT2, SIRT6, and SIRT7, as well as SIRT3, which is mainly mitochondrial but may have nuclear functions in addition (Houtkooper et al., 2012). The mitochondrial SIRT4 and SIRT5, which can act in both the mitochondria and cytosol (Nishida et al., 2015; Park et al., 2013), were not tested. To examine possible C/EBPα-sirtuin interactions, C/EBPα was co-expressed together with one of the FLAG-tagged sirtuins in HEK293T cells. CoIP using an anti-C/EBPα antibody followed by immunoblotting with an anti-FLAG antibody revealed that only SIRT1 interacts with C/EBPα (Figure 3A). The interaction between C/EBPα and SIRT1 was confirmed by reciprocal coIP using an anti-FLAG antibody (Figure 3B). Next we examined the capacity of SIRT1 to deacetylate C/EBPα. HEK293T cells were co-transfected with C/EBPα and p300 expression plasmids to obtain acetylated C/EBPα in the presence of either SIRT1 or SIRT2 expression plasmids or empty vector control. Following C/EBPα IP, immunoblotting with an anti-HA or anti-Ac-K antibody showed binding to p300 and a high level of C/EBPα acetylation, respectively, which are abrogated by co-expression of SIRT1 (Figure 3C). Co-expression of SIRT2, which does not interact with C/EBPα, has no effect on C/EBPα acetylation (Figure 3C). In addition, the ASEB computer algorithm (http://bioinfo bjmu.edu.cn/ huacj; Wang et al., 2012) for prediction of SIRT1-mediated deacetylation lists all the mass spectrometry-identified lysines and K298 as potential SIRT1 deacetylation sites (Table S1). Furthermore, a progressive increase in expression levels of SIRT1 resulted in a progressive decrease in the acetylation level of C/EBPα (Figure 3D), which is accomplished by a progressive decrease in p300-dependent C/EBPα transactivation potential (Figure 3E). To examine whether C/EBPα deacetylation by SIRT1 is attributed to the enzymatic activity of SIRT1, we set up an in vitro deacetylation assay. Purified FLAG-tagged acetylated C/EBPα was obtained by anti-FLAG-IP from HEK293T cells that were co-transfected with C/EBPα-FLAG and p300 expression plasmids. Purified FLAG-tagged SIRT1 was obtained separately by anti-FLAG-IP from HEK293T cells transfected with a SIRT1-FLAG expression plasmid. The deacetylation reaction assay revealed that SIRT1 efficiently deacetylates C/EBPα in the presence of NAD+ in vitro (Figure 3F). Moreover, the deacetylation of C/EBPα by SIRT1 was inhibited in the presence of the sirtuin inhibitor nicotinamide (NAM). Taken together, our data show that lysine residues in C/EBPα can be deacetylated by SIRT1.

Acetylation of C/EBPα Does Not Alter Its Subcellular Localization or DNA Binding
Lysine acetylation of a transcription factor may serve to alter its transcriptional function, its DNA-binding properties, or its subcellular localization (Choudhary et al., 2014). We first examined whether the presence of either p300 or SIRT1 alters the subcellular localization of C/EBPα. Immunofluorescent staining of C/EBPα in HEK293T cells showed no difference in its nuclear localization between hyperacetylated C/EBPα derived from cells co-expressing p300 or hypoacetylated C/EBPα derived from cells co-expressing SIRT1 (Figures 4A and S3A). To determine whether co-expression of p300 or SIRT1 alters the binding of C/EBPα to a DNA recognition sequence, purified (IP) FLAG-tagged C/EBPα wild-type (WT) was incubated with DNA oligonucleotide probes of either a C/EBPα-consensus sequence or a mutated sequence, and DNA-protein complexes were analyzed in an electrophoretic mobility shift analysis (EMSA). SYBR Green DNA and SYPRO Ruby protein staining revealed that there is no difference in the DNA binding of C/EBPα between cells co-expressing p300 or co-expressing SIRT1 (Figure 4B). No DNA binding was detected with the C/EBPα-mutated binding sites. These data show that acetylation status of C/EBPα does not affect DNA binding in a significant way.

To examine the involvement of acetylation of individual C/EBPα lysines on the transactivation activity of C/EBPα, we generated mutations that mimic either acetylation (lysine [K] to glutamine [Q]) or non-acetylation (lysine [K] to arginine [R]) at the acetylated lysines identified by mass spectrometry, K159, K250, K273, K275, K276, and K278, and the established acetylation site, K298. Figure 4C shows that only the single K159Q or K298Q acetylation-mimicking mutations in C/EBPα result in enhanced C/EBPα transactivation capacity compared with the WT C/EBPα, using the C/EBP-binding site reporter. None of the K-to-R acetylation-preventing mutations altered the reporter activity (Figure 4D).

