CHAPTER III

ACETYLATION AND DEACETYLATION OF C/EBPβ AT K39 ALTERS ITS ABILITY TO REGULATE TRANSCRIPTION OF ADIPOCYTE GENES

Abstract

The transcription factor CCAAT/Enhancer Binding Protein (C/EBP) β, an early mediator of adipogenesis, contains multiple acetylation sites. Mutation of C/EBPβ at lysine (K) 39, an acetylation site in the transcriptional activation domain, impairs transcription of C/EBPβ target genes in a dominant negative fashion. Here, K39 of C/EBPβ may be deacetylated by HDAC1, and HDAC1 decreases C/EBPβ-mediated transcription, indicating that acetylation of C/EBPβ at K39 is dynamically regulated to mediate gene transcription. Acetylation of C/EBPβ at K39 is detected in mouse adipose tissue and in 3T3-L1 cells undergoing adipocyte conversion. In addition, mutation of K39 in C/EBPβ impairs activation of the genes for C/EBPα and PPARγ, essential mediators of adipogenesis, and adipocyte genes encoding leptin and Glut4. These findings suggest that acetylation of C/EBPβ at K39 is an important and dynamic regulatory event that contributes to the regulation of functionally relevant genes associated with adipogenesis and adipocyte function.

Introduction

CCAAT/enhancer binding protein (C/EBP) β is a B-ZIP transcription factor that is expressed in adipose, hepatic, and immune cells as well as in a variety of other cell types (1-6). C/EBPβ plays a significant role in adipogenesis (7, 8), as well as in the gluconeogenic pathway (9), liver regeneration (10), and hematopoiesis (11). Transcriptional activation by C/EBPβ appears to involve the recruitment of coregulatory complexes harboring acetyl transferase activity. C/EBPβ interacts with the acetyltransferases p300 (12), CBP (13), and GCN5 (14). P300 and CBP bind C/EBPβ through their E1A pocket regions (12, 13) and serve as coactivators to enhance transcription of C/EBPβ target genes (12, 13, 15).

As with other B-ZIP factors, the activity of C/EBPβ is regulated via posttranslational modifications. We (16) and others (14, 17), have recently demonstrated that C/EBPβ is acetylated at multiple residues including lysine (K) 39 in its transcription activation domain (16). Notably, acetylation of C/EBPβ at K39 appears to be critical for its ability to activate the c-*fos* and C/EBPα (16) promoters. Alanine and arginine substitutions at K39 severely compromise the ability of C/EBPβ to activate these promoters (16), whereas replacement by glutamine, which can mimic acetylated lysine, is well tolerated. This suggests that acetylation of C/EBPβ at K39 is a prerequisite for activation of these genes. In addition to K39, C/EBPβ is acetylated at other sites, where it also appears to serve a positive role in transactivation. As in the case of K39, acetylation of C/EBPβ at

K117 contributes to the activation of C/EBPα (16) and acetylation of C/EBPβ increases Cox-2 expression (18). Similarly, combined mutation of the acetylation targets K98/101/102, promoted interaction of C/EBPβ with the repressive histone deacetylase (HDAC) 1 (14). The activities responsible for setting the acetylation state of C/EBPβ remain poorly understood. We have shown that p300/CBP enhance acetylation of C/EBPβ (16); its acetylation at K39 is induced by growth hormone (16) and at K98/101/102 by glucocorticoids (14). The role of deacetylases is even more unclear although a role for HDAC1 has been proposed (17).

The posttranslational mechanisms that regulate C/EBPβ function are likely to play important roles in controlling the biological responses dependent on C/EBPβ. In this regard, the mechanisms by which C/EBPβ contributes to adipogenesis have become of particular interest in the context of the increasing incidence of obesity worldwide. C/EBPβ is induced early in a cascade of transcription factors that drive adipose differentiation. C/EBPβ, in combination with C/EBPδ, initiate the expression of a second set of transcription factors that include C/EBPα and Peroxisome Proliferator-Activated Receptor (PPAR) γ (19). These two factors are master regulators of adipogenesis (7, 8, 20) and execute the adipocyte differentiation program. Mice lacking both C/EBPβ and C/EBPδ have a significant decrease in adipose tissue mass, even with normal expression of C/EBPα and PPARγ, and cells from C/EBPβ-/- mice are severely compromised in their ability to undergo adipose differentiation (21). Thus, C/EBPβ plays a prominent role in adipose differentiation, particularly through the activation of PPARγ (22). Given the important regulatory consequences of C/EBPβ acetylation, we have turned to the well-

established 3T3-L1 cellular model of adipocyte differentiation to probe the mechanism and impact of C/EBPβ acetylation in the transcription of target genes critical to the adipogenic program.

Materials and Methods

Plasmids and Antibodies

The numbering used to designate residues in C/EBPβ is based on the mouse C/EBPβ sequence (Accession # NM009883). The plasmid HA-C/EBPβ, kindly provided by Dr. J. Cardinaux (University of Lasaunne, Switzerland), encodes C/EBPβ (residues 22-296, also known as LAP2) tagged with HA at the N-terminus (13). Mutations were introduced into C/EBPβ at indicated residues using Stratagene QuikChange XL Site Directed Mutagenesis Kit as described previously (16). All mutations were confirmed by sequencing. The mutated forms of C/EBPβ are referred to as: K39R, K39A, K39Q, K117R, K98R, and K215/216R. To generate C-terminally HIS tagged expression vectors, the mouse cDNA sequence (residues 23 to C terminus) from both the WT and K39R C/EBPβ was amplified by PCR using the forward 5' CCGAGGATCCGGCACCATGG-AAGTGGCCAACTTCTACTACGAGCC 3' and reverse 5' GGTCAAGCTTCTAATG-ATGATGGTGGTGATGGTCGACCGCGCAGTGGCCCGCCGA 3' primers, and inserted as a BamHI and HindIII fragments into the same sites of pcDNA3 p42 HIS (31).

Plasmids for full-length, N-terminally Flag-tagged p300 (p300) were prepared, expressed and purified as previously described (32, 33). Plasmids for WT and mutant H1441A HDAC1 were C-terminally Flag-tagged. Mutation of H144 to A (34) was introduced into HDAC1 using Stratagene QuikChange XL Site Directed Mutagenesis Kit as described previously (16). The plasmid for c-*fos*/luciferase (c-*fos*-luc), which contains the mouse c-fos promoter (–379 to +1) upstream of luciferase, was a gift from Dr. W. Wharton (University of S. Florida) and Dr. B. Cochran (Tufts University) (35). The plasmids for C/EBPα-luciferase (C/EBPα-luc) (36), PPARγ-luciferase (PPARγ-luc) (37), leptin-luciferase (leptin-luc) (38), and Glut-4-luciferase (Glut4-luc) (39) were gifts from Drs. O. MacDougald and M.D. Lane (Johns Hopkins University). pΔODLO2$(CAAT)_4$-Luc contains 4 consensus C/EBP sites upstream of a minimal TATA box and the firefly luciferase coding sequence (31). The plasmid for RSV-β-galactosidase (βgal) was provided by Dr. M. Uhler (University of Michigan). Plasmid vector pcDNA3 was purchased from Clontech, pBR322 was a gift from Dr. M. Lomax (University of Michigan), and Rc/RSV was a gift from Dr. J. Lunblad (Oregon Health Sciences University, Portland, OR).

The following antibodies were used: anti-p300 (Santa Cruz), anti-Flag (Sigma), and anti-C/EBPβ (specific for the C-terminus of C/EBPβ, Santa Cruz) were used at dilutions of 1:100 for immunoprecipitations and 1:1000 for immunoblotting. Anti-acetyl-lysine (anti-Ac-K, monoclonal antibody 4G12 which detects acetylated lysines on histones and p53, Upstate, cat #05-515) was used at a dilution of 1:500 for immunoblotting. An antibody against the C-terminus of PPARγ (anti-PPARγ), an antibody against the C-terminus of C/EBPα (anti-C/EBPα), anti-HA (Covance), anti-HDAC1 (Santa Cruz), and

anti-tubulin (Santa Cruz), were used at a dilution of 1:1000 for immunoblotting. Normal rabbit IgG (Santa Cruz) was used as a control for immunoprecipitations.

Cell Culture

293T cells were provided by Dr. M. Lazar (University of Pennsylvania), and were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 8% calf serum (Invitrogen) in an environment of 5% CO_2/95% air at 37°C. 3T3-LI cells, provided by Dr. O. MacDougald, were maintained in an environment of 10% CO_2/90% air at 37°C, and were differentiated into adipocytes as previously described (40, 41). Briefly, two days after cells reached confluence (day 0), they were incubated with DMEM supplemented with 10% fetal bovine serum, dexamethasone (0.25μM), isomethylbutylxanthine (0.5mM) and insulin (10μg/ml). Two days later (day 2), cells were incubated with DMEM supplemented with 10% fetal bovine serum and insulin. Two days later (day 4), and every other day after that, cells were incubated with DMEM supplemented only with 10% fetal bovine serum. Chinese Hamster Ovary cells expressing rat growth hormone receptor containing the N-terminal half of the cytoplasmic domain (referred to as CHO-GHR cells) were provided by Dr. G. Norstedt (Karolinska Institute, Stockholm, Sweden) and Dr. N. Billestrup (Steno Diabetes Center, Copenhagen, Denmark) (42). They were maintained in Ham's F12 Medium (Gibco/Invitrogen) containing 8% fetal bovine serum and 0.5mg/ml Geneticin (Gibco/Invitrogen) in 5%CO_2/95% air at 37°C. Prior to experiments, CHO-GHR cells were incubated overnight in medium containing 1% bovine serum albumin (BSA, CRG7;

Serological Corp) instead of serum. All media were supplemented with 1mM L-glutamine, 100units/ml penicillin, 100μg/ml streptomycin, and 0.25μg/ml amphotericin. Calcium phosphate transfections were performed as previously described (43), except 50mM HEPES-buffered saline was used instead of BES-buffered saline.