Next we examined subcellular localization of the dual K159Q/K298Q acetylation-mimicking and K159R/K298R non-acetylation mutants of C/EBPα. Neither mutation affected the subcellular localization (Figure 4E). In addition, the mutations do not affect DNA binding in an EMSA (Figure 4F). Furthermore, binding to the C/EBP-binding site in the reporter was not altered by the lysine mutations as was measured by C/EBPα IP and qRT-PCR quantification of bound DNA (Figures 4G and S2B). Finally, chromatin IP (ChiP) experiments showed that there is no
Figure 3. C/EBPα Binds to and Is Deacetylated by SIRT1

(A) Immunoblot analysis of immunoprecipitated (IP) C/EBPα and total lysates (Input) of HEK293T cells ectopically expressing C/EBPα and one of the FLAG-tagged sirtuins. Antibody staining as indicated.

(B) Immunoblot analysis of FLAG-immunoprecipitated (IP) SIRT1 and total lysates (Input) of HEK293T cells ectopically expressing C/EBPα and SIRT1-FLAG or empty vector (E.V.) control. Antibody staining as indicated.

(C) Immunoblot analysis of immunoprecipitated (IP) C/EBPα and total lysates (Input) of HEK293T cells ectopically expressing C/EBPα and p300-HA, and SIRT1-FLAG or SIRT2-FLAG. Antibody staining as indicated.

(D) Immunoblot analysis of immunoprecipitated (IP) C/EBPα and total lysates (Input) of HEK293T cells ectopically expressing C/EBPα and p300-HA and increased amounts of SIRT1-FLAG. Antibody staining as indicated.

(legend continued on next page)
difference in binding between WT C/EBPα, the K159Q/K298Q C/EBPα mutant, or K159R/K298R C/EBPα mutant to natural C/EBP-binding sites in promoters of the endogenous genes G-CSF and PEPCK1 (Figures 4H and S3B). Therefore we conclude that acetylation of the lysines K159/K298 enhanced C/EBPα transactivation without affecting subcellular localization or DNA binding.

**Acetylation of Lysine 298 of C/EBPα Stimulates Acetylation of Subsequent Lysines**

Next we asked whether prevention of acetylation of K159, K298, or all six lysines by K-to-R mutations affects p300 binding and acetylation or the transactivation potential of C/EBPα. K-to-R mutated C/EBPα mutants were co-expressed with p300 in HEK293T cells, and p300 binding and C/EBPα acetylation were analyzed after C/EBPα IP. Notably, the mutation K298R strongly reduced binding to p300, associated with a strong reduction in C/EBPα acetylation (Figure 5A). The K159R single mutation had no effect on p300 binding and C/EBPα acetylation, although in the double mutant K159/298R, the level of C/EBPα acetylation was further decreased (Figure 5A). As expected, mutation of all six lysines (K159, K250, K273, K275, K276, and K298) in the K6R mutant reduced C/EBPα acetylation by p300 to very low levels. In accordance, the transactivation of the C/EBP reporter is similar for co-expression of WT or K159-R/C/EBPα, decreased for K298R-C/EBPα, and further decreased for K159/298R- and K6R-C/EBPα (Figure 5B). Complete complementary results were obtained with the opposite lysine acetylation-mimicking K-to-Q mutants. The K159Q mutant did not significantly improve binding of C/EBPα to p300 or C/EBPα acetylation, while with the K298Q mutant, p300 binding and C/EBPα acetylation were strongly increased, and there was a further increase for the double mutant K159/298Q (Figure 5C). The K6Q mutation also results in enhanced binding of p300 and a stronger acetylation signal, although the anti-L-Ac antibody does not recognize the KQ mutations. This suggests that in the K6Q mutant, acetylation of other lysines increases, which normally are not efficiently acetylated. Co-expression of the K-to-Q C/EBPα mutants, p300, and the luciferase C/EBP reporter resulted in a gradual increase in reporter activity from K159Q- to K298Q- to K159/298Q- and K6Q-C/EBPα (Figure 5D). Finally, increasing amounts of SIRT1 co-expression did not reduce the transactivation potential through deacetylation of either K159/298Q- or K6Q-C/EBPα (Figure S4). Together, these results suggest that K298 acetylation is a priming acetylation event stimulating the recruitment of p300, acetylation of K159, and further acetylation of C/EBPα.