Immunoblotting

293T cells were lysed using Lysis Buffer (420 mM NaCl, 20mM Hepes (pH 7.9), 1mM EDTA, 1mM EGTA, 20% glycerol), supplemented with Inhibitor Cocktail (150mM sodium pyrophosphate, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 10μg/ml aprotinin and leupeptin, 1uM Trichostatin A (TSA) and 50nM nicotinamide (NAM)). Samples were homogenized (50X) using a dounce homogenizer with a tight pestle, centrifuged at 13,000rpm for 10 min at 4°C, and supernatants were collected. 3T3-L1 cells were lysed in SDS lysis buffer (50mM Tris-HCl, 1% SDS, 10mM EDTA, 1mM EGTA, 0.2% Triton X-100), supplemented with Inhibitor Cocktail. An aliquot of lysate was used for immunoblotting. For 3T3-L1 cells, the remaining lysates were diluted and precleared using protein A agarose beads (A beads, RepliGen). Endogenous C/EBPβ was immunoprecipitated using anti-C/EBPβ antibody overnight at 4°C, and immunoprecipitates were collected on beads for 1 hr. Beads were washed three times in Acetylase Buffer (10mM Tris, pH 7.6, 150mM NaCl, 1mM EDTA, and 5% glycerol, 22 mg/ml sodium butyrate (Sigma), 3 mg/ml dithiothreitol (Invitrogen), 1uM TSA, and 50nM NAM). SDS protein dye (50mM Tris, 1% SDS, 0.001% Bromophenol blue, 10% glycerol, 10% β-mercapto-ethanol) was then added and samples were boiled,

separated by SDS-PAGE, and immunoblotted as previously described (44). Bands on immunoblots were visualized and quantified using IRDye 700-coupled anti-mouse IgG (1:10,000) or IRDye 800-coupled anti-rabbit IgG (1:10,000) on an Odyssey infrared scanning system (LI-COR, Inc., Lincoln, NE) as previously described (15). Molecular weight was estimated using Kaleidoscope protein molecular weight standard (Biorad) or Magic Mark XP Western Standard (Invitrogen).

For analysis of acetylation and deacetylation, 293T cells (100mm plates) were transfected with plasmids for WT HA-C/EBPβ or K39R HA-C/EBPβ (4μg), p300 (2μg), the indicated HDAC1 (8μg), or vector. Lysates were collected for immunoblotting as described, separated by SDS-PAGE, and immunoblotted with anti-Ac-K, anti-C/EBPβ, anti-p300, or anti-HDAC1. To analyze the interaction of C/EBPβ with p300, plasmids for WT or mutated C/EBPβ were expressed with p300-Flag or pBR322 vector. 293T cells were lysed using RIPA Buffer (0.5% SDS, 50mM Tris-HCl (pH 7.5), 150mM NaCl, 2mM EGTA), supplemented with Inhibitor Cocktail. p300 was immunoprecipitated using anti-p300 or normal rabbit IgG as control. Samples were separated by SDS-PAGE and immunoblotted with anti-p300 or anti-C/EBPβ. To analyze the interaction of C/EBPβ with HDAC1, plasmids for C/EBPβ were expressed with HDAC1-Flag or Rc/RSV vector. 293T cells were lysed using RIPA Buffer, supplemented with 150mM sodium pyrophosphate, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, and 10μg/ml aprotinin and leupeptin. HDAC1 was immunoprecipitated using anti-Flag or IgG as control. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-Flag (HDAC1) or anti-C/EBPβ.

For in vivo examination of endogenous C/EBPβ, adipose tissue from mixed background WT mice (kindly provided by L. Lopez-Diaz and Dr. L. Samuelson, University of Michigan) was homogenized (100X) using a dounce homogenizer with a tight pestle in Tissue Lysis Buffer (10mM Tris-HCl pH 7.5, 5mM EDTA, 150mM NaCl, 10% glycerol, 0.5%NP-40) supplemented with Inhibitor Cocktail. Lysates were centrifuged at 13,000rpm for 20 min at 4°C, and fat-free supernatants were collected for immunoprecipitation using anti-C/EBPβ, or normal rabbit IgG as control. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-Ac-K or anti-C/EBPβ as described.

Transcription Assays

Plasmids for WT HA-C/EBPβ or HA-C/EBPβ mutated at various residues (each 400 ng/35mm well, unless indicated otherwise) were coexpressed with reporter plasmids c-*fos*-luc, C/EBPα-luc, PPARγ-luc, leptin-luc, or Glut4-luc as indicated (each 400ng/well) in CHO-GHR cells, a reliable system to assess reporter gene activation. The RSV-β-galactosidase (βgal) plasmid (50ng/well) was included to normalize for transfection efficiency. pcDNA3, pBR322, and Rc/RSV vectors were used to normalize the total amount of C/EBPβ, p300 or HDAC1, respectively. 24 hr after transfection, cells were deprived of serum overnight and lysed to assess luciferase and βgal expression, as previously described (45). Luciferase activity was determined using an Opticomp Luminometer. Transcriptional activity is expressed as the ratio of luciferase to β-galactosidase activities. (normalized luciferase activity). Coactivation by p300 was

assessed in cells transfected with plasmids for c-*fos*-luc, WT HA-C/EBPβ or HA-C/EBPβ mutated at indicated residues (10ng/35mm well), in the absence or presence of p300 (0.5μg). To assess the effect of HDAC1 on C/EBPβ-mediated transcription, increasing amounts of plasmids encoding HDAC1 (50, 100, or 200 ng/well) were cotransfected with c-*fos*-luc and C/EBPβ expression vectors. The data in Fig. 1A were obtained by transfection and assay of 293T cells in a 96 well format exactly as described (31) except that the total amount of transfected DNA was 160ng. Each condition was analyzed in triplicate for each experiment. Statistical analysis of results from replicate, independent experiments was performed using 1-way (promoter activation) or 2-way (coactivation) ANOVA and Bonferroni's Multiple Comparison Test (Prism version 3). Statistical significance is indicated in figures by asterisks and brackets. Expression of C/EBPβ in lysates used for luciferase assays was assessed by immunoblotting.

Results

The acetylation state of C/EBPβ is regulated by p300 and HDAC1

C/EBPβ is acetylated at K39, and mutation of K39 to arginine (K39R) severely compromises C/EBPβ-mediated transcription of target genes (16). To investigate the mechanism by which the K39R substitution prevents C/EBPβ-mediated gene expression, we first examined whether coexpression of this mutant can antagonize WT C/EBPβ driven transcription. Interestingly, the K39R mutant can exert a dominant-negative effect since coexpression of increasing amounts of the K39R mutant together with a fixed

amount of WT C/EBPβ led to a dose-dependent inhibition of activity from either a reporter bearing consensus C/EBP sites (Fig 3.1A) or from the C/EBPα promoter (Fig 3.1B).

To understand how the K39R mutant antagonizes the WT activity, we explored multiple scenarios. One possibility is that the mutation leads to mislocalization and exclusion from the nucleus. WT C/EBPβ would then be mislocalized through heterodimerization with the mutant protein. Cellular fractionation studies however, revealed that mutations at K39 do not appear to alter the nuclear/cytoplasmic distribution of C/EBPβ (data not shown). The possibility that the K39R mutation renders C/EBPβ incapable of occupying its target sites is also unlikely since we can readily detect protein/DNA complexes through *in vitro* electrophoretic mobility shift assays as well as at the endogenous *c-fos* promoter by chromatin immunoprecipitation (data not shown). These data therefore suggest some defect downstream of DNA binding that could involve alterations in coactivator function. Cofactors such as p300, which has been shown to enhance the activity of WT C/EBPβ (12, 15), may fail to activate the K39R C/EBPβ mutant. In this view, the K39R mutant may block the WT protein by competing for promoter occupancy, leading to complexes refractory to p300 coactivation. C-*fos* reporter activity driven by WT C/EBPβ is enhanced by coexpression of p300 (Fig 3.2A). In contrast, p300 fails to increase the c-*fos* reporter activity in the case of the K39R mutant. Notably, this effect is specific to K39 since mutation of other lysines known to be acetylated within C/EBPβ (K98 and K215/216) yield complexes that can still be coactivated by p300 (Fig 3.2A, right). The resistance of K39R to p300 coactivation could be due to direct effects on their interaction. Coimmunoprecipitation, (Fig 3.2B) as