**C/EBPα Acetylation Status Determines the C/EBPα-Regulated Transcriptome**

To investigate the consequences of C/EBPα acetylation on global C/EBPα-controlled gene transcription, we generated Hepa1-6 mouse hepatoma cell lines with cumulative-inducible expression of WT, K159Q/K298Q-, or K159R/K298R-C/EBPα-FLAG proteins (Figure 6A). Comparative transcriptome analysis identified 110 upregulated transcripts and 122 downregulated transcripts in the hyperacetylation K159R/K298R-C/EBPα mutant versus hyperacetylation K159Q/K298Q-C/EBPα mutant expressing cells (Figure 6B). We considered genes to be differentially regulated between the hypo- and hyperacetylation C/EBPα mutants only if their expression levels were intermediate in the WT C/EBPα-expressing cells. Ten of each up- or downregulation genes were re-analyzed by qRT-PCR, confirming their regulation shown by the transcriptome analysis (Figure 6C). Gene Ontology (GO) analysis using the DAVID database (Huang et al., 2009) revealed that the upregulated transcripts in the K159R/K298R-C/EBPα mutant-expressing cells are enriched for genes in oxidation-reduction processes and mitochondrial biology, while the downregulated transcripts are enriched for glycoprotein genes (Figure 6D; Table S2). Most of the regulated genes have C/EBPα-associated DNA fragments in the ENCODE database (http://genome.ucsc.edu/ENCODE/; Table S2). C/EBPα is closely related to C/EBPβ, and because they bind to the same recognition sequences, C/EBPβ may substitute for C/EBPα, for which data are not available. In the metabolic context, these results suggest that deacetylation of C/EBPα is involved in the SIRT1-controlled increase in mitochondrial biogenesis and function under conditions of low glucose and low energy.

**Hypoacetylated C/EBPα Enhances Mitochondrial Function**

In line with a role of hypoacetylated C/EBPα in mitochondrial regulation, we found that cumate induction of the K159R/K298R-C/EBPα mutant in Hepa1-6 cells that are cultured in acetylation-favoring high-glucose medium results in increased accumulation of MitoTracker fluorescent dye as a measure for mitochondrial mass, compared with the hyperacetylation K159Q/K298Q- or WT C/EBPα (Figures 7A and S5A). In addition, under low-glucose deacetylation-favoring conditions (2.5 mM), WT reaches similar mitochondrial mass compared with hypoa- cytetation K159R/K298R-C/EBPα, while the acetylation-mimicking K159Q/K298Q-C/EBPα fails to increase mitochondrial mass (Figure 7A). The relative mtDNA copy number did not change upon expression of the C/EBPα variants (Figure S5B). To examine whether C/EBPα is required for SIRT1-dependent induction of mitochondrial mass, we stimulated SIRT1 activity by treatment with SIRT1 activator II and compared mitochondrial mass of cells with short hairpin C/EBPα (sh-C/EBPα) knockdown to control short hairpin RNA (shRNA). Treatment with SIRT1 activator II resulted in a clear increase in mitochondrial mass in control cells that was almost completely abrogated in C/EBPα knockdown cells (Figure 7B). Taken together, these data show
that deacetylation of C/EBPα is required for the SIRT1-induced increase in mitochondrial mass.

To investigate whether mitochondrial function is affected by C/EBPα acetylation, we measured using the Seahorse XF extracellular flux analyzer basal oxygen consumption rate (OCR), maximal OCR (treatment with mitochondrial uncoupler 2,4-dinitrophenol [DNP]), and spare respiratory capacity (SRC) as indicators of mitochondrial respiration. In addition, we measured extracellular acidification rate (ECAR) and maximal ECAR (treatment with oligomycin) as measurement of glycolysis. Under high-glucose (25 mM) acetylation-favoring conditions, expression of the hypoacetylation K159/298R-C/EBPα mutant results in an increase in basal OCR, maximal OCR, and SRC (Figures 7C and SSC). This indicates that the hypoacetylated C/EBPα induces mitochondrial respiration. In addition, the hypoacetylation K159/298R-C/EBPα mutant increases basal and maximal ECAR (Figures 7D and SSD).

Under low-glucose (2.5 mM) deacetylation-favoring conditions, expression of WT C/EBPα increased mitochondrial respiration (basal OCR, maximal OCR, and SRC) to similar extents as the hypoacetylation K159/298R-C/EBPα mutant. Expression of the hyperacetylation K159/298Q-C/EBPα mutant did not result in a comparable increase in respiration (Figures 7E and SSE). Induction of the hypoacetylation K159/298R-C/EBPα did not increase ECAR compared with WT C/EBPα, while the K159/298Q-C/EBPα mutant mildly decreased the maximal ECAR (Figures 7F and S5F). These data indicate that induction of respiration by C/EBPα requires its lysine residues either to be available for deacetylation or being mutated to mimic hypoacetylation. To test whether SIRT1 activation is required for the induction of respiration by WT C/EBPα under low-glucose conditions, the cells were treated with the SIRT1 inhibitor Ex-527 (selisistat), which completely inhibited the WT C/EBPα-induced basal OCR, maximal OCR, and SRC under the low-glucose (2.5 mM) deacetylation-favoring condition (Figures 7G and S5G).

Taken together, our data suggest that deacetylation of C/EBPα is part of the SIRT1-controlled increase in mitochondrial biogenesis and function.

**DISCUSSION**

In this study, we demonstrate that C/EBPα is acetylated by p300 and deacetylated by SIRT1 and that the acetylation status of C/EBPα determines its transcriptional functions. By using acetylation-mimicking (KQ) or acetylation-preventing (KR) mutations, our data suggest that acetylation of lysine residue K298 primes for p300-catalyzed acetylation at various additional lysines and that the K159/298Q dual mutation can substitute for maximal acetylation levels. We show that the acetylation status of C/EBPα modified by p300, SIRT1, and K159/298Q mutations or by K159/298R mutations does not alter its cellular localization or DNA binding. Whole coding transcriptome analysis revealed that the hypoacetylation K159/298R-C/EBPα mutant induces transcripts involved in mitochondrial function and oxidation-reduction processes. Accordingly, expression of K159/298R-C/EBPα increases mitochondrial mass and respiration whereas C/EBPα knockdown abrogates the increase in mitochondrial mass induced by SIRT1 activation. Furthermore, inhibition of SIRT1 blunts WT C/EBPα-induced mitochondrial respiration under low-glucose conditions. Our data fit into a model in which C/EBPα functions downstream of SIRT1 to transcriptionally adapt mitochondrial function in response to alterations in the cellular energy and nutrition state. The more subtle increase in ECAR upon K159/298R-C/EBPα induction, suggesting an increase in glycolysis, is observed only under high-glucose conditions. Possibly, the higher metabolic (respiration) rate of the K159/298R-C/EBPα-expressing cells allows more glucose uptake under high-glucose conditions that is constrained by low-glucose availability.

The results of the transcriptome analysis, revealing differential up- or downregulation of distinct endogenous genes by the hypoacetylated K159/298R-C/EBPα mutant versus the hyperacetylated K159/298Q-C/EBPα mutant (Figure 6B), are seemingly contradictory to the results obtained with the naked (non-chromatinized) promoter-reporter assays. The reporter used in our study is activated by WT C/EBPα, and its activation is further increased by co-transfection of p300 (Figure 1D). The reporter is also strongly activated by the transfection of hyperacetylated
K298Q-C/EBPα (Figures 4C and 4D) or K159/298Q-C/EBPα mutants (Figures 5B and 5D), while the effect of the hypoacetylated K298R- and K159/298R-C/EBPα mutants is similar to WT C/EBPα in the absence of p300 and inhibitory in the context of p300 co-transfection. This suggests that acetylation of C/EBPα increases its transcriptional activity, but it also shows that hypoacetylated K298R- and K159/298R-C/EBPα mutants still have a transactivation potential that is similar to WT C/EBPα in the absence of p300. Promoter-reporters have the limitation that the readout is determined by a selected naked DNA fragment; in our case it solely contains two natural C/EBP-binding sites of the cMGF promoter (Sterneck et al., 1992). Therefore, they cannot fully substitute for the more complex regulation of endogenous transcription that is influenced by chromatin modifiers and by other transcription factors that bind at the promoter and enhancer regions. Although we do not know whether the observed changes in the transcriptome are a result of direct promoter regulation through C/EBPα or of an indirect effect, the presence of C/EBP-binding sites in most of the genes speaks in favor of direct regulation (Table S3). Thus, the acetylation state of C/EBPα might discriminate between interaction partners and/or co-factors and thereby affect different endogenous promoters in opposite ways. The finding that upregulated genes in cells expressing the hyperacetylation K159/298Q-C/EBPα mutant fall into different GO categories compared with those induced by the hypoacetylation K159/298R-C/EBPα mutant supports such a scenario.

The C/EBPα acetylation switch involving p300 and SIRT1 is reminiscent of the acetylation of C/EBPα regulated by these same factors (Bartels et al., 2015). C/EBPα is expressed exclusively in myeloid cells, and acetylation of two lysines (K121 and K198) is indispensable for C/EBPα-induced terminal neutrophil differentiation. C/EBPα-K121 is homologous to K159 of C/EBPα, and both are subject to sumoylation, and C/EBPα-K198 is homologos to K276, which we found acetylated in C/EBPα, further supporting the similarities in the acetylation of both proteins. In agreement with our results, p300-mediated acetylation of C/EBPα enhances transactivation of a C/EBPα-binding site containing M-CSFR-promoter reporter, and the acetylation status does not affect cellular localization of C/EBPα. In contrast to our findings obtained with deacetylated C/EBPα, non-acetylated C/EBPα mutations are shown to reduce DNA binding, but DNA binding of WT C/EBPα upon co-transfection with p300 or SIRT1 was not investigated (Bartels et al., 2015).

It has been shown that C/EBPα expression is essential for mitochondrial biogenesis and proper expression of both nuclear and mitochondrial genome-encoded genes in brown fat (Carmona et al., 2002). Our study shows that this function of C/EBPα depends on the hypoacetylated state of C/EBPα, which is provided by the energy-sensing deacetylase SIRT1, suggesting that C/EBPα mediates effects of SIRT1 on mitochondrial function. This is corroborated by the finding that the reduction of glucose concentration can induce mitochondrial respiration in WT C/EBPα-expressing cells but not in cells expressing either the acetylation-mimicking K159/298Q-C/EBPα mutant or the hypoacetylated K159/298R-C/EBPα mutant; while K159/298R

**Figure 5. Acetylation of Lysine 298 of C/EBPα Stimulates Acetylation of Subsequent Lysines**

(A) Immunoblot analysis of immunoprecipitated (IP) C/EBPα and total lysates (Input) of HEK293T cells ectopically expressing WT or one of the KR-C/EBPα mutants C/EBPα and p300-HA. Antibody staining as indicated.