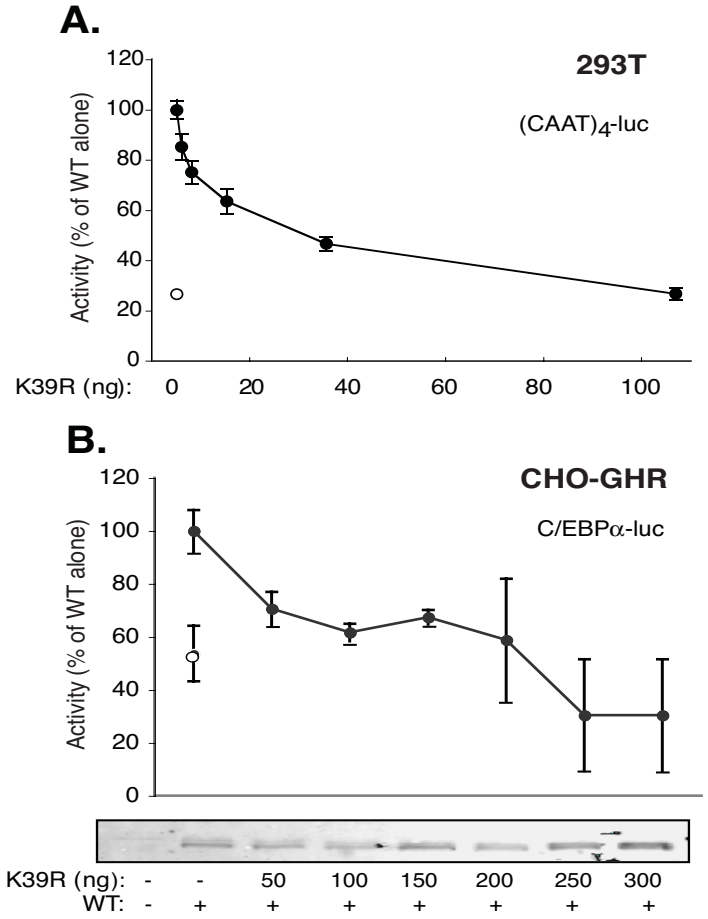

Fig 3.1. Expression of the acetylation site mutant K39R C/EBPβ impairs the transcriptional activity of its WT counterpart. **A**, 293T cells were cotransfected with plasmids $(CAAT)_4$-luc (30ng), βgal (10ng), and the indicated amounts of expression vector for K39R C/EBPβ HIS in the absence (open circle) or presence (closed circles) of plasmid encoding WT C/EBPβ HIS (10 ng). WT C/EBPβ driven activity was 5.16 ± 0.86. **B**, Plasmids for WT HA-C/EBPβ, with increasing amounts of K39R C/EBPβ (closed circles), or pcDNA3 vector (open circle) were cotransfected with C/EBPα-luc in CHO-GHR cells. Data represent the mean (±) of three independent experiments performed in triplicate or quadruplicate and are expressed as % of the normalized luciferase activity for WT alone. Expression of WT and K39R C/EBPβ in CHO-GHR cells is shown in the lower panel.

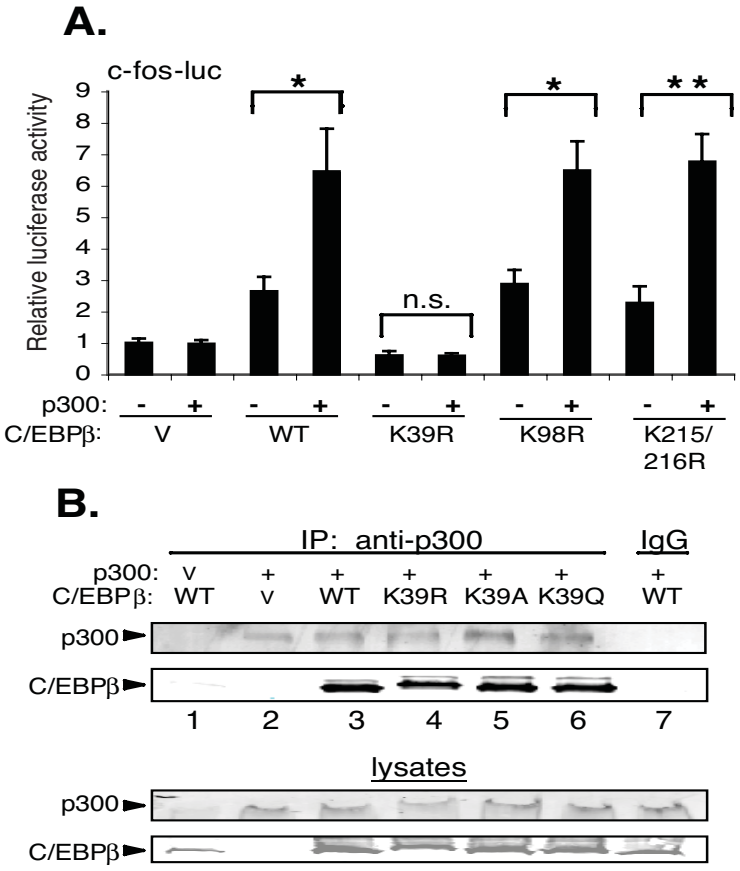

Fig 3.2. The transcriptional impairment of K39R C/EBPβ is independent of altered association with p300. A, CHO-GHR cells were transfected with plasmids for c-*fos*-luc and either WT C/EBPβ or C/EBPβ mutated at the indicated lysines, in the absence (-) or presence (+) of an expression vector for p300. Bars represent mean ± SEM, and transcriptional activity is expressed relative to control, which was set to 1 (n=3 independent experiments). Asterisks represent significant changes for the indicated comparisons (* $p<0.05$; ** $p<0.01$). **B**, Plasmids for WT or mutated HA-C/EBPβ, or pcDNA3 vector (V) were cotransfected with (+) or without (V) p300-Flag in 293T cells. Samples were immunoprecipitated with anti-p300 (top panels) and probed by immunoblotting with anti-p300 (upper panel) or anti-HA (second panel) antibodies. Lysates (bottom panels) were immunoblotted with anti-p300 (upper) or anti-C/EBPβ (lower) antibodies. In the absence of exogenous p300, WT C/EBPβ was not detectable in p300 immunoprecipitates (lane 1). Neither p300 nor C/EBPβ was detected when IgG was used instead of anti-p300 for immunoprecipitation (lane 5). Similar results were obtained in two other experiments.

well as *in vitro* GST-pulldown (data not shown) assays however, indicate that p300 interacts with both WT and K39 mutants, including K39R C/EBPβ. Taken together, these data are consistent with a model where an acetylatable lysine residue at position 39 is critical for C/EBPβ activity and that disruption of acetylation leads to complexes that are resistant to coactivation.

One possible mechanism of regulating the acetylation state of K39 is by deacetylation. As reported previously (14), we find that C/EBPβ interacts with HDAC1 (Fig 3.3A). Whether HDAC1 regulates the acetylation status of K39 C/EBPβ is unresolved however. Using a c-*fos* reporter assay, we show that expression of HDAC1, but not its deacetylation deficient mutant (H141A), represses C/EBPβ-mediated c-*fos* reporter activity (Fig 3.3B). By taking advantage of an anti-acetyl-lysine antibody that recognizes acetylated K39 C/EBPβ (16), we have examined the influence of HDAC1 on K39 C/EBPβ acetylation. In a transfection assay using 293T cells, coexpression of p300 enhances C/EBPβ acetylation (Fig 3.3C lanes 1 and 2) (16). Notably, further coexpression of HDAC1 (Fig 3.3C, lane 3), but not its deacetylation deficient mutant (lane 4), reduced K39 C/EBPβ acetylation. These results suggest that HDAC1 decreases C/EBPβ-mediated transcriptional activation, and that acetylation of C/EBPβ is dynamically regulated by both p300 and HDAC1.

The acetylation of C/EBPβ at K39 modulates transcription of adipocyte genes

C/EBPβ plays a critical role during adipogenesis by setting in motion a cascading program of gene expression. Early in differentiation, C/EBPβ induces the expression of

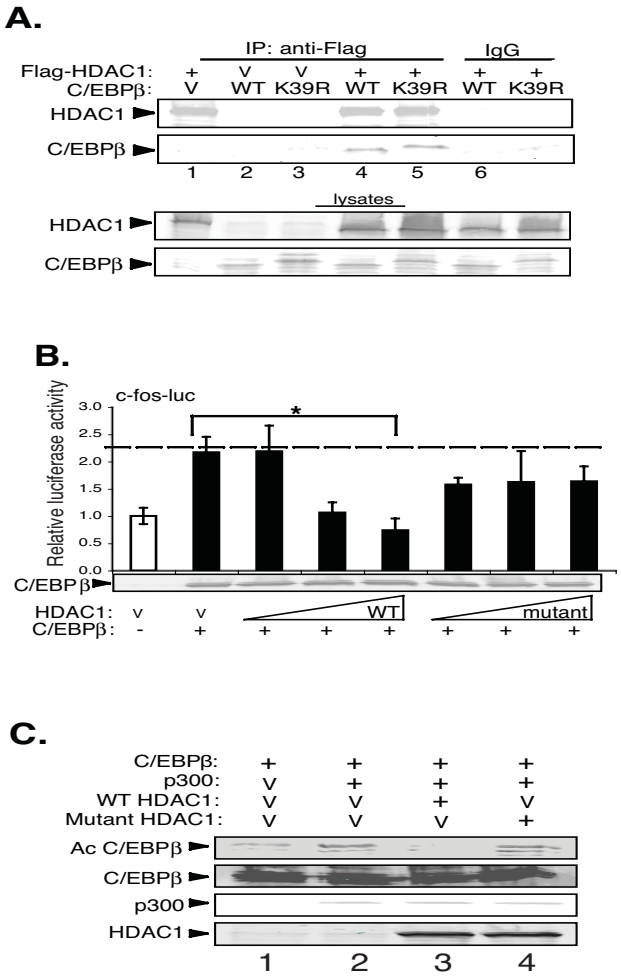

Fig 3.3. **HDAC1 deacetylates C/EBPβ and impairs C/EBPβ-mediated transcription.** **A**, Plasmids for WT C/EBPβ or K39R C/EBPβ were cotransfected with an expression vector for HDAC1 or empty RC/RSV vector (V) in 293T cells. HDAC1-Flag was immunoprecipitated with anti-Flag antibodies and immunoprecipitates (top panels) were analyzed by immunoblotting with anti-C/EBPβ or anti-Flag (HDAC1) antibodies. Lysates (bottom panels) indicate expression of C/EBPβ and HDAC1. Similar results were obtained in two other experiments**.** **B**, CHO-GHR cells were cotransfected with plasmids for c-*fos*-luc and WT C/EBPβ, and either WT or inactive mutant (H141A) HDAC1 (50, 100 or 200 ng/per well), or Rc/RSV vector (V) (n=3). A significant decrease (*$p < 0.05$) in C/EBPβ-mediated activation of c-*fos*-luc was observed with 200ng of WT HDAC1. No significant change was observed with the mutant HDAC1. **C**, Plasmids for WT C/EBPβ were transfected alone, or with the indicated HDAC1 in the absence (V) or presence (+) of p300 in 293T cells. Lysates were analyzed by immunoblotting with anti-Ac-K (Ac C/EBPβ), anti-C/EBPβ, anti-p300 or anti-HDAC1. Similar results were obtained in two other experiments.