(B) HEK293T cells were transiently transfected with luciferase C/EBP-responsive firefly reporter, Renilla expression vector for normalization, p300-HA, and either WT or one of the KR-C/EBPα mutant expression vectors. Luciferase activity was measured 48 hr later (n = 3).

(C) Immunoblot analysis of immunoprecipitated (IP) C/EBPα and total lysates (Input) of HEK293T cells ectopically expressing WT or one of the KQ-C/EBPα mutants C/EBPα and p300-HA. Antibody staining as indicated.

(D) HEK293T cells were transiently transfected with luciferase C/EBP-responsive firefly reporter, Renilla expression vector for normalization, p300-HA, and either WT or one of the KR-C/EBPα mutant expression vectors. Luciferase activity was measured after 48 hr (n = 3).

Statistical differences were analyzed using Student’s t tests. Error bars represent ±SD. *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant. K6, K159, K250, K273, K275, K276, and K298.
mutant has already increased mitochondrial respiration at high-glucose concentrations compared with WT C/EBPα, the respiration stays at a low level in the K159/298Q mutant-expressing cells.

SIRT1 is known to control mitochondrial biogenesis and gene expression by deacetylating the transcriptional coactivator PPARγ coactivator 1-alpha (PGC1α) (Houtkooper et al., 2012; Rodgers et al., 2005; Gerhart-Hines et al., 2007; Nemoto et al., 2009).

Figure 6. C/EBPα Acetylation Status Determines the C/EBPα-Regulated Transcriptome
(A) Immunoblot analysis of C/EBPα-FLAG and total lysates (Input) of Hepa1–6 cells expressing WT, K159/298Q-, and K159/298R-C/EBPα-FLAG cumate-inducible constructs or empty vector (E.V.) control. Antibody staining as indicated.
(B) Heatmap of 232 differentially expressed genes (DEGs) in cumate-induced Hepa1–6 cells expressing K159/298R-C/EBPα-FLAG compared with the cells expressing K159/298Q-C/EBPα-FLAG as measured by RNA sequencing (RNA-seq). Low expression is shown in cyan, and high expression is in yellow. False discovery rate [FDR] adjusted p value < 0.01, and the medians in the WT condition are located between the medians of K159/298Q and K159/298R. See Table S2 for a complete list of DEGs.
(C) Relative mRNA expression levels (qRT-PCR) of ten upregulated (left) and ten downregulated (right) genes in cumate-induced Hepa1–6 cells expressing K159/298R-C/EBPα-FLAG compared with the cells expressing K159/298Q-C/EBPα-FLAG (n = 3). Corresponding p values are depicted as determined using Student’s t test. Error bars represent ±SD.
(D) Representative functional annotation clusters of upregulated and downregulated genes in the 232 DEGs (Davis analysis adjusted enrichment score > 1.3). See Table S2 for the list of clustered genes.
Figure 7. Hypoacetylated C/EBPα Enhances Mitochondrial Function

(A) Cumate-induced Hepa1–6 cells expressing WT, K159/298Q-, or K159/298R-C/EBPα-FLAG were cultured in either high-glucose (25 mM) or low-glucose (2.5 mM) glucose medium, and mitochondrial mass was measured using MitoTracker fluorescent dye.

(B) Hepa1–6 cells with C/EBPα-KD (shC/EBPα) or control cells (shCTRL) were treated overnight with either DMSO as solvent or SIRT1 activator II. Mitochondrial mass was measured using MitoTracker fluorescent dye. Immunoblots of C/EBPα and β-actin loading control are shown at the right.

(C, E, and G) Basal and maximal OCR and SRC in cumate-induced Hepa1–6 cells expressing WT, K159/298Q-, or K159/298R-C/EBPα-FLAG proteins and cultured in medium with 25 mM glucose (C), 2.5 mM glucose (E), or 2.5 mM glucose (G) and treated with the SIRT1 inhibitor Ex-527 (selisistat) 16 hr before the measurement.
SIRT1 regulates adiponectin gene expression through stimulation of a FOXO1-C/EBPβ transactivation complex (Ciao and Shao, 2006). Here, FOXO1 is thought to be the target and deacetylated by SIRT1, but deacetylation of C/EBPβ was not investigated in this study. By using a hypoacetylation (K159/298R) mutant, we demonstrate that C/EBPβ deacetylation alone is sufficient for stimulating mitochondrial function. Whether deacetylated C/EBPβ induces PGC1α expression (eventually in collaboration with FOXO transcription factors), collaborates with PGC1α in the activation of mitochondrial genes, or acts independently of PGC1α remains to be analyzed in future experiments.