the key adipogenic factors PPARγ and C/EBPα (8). Given the important regulatory consequences of K39 acetylation on C/EBPβ, this modification is likely to have substantial influence on C/EBPβ-mediated regulation of PPARγ and C/EBPα gene expression during adipogenesis. To examine whether this modification occurs in the context of adipose tissue, we have carried out immunoprecipitation experiments. In Figure 3.4A, acetylation of K39 C/EBPβ is detectable in mouse adipose tissue. Furthermore, in the 3T3-L1 *in vitro* model of adipocyte differentiation (8), acetylation of K39 C/EBPβ is detected starting at day 2 after adipogenesis induction (Fig 3.4B, upper panels); the appearance of the acetylation of C/EBPβ at K39 parallels the expression of C/EBPβ and briefly precedes the upregulation of C/EBPα and PPARγ (Fig 3.4B, lower panels).

To probe the impact of K39 C/EBPβ acetylation in 3T3-L1 cells, we have examined the ability of WT and mutant forms of C/EBPβ to activate key adipogenesis target genes. Consistent with our previous results in CHO cells (16), we find that K39R C/EBPβ fails to activate the C/EBPα promoter (not shown). When we extend this analysis to the promoter for PPARγ, the other key regulator of adipogenesis, we find that arginine or alanine substitutions at position 39 compromise C/EBPβ-mediated activation of the PPARγ promoter whereas a glutamine substitution, which can mimic the function of acetyl-lysine (23) does not (Fig 3.5A). The reduction in basal activity in the presence of the K39R mutant is consistent with the dominant-negative effect of this substitution. Notably, this effect is specific for K39, as C/EBPβ forms bearing mutations of other known acetylation sites activate PPARγ as effectively as WT C/EBPβ (Fig 3.5A,B). In

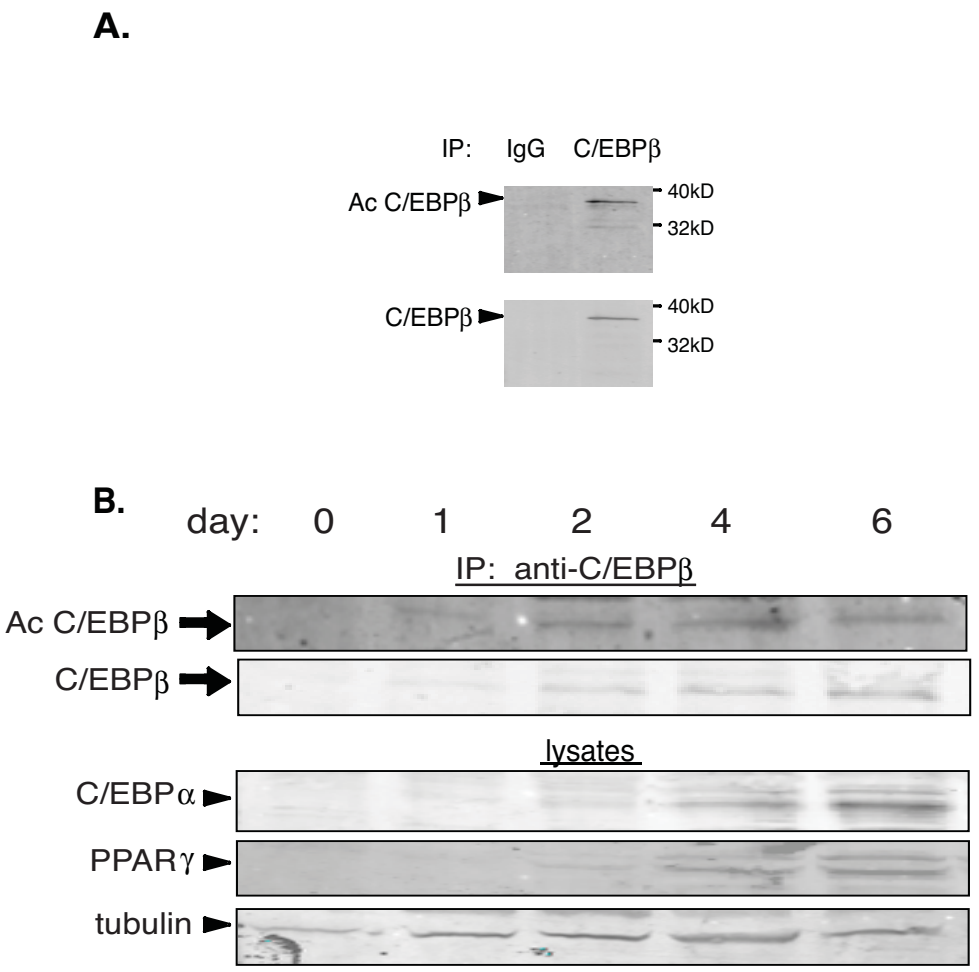

Fig 3.4. C/EBPβ is acetylated at K39 in mouse adipose tissue and 3T3-L1 adipocytes. **A**, Mouse adipose tissue was homogenized as described in Methods and samples were subjected to immunoprecipitation with anti-C/EBPβ or normal IgG antibodies. Immunoprecipitates were separated by SDS-PAGE and used for immunoblotting with anti-Ac-K (Ac C/EBPβ, top) or anti-C/EBPβ (bottom). Similar results were obtained in two other experiments. **B**, Confluent 3T3-L1 cells were induced to convert to adipocytes on day 0 and lysates were prepared at the indicated times. In upper panels, samples were immunoprecipitated (IP) with anti-C/EBPβ antibodies, separated by SDS-PAGE and used for immunoblotting with anti-Ac-K (Ac C/EBPβ, top) or anti-C/EBPβ (bottom) antibodies. Arrows indicate C/EBPβ. In the lower panels, lysates were analyzed by immunoblotting using anti-C/EBPα (top), anti-PPARγ (middle), or anti-tubulin (bottom) antibodies. Similar results were obtained in two other experiments.

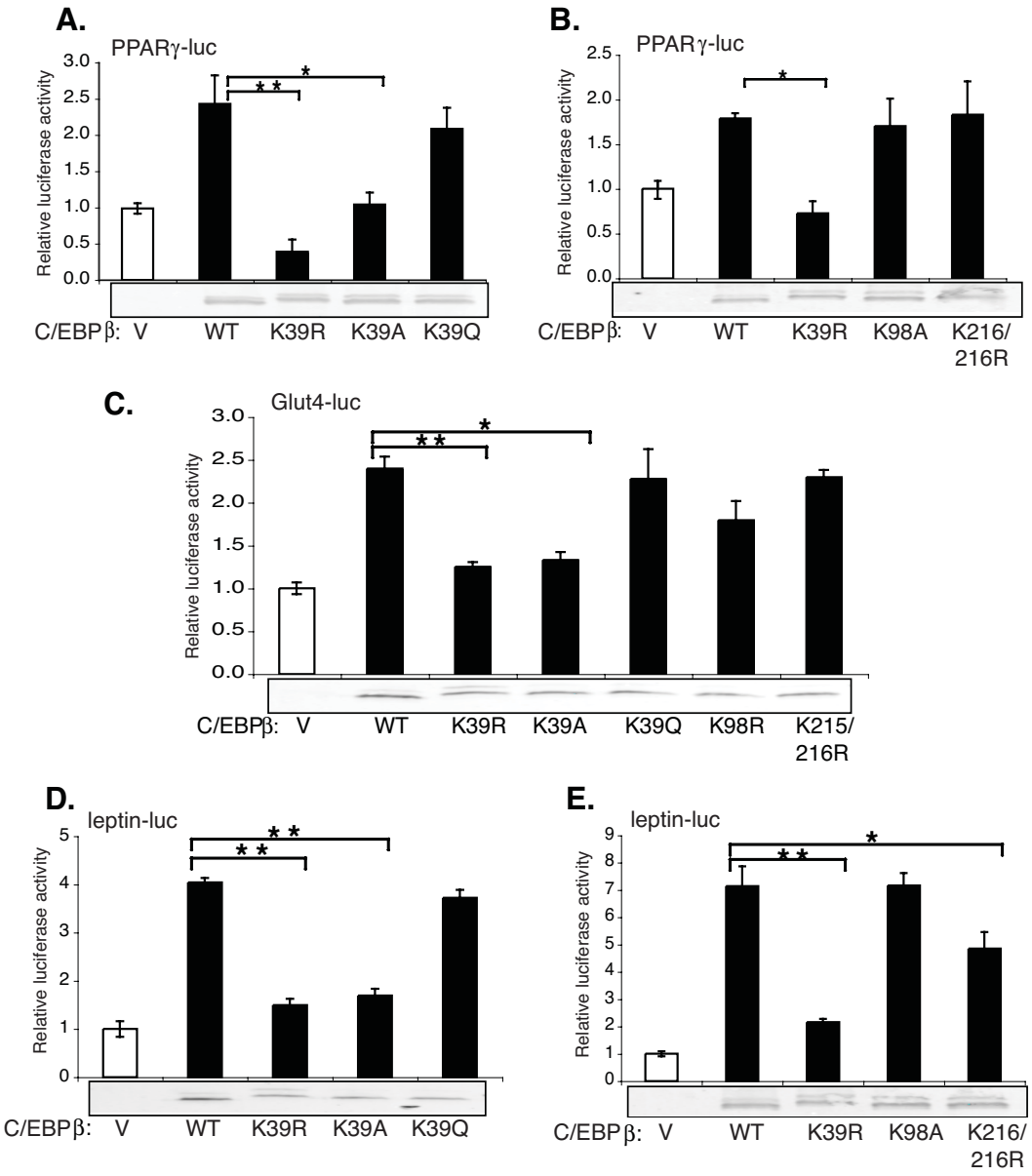

Fig 3.5. Acetylatable K39 in C/EBPβ contributes to activation of adipogenic genes. CHO-GHR cells were transfected with plasmids for expression of WT HA-C/EBPβ, the indicated C/EBPβ lysine mutant, or pcDNA3 vector (V) together with PPARγ-luc (panels **A** and **B**), Glut4-luc (panel **C**), or leptin-luc (panels **D** and **E**) plasmids. Cells were lysed 48 hr later and normalized luciferase activity was measured (n=3 for each panel). For all reporters, transcriptional activation by WT C/EBPβ is significantly greater than control ($p<0.05$ for PPARγ-luc, $p<0.01$ for Glut4-luc and leptin-luc). The activity of all mutants was compared to WT C/EBPβ and brackets indicate significant differences (* $p<0.05$; ** $p<0.01$). A representative immunoblot of lysates used for luciferase assay, probed with anti-HA antibodies, illustrates the relative expression of C/EBPβ proteins.

addition to driving the expression of C/EBPα and PPARγ, C/EBPβ also contributes to the expression of adipocyte genes such as Glut4 and leptin. As in the case of the transcription factor genes, we find that acetylation of K39 is an important requirement for effective activation of the Glut 4 (Fig 3.5C) and leptin promoters (Fig 3.5D, E). Notably, whereas K39 acetylation is important for the regulation of all the genes examined, the effect of substitutions at the other C/EBPβ acetylation sites we have examined is not as consistent or substantial. Taken together, these results indicate that acetylation at K39 controls the ability of C/EBPβ to regulate multiple genes central to adipocyte differentiation and implicates this posttranslational modification as a potential regulatory point during adipogenesis.

Discussion

Acetylation and deacetylation of C/EBPβ modulate activation of adipocyte genes

Posttranslational modification of C/EBPβ is an effective means of regulating the activity of this transcription factor. We have provided evidence that acetylation of C/EBPβ at K39 occurs in differentiated 3T3-L1 adipocytes and mouse adipose tissue. Further, disruption of C/EBPβ acetylation at K39 renders it refractory to p300 coactivation and impairs activation of adipocyte genes encoding C/EBPα, PPARγ, Glut4, and leptin. These observations are consistent with a model in which alterations in the acetylation state of C/EBPβ at K39 modulate its ability to mediate activation of target genes essential for adipose differentiation. The steady-state acetylation of C/EBPβ at K39 reflects the interplay between acetylase and deacetylase activities. Based on our data, both p300 and

HDAC1 contribute to this balance. The observation that during adipose conversion of 3T3-L1 and 3T3-F442A cells, p300 levels increase; whereas HDAC levels decrease (24) is consistent with the positive role played by this modification in the C/EBPβ regulation of adipogenesis genes. Further, HDAC1 overexpression decreases adipocyte conversion in 3T3-L1 cells (24, 25). While the present analysis focuses on acetylation at K39, acetylation at other residues of C/EBPβ can clearly contribute to adipogenesis. The recent work of Wiper-Bergeron et al. indicates that acetylation at K98/101/102 in C/EBPβ by the acetyltransferase GCN5 prevented HDAC1 dissociation from C/EBPβ on the C/EBPα promoter during adipose differentiation (14). Moreover, mutation of all three residues simultaneously (K98/101/102R) decreased the ability of C/EBPβ to mediate adipose conversion in NIH-3T3 cells (14). Further studies may address whether acetylation at K39 and K98/101/102 within C/EBPβ work in concert to regulate the transcriptional activity of C/EBPβ during adipose conversion.

A dynamic balance between acetylation and deacetylation at K39 in C/EBPβ

Our experiments provide evidence that HDAC1 reduces acetylation of C/EBPβ at K39 in a manner that requires its deacetylase activity. C/EBPβ associates with HDAC1, and both HDAC1 and C/EBPβ have been observed to occupy the C/EBPα promoter by ChIP analysis (14). The dissociation of HDAC1 from C/EBPβ was suggested to facilitate activation of the C/EBPα promoter (14). Our data suggest that, in addition to the loss of the well-known repressive HDAC complex, release of HDAC1 may contribute to activation by permitting the accumulation of K39 acetylation in C/EBPβ. Since C/EBPβ

is an early inducer of adipogenesis, deacetylation of C/EBPβ at K39 or other residues by HDAC1 may be an important site of regulation during adipogenesis.

It is important to recognize that lysines can be subject to multiple modifications such as SUMOylation, ubiquitination, or methylation (26). Although K39 in C/EBPβ may be a site for other modifications, our finding that replacement of K39 by glutamine, which can mimic an acetylated lysine, resembles the WT protein supports the idea that the phenotype of the K39R and K39A substitutions is likely due to loss of acetylation. Furthermore, preliminary data suggest that K39 is not a site of SUMOylation and mutations at this site do not appear to affect ubiquitination (L. Subramanian, unpublished data). It is also interesting to note that the K39R substitution leads to a consistent upward shift in the electrophoretic migration of C/EBPβ (see Fig 3.3A for example). Loss of K39 acetylation may therefore lead to alterations in other posttranslational modifications such as phosphorylation. Although our previous findings indicate that acetylation at K39 in C/EBPβ is independent of phosphorylation at S184 or T188 (16), whether acetylation at K39 alters phosphorylation at other sites remains to be determined. Our recent observation that treatment of K39 C/EBPβ with alkaline phosphatase can reduce its slight upward shift in mobility (not shown) argues that exploring in the future the phosphorylation state of this mutant is likely to be revealing.

The acetylation state of K39 modulates C/EBPβ-mediated transcription

The acetylation state of C/EBPβ at K39 contributes to its role in transactivation of target genes. The strong functional effects of K39 mutations are rather remarkable. C/EBPβ harbors multiple transcriptional activation functions and the redundant nature of

activation domains makes them rather tolerant to mutations (27). The fact that a single substitution at K39 is sufficient to severely compromise C/EBPβ activity argues that acetylation at this position is a "gatekeeper" modification required for the function of C/EBPβ. Studies of C/EBPβ (28) and the related C/EBPα indicate that the N and C terminal regions are in close proximity (29). Perhaps K39 acetylation is required for the opening of an otherwise closed and inactive form of C/EBPβ.

Remarkably, our data indicates that the functional effects of K39 substitutions are not reflected in detectable alterations in the interaction between C/EBPβ and either p300 or HDAC1. Acetylation of C/EBPβ may nonetheless alter its association with other coactivators or corepressors, as in the case of *c-myb*. When acetylated, this protein shows increased affinity for CBP (30). Such alterations in coregulator interaction may contribute to the mechanism by which K39R C/EBPβ exerts its dominant negative effects.

Taken together, these studies indicate that deacetylation by HDAC1, and p300 acetylation at K39 in C/EBPβ modulates its ability to mediate activation of target genes. The dynamic interplay between these opposing activities appears to be especially pertinent for adipocytes where this modification is readily detected and exerts an important influence on C/EBPβ mediated activation of critical target genes for adipogenesis and adipocyte function. We anticipate that, by serving as a key regulatory modification, acetylation of C/EBPβ at K39 may also exert important effects in other metabolic and differentiation events regulated by this transcription factor, such as those occurring in the liver and the hematopoietic compartment.

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CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

The goal of my dissertation research has been to establish whether C/EBPβ is acetylated and to characterize the role of the acetylation of C/EBPβ in its function. The transcription factor C/EBPβ plays a central role in a variety of cellular processes, such as adipose differentiation (1) and hepatocyte proliferation (2), so that insight into how its function is controlled would be of interest in many physiological and potentially therapeutic contexts related to obesity and cancer. This study built on findings that C/EBPβ interacts with the acetyltransferase p300 (3) and that C/EBPβ is coactivated by p300 on c-*fos* (4). Thus, we hypothesized that C/EBPβ is acetylated and that acetylation of C/EBPβ may contribute to its role in adipogenesis.

It was essential first to determine whether C/EBPβ was acetylated. In Chapter II, I found that the acetyltransferases p300 and P/CAF acetylate C/EBPβ both *in vitro* and in living cells. I identified novel acetylation sites, lysine (K) 39, K117, and K215/216. While these studies were in progress, another group reported that C/EBPβ is acetylated at K215/216 (5). Acetylation of C/EBPβ at K39 is of particular interest because K39 lies in the transcriptional activation domain. We were able to move investigation of K39 acetylation forward using an antibody against acetylated lysines that detects acetylation

of C/EBPβ at K39, but not K98, K117 or K215/216 under conditions of these experiments. Both p300 and P/CAF were found to acetylate C/EBPβ at K39. Acetylation of C/EBPβ at K39 is independent of phosphorylation at T188, although a relationship with other phosphorylation sites within C/EBPβ has not been ruled out. Mutation of K39 in C/EBPβ to arginine or alanine impairs its ability to activate several target genes, including c-*fos*. Further, growth hormone (GH) increases acetylation of C/EBPβ at K39, and when K39 is mutated, GH-induced activation of c-*fos* is blunted, suggesting that acetylation of C/EBPβ at K39 contributes to GH-mediated activation of c-*fos* transcription.