Recently, Bararia et al. (2016) showed that C/EBPβ is acetylated by the KAT GCN5 at lysines K298 and K302 in the DNA-binding domain and K326 in the leucine zipper dimerization domain by using in vitro acetylation of short C/EBPβ peptides and confirmation by mass spectrometry and western blotting using specific antibodies raised against acetylated C/EBPβ. In the latter study, acetylated C/EBPβ was found to be enriched in human myeloid leukemia cell lines and primary acute myeloid leukemia (AML) samples, and the data show that C/EBPβ acetylation results in impaired DNA binding and thus loss of transcriptional activity, resulting in inhibition of C/EBPβ granulopoietic function. We did not observe effects on DNA binding per se between hypo- or hyperacetylated C/EBPβ. These differences may be the result of the different mutations used and the different experimental systems, hematopoietic cells in the study of Bararia et al. (2016) versus HEK293T and liver Hepa1-6 cells in our study. Bararia et al. (2016) showed loss of DNA binding and transactivation activity using dual K298Q/K302Q or triple K298Q/K302Q/K326Q mutants, which all reside in the basic leucine-zipper (bZIP) DNA-binding domain. Importantly, they reported that single acetylation-mimicking mutants of one of the three lysines showed no effect on DNA binding and transactivation. In our mass spectrometry analysis, K298Q, K302Q, and K326Q were not covered. K298 is predicted to be acetylated by p300 and deacetylated by SIRT1 (Table S1) and was identified as a p300 acetylation site by using Ac-K298-specific antibodies. We did not include K302 and K326, because they are not predicted as targets for p300 or SIRT1 (Table S1). Here we examined the dual K159Q/K298Q mutation, of which K159 lies outside the bZIP domain. Because we also do not see any effect on DNA binding with co-transfection of p300 and rather a stimulation of reporter promoter activity, we believe that at least in the experimental systems we use, acetylation of C/EBPβ does not alter DNA binding. Bararia et al. (2016) found that co-transfection of p300 and C/EBPβ resulted in stimulation of a C/EBP-binding site reporter, while co-transfection with GCN5 repressed the reporter. Similar to these results and to other studies (Erickson et al., 2001; Bararia et al., 2016), we also found that co-expression of p300 and C/EBPβ stimulates a C/EBP-dependent promoter reporter, but in our system, GCN5 did not alter the reporter activation in a dose-dependent manner (although GCN5 binds C/EBPβ). Possibly, in different cellular systems, acetylation of C/EBPβ can occur at different lysine residues by different KATs with different outcomes on DNA binding and/or transactivation. Different KAT regulatory pathways, C/EBPβ-interacting proteins, or other PTMs of C/EBPβ might influence this process. Overall, our data are more in agreement with the effects of acetylation and deacetylation of C/EBPβ by p300 and SIRT1 (Bartels et al., 2015), as discussed above.

C/EBPβ is subject of extensive PTMs, including phosphorylation, methylation, sumoylation, and ubiquitination (Leutz et al., 2011; Nerlov, 2008). Sumoylation of C/EBPβ at lysine residue K159 reduces C/EBPβ transactivation of the albumin gene in fetal primary hepatocytes and abrogates the interaction with Brahma-Related Gene-1 (BRG1), resulting in reduced inhibitory effect on cell proliferation (Kim et al., 2002; Sato et al., 2006). Acetylation and sumoylation at K159 are obviously mutually exclusive, and prevention of sumoylation by acetylation could be involved in the observed higher transcriptional efficacy of the K159Q mutant measured with the C/EBPβ-binding site reporter. However, the K159R that similarly prevents sumoylation at this site shows no enhanced activity, suggesting that lysine acetylation modulates the transcriptional activity of C/EBPβ through other mechanisms.

Taken together, our results suggest that C/EBPβ acetylation depends on nutrient (glucose) availability and is negatively controlled by the class III KDAC SIRT1. Our observations that hypoacetylation-mimicking C/EBPβ mutant-expressing cells show increased expression of mitochondrial genes, higher mitochondrial mass, and mitochondrial respiration suggest that C/EBPβ is a critical downstream mediator of SIRT1 mitochondrial function.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**

The pcDNA3-based full-length (p42) rat C/EBPβ and rat C/EBPβ-FLAG have been described earlier (Müller et al., 2010). Cloning details are available upon request. See Supplemental Experimental Procedures for other plasmids used.

**Cell Culture, Transfection, and Immunofluorescence**

All cells were cultured in DMEM plus 10% fetal calf serum (FCS) (Invitrogen) and penicillin/streptomycin at 5% CO2 and 37°C. HEK293T cells were seeded at 2.5 × 10⁶ cell in 10 cm dishes and transfected the next day with 5 μg expression vectors using calcium phosphate. The immunofluorescence staining protocol was described previously (Müller et al., 2010). The primary antibodies used were anti-C/EBPβ (14A: Santa Cruz Biotechnology), anti-FLAG (M2, F3165; Sigma-Aldrich), and anti-HA (MMS-101R; Convace). Secondary antibodies were Alexa Fluor 488 or 568 conjugated (Invitrogen), p300 inhibitor G646 (CAS 328968-36-1; Sigma-Aldrich) was used at final concentration of 10 μM.