As demonstrated in Chapter III, I found that K39R C/EBPβ exhibits properties of a dominant negative protein. Mutation at acetylation site K39 in C/EBPβ inhibits transcriptional activation even in the presence of p300. Such inhibition was independent of changes in association of C/EBPβ with p300. Conversely, the deacetylase HDAC1 is reported to associate with C/EBPβ (6). I independently adapted an approach, similar to one recently reported to detect deacetylation of MEF2 (7), in which I expressed relatively large amounts of HDAC1 to overcome HDAC inhibitors, which were necessary initially to allow acetylation. HDAC1, but not a deacetylase-deficient mutant of HDAC1, was found to deacetylate C/EBPβ. In agreement with this finding, WT HDAC1 decreased C/EBPβ-mediated activation of c-*fos*, presumably through deacetylation at K39, while deacetylase-deficient mutant HDAC1 had no effect. Together these findings demonstrate the dynamic effects of p300 and HDAC1, which modulate acetylation state, on C/EBPβ function. In this chapter, I also describe activation by C/EBPβ of promoters for

adipocyte target genes C/EBPα, PPARγ, as well as leptin and Glut-4, which is inhibited when its acetylation site K39 is mutated. Endogenous C/EBPβ is shown to be acetylated at K39 in mouse adipose tissue, as well as in differentiated 3T3-L1 adipocytes. The detection of endogenous acetylated C/EBPβ at K39 is consistent with our hypothesis that acetylation at this residue has functional relevance in activation of adipocyte genes.

A model has been developed to incorporate the findings of this thesis work (described in legend of Fig 4.1) into the current understanding of regulation of C/EBPβ function. Acetylation of C/EBPβ is mediated by p300 or other acetyltransferases, while HDAC1 deacetylates C/EBPβ. Acetylation occurs at K39, possibly in cooperation with acetylation and other modifications of other sites, and contributes to activation of adipocyte target genes, including C/EBPα and PPARγ.

Acetylation of C/EBPβ is a regulated event

Although these studies demonstrate that acetylation of C/EBPβ is a dynamic event, it is unclear at present how p300-mediated acetylation of C/EBPβ is induced. We observe that GH increases acetylation of C/EBPβ at K39, which is consistent with the role of C/EBPβ-mediated activation of c-*fos* by GH. Others have found that acetylation of C/EBPβ is increased in response to dexamethasone, which is a component in MDI adipose differentiation medium, in 3T3-L1 cells (6), and in response to lipopolysaccharide (LPS) in murine macrophage RAW264.7 cells (8). Dexamethasone, which increases C/EBPβ binding to DNA and nuclear localization in H4IIE cells (9), and LPS, which increases C/EBPβ mRNA levels in several tissues (10), are both reported to

stimulate C/EBPβ activity. It is tempting to speculate that effects observed with dexamethasone and LPS may be at least partially due to C/EBPβ acetylation.

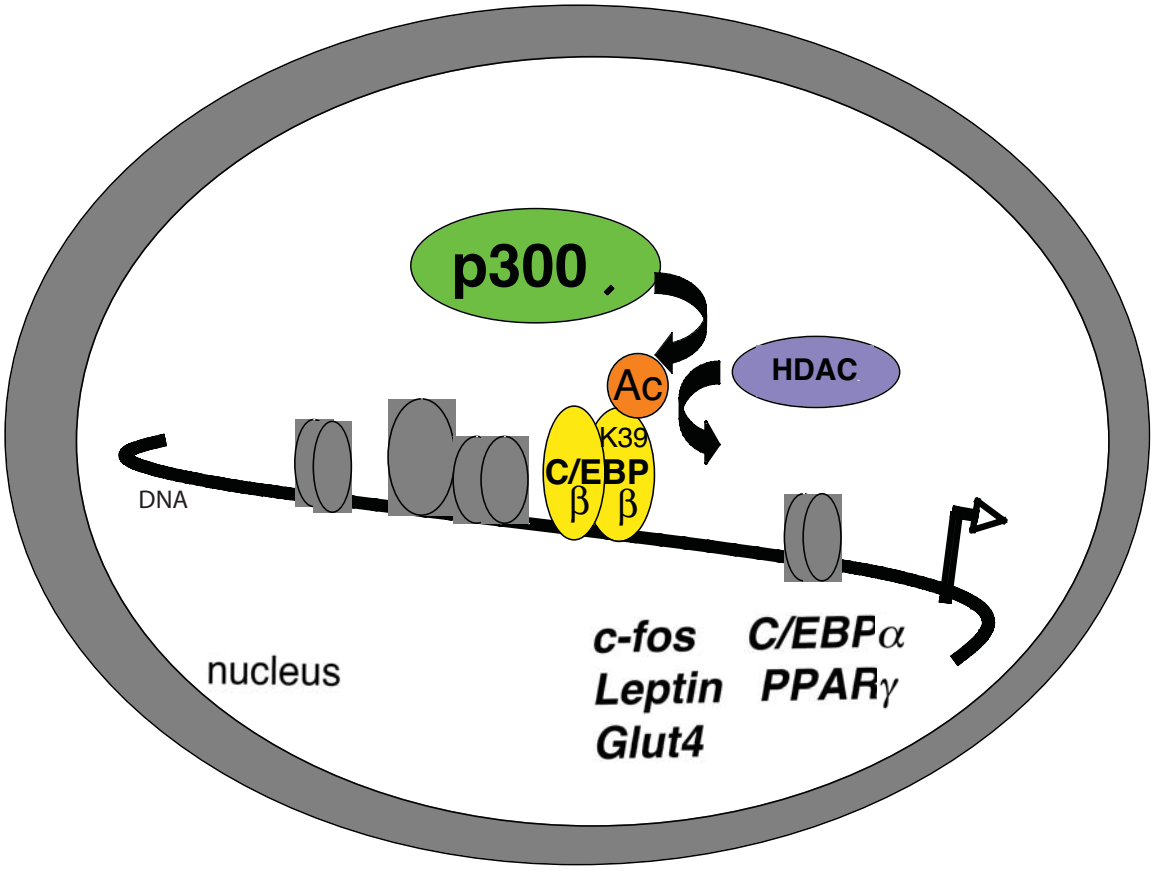


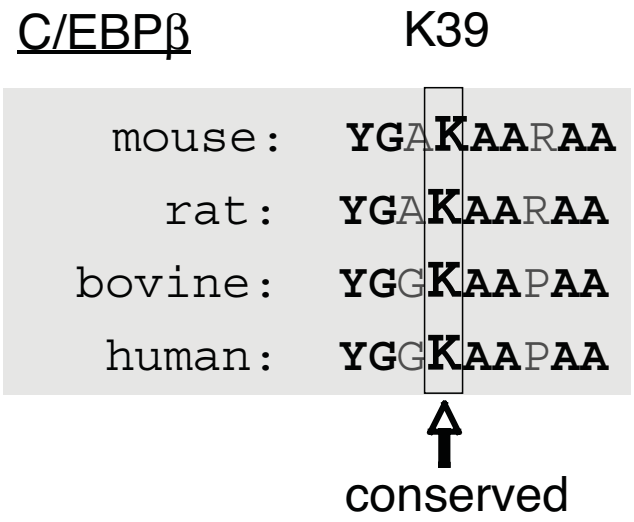
Fig 4.1. Model of C/EBPβ action. A model has been developed to incorporate the findings of this thesis work into the current understanding of regulation of C/EBPβ function in the cell. In the nucleus, C/EBPβ is one of many proteins that bind to DNA to regulate transcription. Acetylation (Ac) of C/EBPβ is mediated by p300 or other acetyltransferases, and HDAC1 mediates deacetylation of C/EBPβ. Acetylation of

C/EBPβ occurs at K39, possibly in cooperation with acetylation of other residues and/or other posttranslational modifications (not shown), and contributes to activation of adipocyte target genes, including C/EBPα and PPARγ. Other C/EBPβ target genes shown are c-*fos*, leptin, and Glut-4.

Some acetylated lysines in C/EBPβ, including K39, are conserved

Acetylated lysines K39, K98, K117 are specific to C/EBPβ, and are not conserved in C/EBPα, C/EBPδ, or C/EBPε; K215/216 are conserved in C/EBPα, C/EBPβ and C/EBPε. These differences suggest that C/EBPs are not regulated similarly by N-terminal acetylation, including at K39. This is consistent with the fact that C/EBP family members display the least homology at their N-termini (11), and play distinct roles in adipose and hepatocyte differentiation (1, 12).

K39 is conserved in C/EBPβ from mouse, rabbit, bovine, and human (Fig. 4.2), as are lysine residues at acetylation sites K98/101/102 and K215/216, suggesting their biological relevance; in contrast, acetylation site K117 is conserved only in mouse and rat C/EBPβ. K215/216 are the only lysines in C/EBPβ that fit precisely into the K/R-X-K-K acetylation motif described previously for the transcription factor p53 (13). Interestingly, in mouse C/EBPβ, K39, K98, and K117, but not K215/216, are all contained within a G-A-K sequence (Fig. 4.2), suggesting that G-A-K or G-X-K is a potential acetylation motif in C/EBPβ. Several acetylation sites in human histones conform to this G-X-K motif, including K15 in histone H2B (G-S-K), K14 in histone H3 (G-G-K), K8 in histone H4 (G-G-K), and K16 in histone H4 (G-A-K) (14). Two proposed acetylation sites within E2F1, K120 (G-V-K) and K125 (G-E-K), also share this putative G-X-K motif (15). Similarly, others report G-K (16) or G-K-X-X-P (17) as potential acetylation motifs. It should be noted, however, that there are also examples of acetylated residues that do not conform to any known acetylation motifs (14, 18).



Mouse C/EBPβ

(K39)	aa34-42:	LAY-GAK-AARA
(K98)	aa93-102:	DDY-GAK-PSKK
(K117)	aa112-121:	GRA-GAK-AAPP

Fig 4.2. Conserved acetylation sites within C/EBPβ. K39 in C/EBPβ is conserved in mouse, rat, bovine, and human (top). Acetylated residues K39, K98, and K117 in mouse C/EBPβ are all contained within a G-A-K sequence (bottom). G-A-K may be an acetylation motif.

Anti-acetyl lysine antibody that detects acetylation of C/EBPβ at K39

During the course of our experiments, we used an anti-acetyl lysine antibody that was originally generated against acetylated histones, and also recognizes acetylated p53, auto-acetylated p300, and auto-acetylated P/CAF (5). Interestingly, this antibody was used to detect acetylation of C/EBPβ at K215/216 in immune cells (5). In 293T cells, we were unable to detect a change in acetylation of C/EBPβ when K215/216 was mutated. This anti-acetyl-lysine antibody detected a significant decrease in acetylation only when K39 was mutated in C/EBPβ, but not when R42, K98, K117, or K215/216 was mutated (see Fig 2.4). Thus, although this antibody is not specific for acetylated K39 in C/EBPβ, it seems to be able to detect acetylation changes at this site under conditions tested. However, in order to determine the specificity of this antibody (i.e. Is K39 the only acetylated lysine residue in C/EBPβ recognized by this antibody?), acetylated and non-acetylated peptides containing all lysine candidates in C/EBPβ would have to be compared. Although attempts to produce a specific antibody have proven unsuccessful thus far, a specific antibody would help further these studies involving acetylation of C/EBPβ at K39.

Acetylation of C/EBPβ modulates adipose differentiation

We found that mutation of K39R in C/EBPβ impaired activation of adipogenic target genes C/EBPα and PPARγ. In a recent report, Wiper-Bergeron *et al.* found that C/EBPβ is acetylated at K98/101/102 by GCN5, and that mutating these sites inhibited adipose

differentiation in NIH-3T3 cells, as determined by Oil Red O staining and C/EBPα expression (6). Further, acetylation at these sites in C/EBPβ causes the disassociation of HDAC1 from C/EBPβ on the C/EBPα promoter, which the authors postulate contributes to C/EBPβ-mediated increase in C/EBPα expression, and thus adipose differentiation. In our studies using peptides in an acetylation assay, we were unable to detect acetylation by p300 of a peptide that contained K98/101/102 (see Fig 2.4A) and mutation of K98 did not impair transcriptional activation of the variety of target genes tested, including C/EBPα promoter. The reason for these inconsistencies is not clear, but raises questions (see below).

To characterize the role of acetylation of C/EBPβ at K39 in adipose differentiation, we stably expressed WT, K39R and K39Q C/EBPβ in 3T3-L1 and NIH-3T3 cells, and evaluated rates of adipose differentiation. Cells expressing WT C/EBPβ differentiated into adipocytes similarly to cells expressing K39R C/EBPβ and K39Q C/EBPβ (Appendix A). Considering the decrease in acetylation that we observed with K39R, the dramatic decreases in transcriptional activation of target genes (including C/EBPα and PPARγ) with K39R in promoter luciferase assays, and the data that support that K39R is a dominant negative protein, we had predicted that there would be a decrease in adipose differentiation in NIH-3T3 or 3T3-L1 cells stably expressing K39R C/EBPβ. Closer scrutiny of the Wiper-Bergeron *et al.* report that acetylation of K98/101/102 mediates dexamethasone-induced adipogenesis raises two major concerns with their findings: First, they did not observe acetylation of C/EBPβ in the presence of p300 *in vitro*, an observation that has been consistently observed by others (5), as well as here (Fig 2.1). Nevertheless, since these authors were able to detect acetylation of

histones by full-length p300, p300 was indeed enzymatically active. Second, when examining adipose differentiation in cells expressing WT vs K98/101/102R C/EBPβ, they use expression of C/EBPα as an indicator of adipose differentiation, where WT C/EBPβ induced expression of C/EBPα and K98/101/102R failed to do so. The problem with this finding is that NIH-3T3 cells do not express C/EBPα upon induction of adipose differentiation (19). Thus the decrease in C/EBPα expression in NIH-3T3 cells observed with K98/101/102R C/EBPβ is still unresolved. Oil Red O staining indicates that C/EBPβ mutant K98/101/102R was able to inhibit adipose differentiation (6), while our studies indicate that K39R C/EBPβ does not. This difference suggests a more robust role for acetylation of K98/101/102 as compared to K39 in C/EBPβ function in adipogenesis. There appears to be disassociation between altered expression of adipogenic genes (C/EBPα and PPARγ) and detection of changes in cellular lipid accumulation, however. For example, given that mutant T188A C/EBPβ has been shown to inhibit C/EBPα activation robustly, but impairment in adipose differentiation is not detected in Swiss3T3 cells stably expressing T188A C/EBPβ (20), perhaps it is not too surprising that expression of K39R C/EBPβ does not detectably impair adipose differentiation.

FUTURE DIRECTIONS

Mechanism of action of K39R C/EBPβ

While the present studies identify critical consequences of acetylation of C/EBPβ, many questions remain with regard to the mechanism by which acetylation of C/EBPβ at K39

alters its function. K39R C/EBPβ appears to have dominant negative properties: expression of an increasing amount of K39R with a fixed amount of WT C/EBPβ led to a dose-dependent impairment in WT activity (Fig 3.1). K39R C/EBPβ may inhibit WT C/EBPβ function through changes in (a) degradation, (b) cellular localization, (c) binding to DNA, or (d) association with coactivators, corepressors, or the transcriptional machinery. It is unlikely that K39R C/EBPβ alters (a) degradation since no changes in protein stability could be detected between acetylated and nonacetylated C/EBPβ on immunoblots under the conditions tested. One way to test this further would be to treat WT C/EBPβ and K39R C/EBPβ with cycloheximide to study protein stability with radiolabeling (as an indicator of half-life) (15). K39R C/EBPβ did not differ in (b) cellular localization from WT, as determined by cell fractionation or immunocytochemistry (data not shown). Similar to WT C/EBPβ, K39R C/EBPβ could still (c) bind to DNA, as determined by EMSA and ChIP analysis (data not shown). (d) Interactions of C/EBPβ with both p300 and HDAC1 are not detectably altered by mutation at K39 (Fig 3.3). Since the N-terminus of C/EBPβ is reported to interact with other coregulatory factors, including the SMRT complex (9) and the Swi/Snf complex (21), the possibility that interactions are changed by mutation at K39 requires further investigation.

While our evidence does not support impaired function of K39R C/EBPβ prior to binding to DNA (pre-DNA binding), the possibility remains that mutant K39R C/EBPβ may have consequences downstream (post-DNA binding). Earlier studies of C/EBPβ (22) and C/EBPα (23) suggest that the N- and C-termini may be in close proximity once

bound to DNA. One tempting theory is that K39R, which is situated at the N-terminus, disrupts the folding of the C/EBPβ molecule. This could be tested by comparing the distance between the N- and C- termini of both WT and K39R C/EBPβ using Fluorescent Resonance Energy Transfer (FRET), as previously described for WT and phosphorylation mutant S21A C/EBPα (23). Further studies may elucidate the exact mechanism, and whether folding is involved, in K39R C/EBPβ action.

Cooperation among posttranslational modifications of C/EBPβ

We also considered possible interplay between the phosphorylation and acetylation of C/EBPβ, analogous to the "histone code" theory proposed by Jenuwein and Allis (24), which posits that specific combinations of posttranslational modifications on histones work together to regulate histone function. A similar idea has been postulated for transcription factor p53 (25, 26).

We were intrigued by the possible interplay of phosphorylation and acetylation in C/EBPβ because we consistently observe a slight upward shift in migration of K39R C/EBPβ relative to WT C/EBPβ in immunoblots. We considered whether the slight upward shift reflects changes in phosphorylation of C/EBPβ. When lysates of cells expressing the K39R mutant were treated with alkaline phosphatase, the upward shift of K39R C/EBPβ receded to that of WT C/EBPβ, consistent with a loss in phosphorylation (Appendix B). This suggests that mutating K39 to R may alter C/EBPβ in such a way that its phosphorylation state becomes modified, which may in turn contribute to the mechanism by which K39R C/EBPβ decreases transcriptional activation of target genes.

C/EBPβ contains multiple phosphorylation sites including GSK3 substrate site S184, MAPK/cdk2 substrate site T188, and RSK2 substrate site T217 (Fig 1.1).

We also investigated potential interplay between acetylation and phosphorylation using C/EBPβ mutated at critical residues susceptible to acetylation or phosphorylation, and found that C/EBPβ is acetylated at K39 despite mutations at phosphorylation sites S184 and T188 (Fig 2.5). Further, p300-mediated acetylation was not impaired in mutants S184A C/EBPβ and T188A C/EBPβ. Phosphorylation of C/EBPβ at T188 was not altered in the presence of p300, as detected with an antibody specific for phosphorylation at T188. Conversely, mutations at acetylation sites K39, K117, or K215/216, alone or in combination, did not disrupt phosphorylation at T188 (Supplemental Fig S2.2).