(D and F) Basal and maximal ECAR in cumate-induced Hepa1–6 cells expressing WT, K159/298Q-, or K159/298R-C/EBPβ-FLAG proteins and cultured in medium with 25 mM (D) or 2.5 mM (F) glucose.

For all experiments (n = 5), statistical differences were analyzed using Student’s t tests. Error bars represent ± SD. *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.
C/EBP

CoIP was performed as described previously (Müller et al., 2010). Anti-C/EBPα (14AA; Santa Cruz Biotechnology), anti-FLAG (M2, F3165; Sigma-Aldrich), anti-HA (MMS-1011R; Convance), and anti-Tip60 (NB20-20647; Novus Biologicals) were used for precipitation as indicated. To detect the acetylation of C/EBPα in Fao cells, endogenous level, or in transiently transfected HEK293T cells, the cells were treated with the deacetylase inhibitors 1 μM TSA (T8552; Sigma-Aldrich) and 5 mM NAM (4786SU; Sigma-Aldrich) 8 hr before harvesting. The IP lysis buffer and IP wash buffer were supplemented with these inhibitors as well.

Immunoblotting

Western blotting was performed following a general protocol. The following antibodies were used: anti-C/EBPα (14AA), anti-SIRT1 (H-300), anti-α-tubulin (TU-02), anti-p300 (C-20), and anti-P/CAF (H-369) (Santa Cruz Biotechnology); anti-acetyl-Lys (05-515, clone 4G12; Millipore); anti-FLAG (M2, F3165; Sigma-Aldrich); anti-HA (MMS-1011R; Convance); anti-β-actin (clone C4, 691001; MP Biomedicals), anti-Tip60 (NB20-20647; Novus Biologicals), and anti-Ac-K298-C/EBPα (Barania et al., 2016). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham Life Technologies. The bands were visualized by chemiluminescence (ECL; Amersham Life Technologies).

Luciferase Assay

The luciferase construct containing two consensus C/EBPα-binding sites (pM82; lacking the AP-1-binding site) was described previously (Sterneck et al., 1992). For the luciferase assay, 25,000 HEK293T cells per well were seeded in 96-well plates. After 24 hr, cells were cotransfected with the luciferase reporter, Renilla expression vector, and other expression vectors as indicated using FuGENE HD (Promega). After 48 hr, luciferase activity was measured by Dual-Glo Luciferase Assay System (2920; Promega) following the manufacturer’s protocol using a GloMax-Multi Detection System (Promega).

In Vitro Deacetylation

In vitro deacetylation assay was performed as previously described (Li et al., 2008). Acetylated C/EBPα was obtained by co-transfecting HEK293T cells with C/EBPα-FLAG and p300 expression plasmids. Cells were treated with 10 μM TSA and 5 mM NAM 8 hr before harvest. Anti-FLAG M2 beads (M8823; Sigma-Aldrich) were used for precipitation, and 3X-FLAG peptide (F4799; Sigma-Aldrich) was used for elution.

Lentiviral Transduction and Cumate-Inducible System

Hepa1–6 cells were infected with SparQ All-in-One Cumate Switch Vector (QM812B-1; System Bioscience) containing WT rC/EBPα cDNA, K298-C/EBPα, and K159/298R-C/EBPα expression vectors for the C/EBP-binding site IP. ChIP assay was performed with 5 × 10⁶ cells using a Bioruptor (Diagenode) for sonication (details are available on request). ChIP antibodies were against FLAG (M2, F3165; Sigma-Aldrich) and non-specific mouse IgG (Santa Cruz Biotechnology). The fold enrichment was calculated relative to the background detected with non-specific rabbit IgG. For the semi-quantitative PCR, 1/50 (1 μ) of DNA obtained from the ChIP assay was used as template in a PCR with 28 cycles. Primer pairs are listed in Table S3.

Mitochondrial Content and mtDNA Copy Number

Mitochondrial mass was measured using the Mitotracker Red 480 kit following the manufacturer’s protocol (M22425; Thermofisher Scientific). Fluorescence was measured using a GloMax-Multi Detection System (Promega). SIRT1 activator II (CAS 374922-43-7; 566313; Merck) was used at final concentration of 10 μM. Mitochondrial DNA was co-purified with genomic DNA from Hepa1–6 cells using standard protocol, Ct values were determined for cytochrome b gene encoded by mtDNA and β-actin gene encoded by the nuclear DNA, and the relative mtDNA copy number was calculated by normalizing to β-actin gene copy number. Primer pairs are listed in Table S3.

Mice

C57BL/6 male mice were housed individually with a standard 12-hr light/dark cycle at 22°C in a pathogen-free animal facility and were used for all experiments. Numbers of mice used in the separate experiments are given in the figure legends. Single caged mice 3 months of age were fed ad libitum or fed calorie restricted (70% of normal food intake) for 4 weeks. For the other experiment, mice were fed a HFD or normal control diet (Research Diets; product D12492: 60% fat, 20% carbohydrates, 20% protein; control diet D12450B: 10% fat, 70% carbohydrates, 20% protein) for 20 weeks. Mice were sacrificed by isoflurane at the end of each study. All animal experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee.

CoIP

C/EBPα DNA-binding affinity was analyzed using EMSA (EMSA) kit, with SYBR Green and SYPRO Ruby EMSA stain (E33075; Thermo Fisher Scientific), following the manufacturer’s protocol.

Measurement of OCR

OCR and ECARs were determined using a Seahorse XF96 Extracellular Flux analyzer (Seahorse Bioscience). Cumate-induced Hepa1–6 cells (2.5 × 10⁶ per well) were seeded into a 96-well XF cell culture microplate 24 hr prior to the assay (Supplemental Experimental Procedures).

Mass Spectrometry Analysis

HEK293T cells were transiently transfected with C/EBPα and p300-HA expression vectors. C/EBPα was immunoprecipitated using rabbit anti-C/EBPα antibody followed by SDS-PAGE and the proper C/EBPα protein band cut and used for further mass spectrometry (MS) protocol (Supplemental Experimental Procedures).

RNA Sequencing Analysis

Transcriptome analysis was done in triplicates. Hepa1–6 cells treated for 3 days with cumate solution to express WT, K159/298Q-, and K159/298R-C/EBPα proteins were harvested, and the total RNAs were isolated using RNeasy Plus Mini Kit (74136; QIAGEN) according to the manufacturer’s protocol (Supplemental Experimental Procedures).

Real-Time qPCR Analysis

Total RNA was isolated using the RNeasy Kit (QIAGEN). For cDNA synthesis, 1 μg RNA was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using Oligo(dT) primers. qRT-PCR was performed using the LightCycler 480 SYBR Green I Master Mix (Roche). Primer pairs are listed in Table S3.

Chromatin and Reporter C/EBP-Binding Site IP

HEK293T cells were transfected with WT, K159/298Q-, or K159/298R-C/EBPα expression vectors for the ChIP. HEK293T cells were cotransfected with C/EBP-binding site reporter construct and WT, K159/298Q-, or K159/298R-C/EBPα.FLAG expression vectors for the C/EBP-binding site IP. ChIP assay was performed with 5 × 10⁶ cells using a Bioruptor (Diagenode) for sonication (details are available on request). ChIP antibodies were against FLAG (M2, F3165; Sigma-Aldrich) and non-specific mouse IgG (Santa Cruz Biotechnology). The fold enrichment was calculated relative to the background detected with non-specific rabbit IgG. For the semi-quantitative PCR, 1/50 (1 μ) of DNA obtained from the ChIP assay was used as template in a PCR with 28 cycles. Primer pairs are listed in Table S3.

EMSA

HEK293T cells were transfected with expression vectors using the calcium phosphate method. Anti-FLAG M2 beads (M8823; Sigma-Aldrich) were used for precipitating C/EBPα-FLAG, and 3X-FLAG peptide (F4799; Sigma-Aldrich) was used for elution. Purified C/EBPα-FLAG was incubated with double-strand oligodeoxynucleotides containing either C/EBPα consensus binding site or mutated one. The sense and antisense sequences are as follows: C/EBPα consensus: sense 5’-CTA GCA TCT GCA GAT TGC GCA TGC AC-3’; antisense 5’-CTG AGT GCA GAT TGC GCA GAT TGC AGA TG-3’; mutant C/EBPα consensus: sense 5’-CTA GCA TCT GCA GAT TGC GCA TGC AGA TG-3’; antisense 5’-CTG AGT GCA GAT TGC GCA TGC AGA TG-3’. The C/EBPα consensus and mutant sequences are underlined.

Cell Reports 22, 497–511, January 9, 2018 509
Data were analyzed using two-tailed independent-samples Student’s t tests for comparisons between two different groups and are expressed as mean ± SD. The data met the assumptions of this test. Differences were considered to be significant when p < 0.05. The RNA-seq Analysis section in Supplemental Experimental Procedures contains details of statistical methods. No statistical methods were used to determine sample size, and randomization was not used for analyses.

The accession number for the transcriptome RNA sequencing data reported in this paper is ArrayExpress: E-MTAB-6323.

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.061.

We thank Daniel Tenen, SCI Singapore/Harvard Medical School, for providing the anti-Ac-K298-C/EBPα antibody; Tony Kouzarides, Cambridge University, for providing the PCAF-FLAG expression vector; Junjie Chen, University of Zurich, for providing the p300-HA and ΔKAT-p300-HA expression vectors. M.A.Z. and T.A. were supported by the Leibniz Graduate School on Aging and Age-Related Diseases (LGSA; http://www.leibniz-fli.de/career-development/graduates) and the University Medical Center Groningen (UMCG). G.H. was supported by the LGSA and Deutsche Krebshilfe e.V. through a grant (110193) to C.F.C.


The authors declare no competing interests.

Received: June 28, 2017
Revised: October 26, 2017
Accepted: December 15, 2017
Published: January 9, 2018

REFERENCES