To further examine whether phosphorylation at S184, T188, or T217 contribute to the upward shift in migration observed with K39R C/EBPβ, mutations at these residues were analyzed in combination with K39R (Appendix C). It was reasoned that if the upward shift were relieved by combined mutation of K39 with any of these phosphorylation site mutations (K39R/S184A, K39R/T188A, or K39R/T217A C/EBPβ), then this relief would suggest that mutation to R in C/EBPβ facilitates modulation of C/EBPβ phosphorylation at S184, T188, or T217. However, C/EBPβ with any of these combined mutations migrated similarly to K39R C/EBPβ (Appendix C), suggesting that impaired phosphorylation at these sites does not relieve the upward shift (putative hyperphosphorylation) observed with K39R C/EBPβ. Further, acetylation of K39R/S184A, K39R/T188A, or K39R/T217A was disrupted (data not shown), as observed previously when any of these other residues was mutated in combination with

K39. Although acetylation of C/EBPβ at K39 and its phosphorylation at T188, S184, or T217 appear to be independent of each other, other phosphorylation sites may be interdependent with acetylation of C/EBPβ. The posttranslational modification described on C/EBPβ that is closest to K39 is phosphorylation at S64 (27, 28): Ras-mediated phosphorylation of rat C/EBPβ at S64 (also conserved in mouse) is reported to occur in response to LPS. Because LPS has also been reported to increase acetylation of C/EBPβ (8), the proximity of S64 to K39 in the transcriptional activation domain, and the fact that antibodies exist against phosphorylated S64, makes this modification a promising target for future study of its possible interplay with acetylation at K39.

Liver as a model system to study the functional consequences of acetylation of C/EBPβ at K39

Adipocytes may not be the only, or the most, responsive system for examining the functional consequences of acetylation of C/EBPβ at K39. In C/EBPβ (-/-) knockout mice, few, if any, defects in adipose tissue mass were detectable, although a decrease in adipose differentiation could be detected in cells derived from C/EBPβ (-/-) mice (29). In C/EBPβ (-/-) mice, it was only in combination with C/EBPδ (-/-) that defects in adiposity could be detected. Overexpression of WT C/EBPβ in several cell lines, including 3T3-L1 cells, has been shown to enhance adipose differentiation (30). As described above, when WT or K39R C/EBPβ was stably expressed in 3T3-L1 or NIH-3T3 cells, adipose differentiation was enhanced similarly with both WT and K39R C/EBPβ. In hindsight, this was not surprising considering that important regulatory posttranslational modifications of C/EBPβ such as phosphorylation at T188 do not have robust effects on

adipocyte differentiation: Similar to present findings with K39R C/EBPβ, Swiss3T3 cells stably expressing C/EBPβ phosphorylation site mutant T188A did not exhibit differences in adipose conversion when compared to WT C/EBPβ, even though T188A did impair expression of adipocyte genes C/EBPα and adiponectin (20). One exception, mutation of K98/101/102R, exhibited impaired adipose differentiation when stably expressed in NIH-3T3 cells (6), although concerns in the interpretation of these observations are described above.

An interesting alternative readout may be provided in the liver, since one posttranslational modification of C/EBPβ, phosphorylation at T217, did display robust consequences in hepatocytes. C/EBPβ mediates hepatocyte proliferation induced by TGFα (31), but proliferation is inhibited when RSK2-substrate site T217 is mutated to alanine. When T217 is replaced with aspartate, a residue that mimics phosphorylation, C/EBPβ could then induce proliferation independent of TGFα. Using liver to assess consequences of acetylation of C/EBPβ is promising in light of our preliminary observation of acetylation of endogenous C/EBPβ in mouse liver samples (data not shown). Additionally, other C/EBPβ-dependent cells, such as macrophages and pituitary cells, may also provide sensitive systems to discern the function of acetylation of C/EBPβ.

Both an increase in cell size and cell number contribute to obesity, or the overabundance of adipocytes (1, 32). Since acetylation of C/EBPβ at K39 contributes to activation of adipocyte target genes, this may likely have consequences on adipogenesis, and possibly obesity. Mutation of C/EBPβ at K98/101/102 has already been shown to disrupt not only activation of adipocyte target genes, but also adipose differentiation (6).

Further studies will be necessary to determine the role of acetylation at K39 in adipogenesis.

Implications for drug targeting of acetylation of C/EBPβ

A potential future outcome of the present work would be the identification of pharmacological agents that can exclusively target acetylation of C/EBPβ at K39, and as a result modulate C/EBPβ function. The difficulty lies in developing a pharmacological agent that targets any specific posttranslational modification on a distinct transcription factor.

A less specific approach to control acetylation has been reported using HDAC inhibitors. In adipose differentiation, HDAC inhibitors have an inconsistent role (see Introduction: Deacetylases). However, the HDAC inhibitor TSA induces cell cycle arrest in rat hepatocytes (33) and hepatoma cells (34, 35), which is in agreement with its established anti-proliferative properties (36). TSA was able to maintain functional differentiation of primary cultured rat hepatocytes, as indicated by increased cell viability and albumin secretion vs control cells; expression of C/EBPα, which is antimitotic, also increased (37). Thus, HDAC inhibitors appear effective for inhibiting proliferation in liver.

In parallel to its role in differentiation of adipocytes (1), C/EBPβ is critical for liver regeneration and proliferation, while C/EBPα maintains hepatocytes in the differentiated state (12). In the liver, inhibition of proliferation by TSA may depend on

what point in the cell cycle it is added, such that if TSA is added early (during hepatocyte proliferation), proliferation may be inhibited, while if TSA is added later (during hepatocyte maintenance), differentiation may be sustained or enhanced (33-35). Although this is an oversimplification, lessons can be learned from the liver when interpreting HDAC inhibitor effects in adipose differentiation. One would expect HDAC inhibitor treatment early in adipose differentiation to be inhibitory because HDAC inhibitors are antimitotic (36), and mitotic clonal expansion is suggested to be necessary for early adipose differentiation (38), and is dependent on C/EBPβ (39). Based on this reasoning, if HDAC inhibitors are added later (after mitotic clonal expansion), then one would expect that adipose differentiation would be maintained or even enhanced, as it is in the liver. Consistent with these data, we (data not shown) and others (40) found that early treatment of adipocytes with TSA (simultaneous with induction of adipogenesis) did indeed inhibit adipogenesis. However, as described in the Introduction, some reports have indicated that treatment of preadipocytes with HDAC inhibitors, even at early time points in differentiation, can enhance adipogenesis (41-43). Future studies will have to elucidate how HDAC treatment in early stages of adipogenesis can yield opposite results in 3T3-L1 cells in different studies.

In considering clinical applications of HDAC inhibitors, the HDAC inhibitor valproic acid actually increases weight gain (44). This is consistent with the idea that HDAC inhibitors can maintain or enhance adipose differentiation once adipose tissue is established. It is important to note, however, that valproic acid, which is used as an anticonvulsant and mood stabilizer, most likely has multifaceted effects. Overall, these

studies suggest that use of HDAC inhibitors in treating obesity will not be straightforward.

Another possibility for inhibiting C/EBPβ function is to target C/EBPβ directly with small molecule inhibitors. As described for specific targeting of p53, small molecules interfere with p53-MDM2 binding, which would otherwise inhibit p53 function (45, 46). Perhaps controlling C/EBPβ function with small molecules will become one component of treatments for obesity. More likely, control of C/EBPβ, and/or the acetylation state of C/EBPβ, will have to be managed prior to the establishment of differentiated adipocytes in order to inhibit adipocyte proliferation effectively.

CONCLUSIONS

These studies have identified and characterized a novel acetylation site in the transcription factor C/EBPβ, and provide insight into regulation of C/EBPβ-dependent mechanisms in adipose differentiation. These findings support a model in which dynamic regulation of the acetylation of C/EBPβ contributes to its ability to mediate transcriptional activation of target genes associated with adipogenesis. Such insights add to our ability to address mechanisms contributing to obesity, and may suggest new approaches for treating obesity and related health problems.

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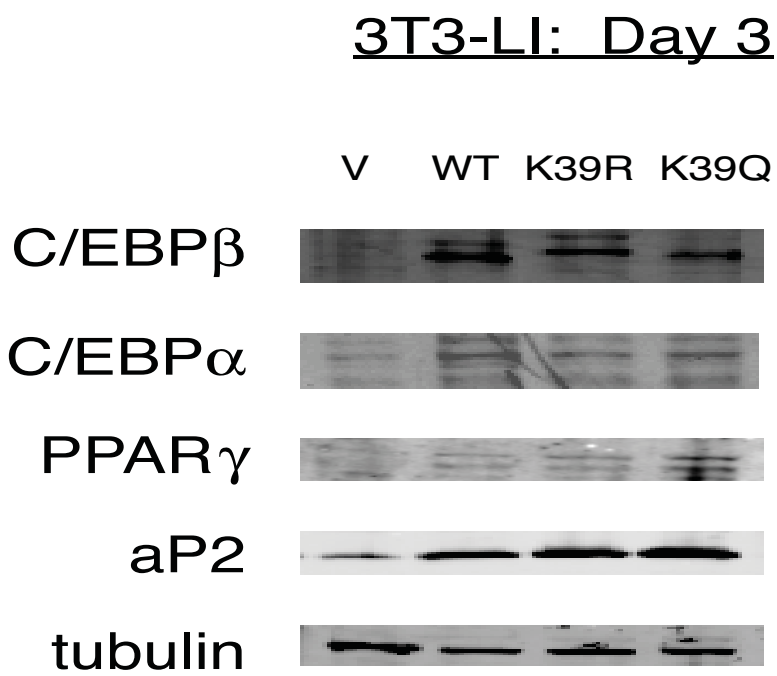
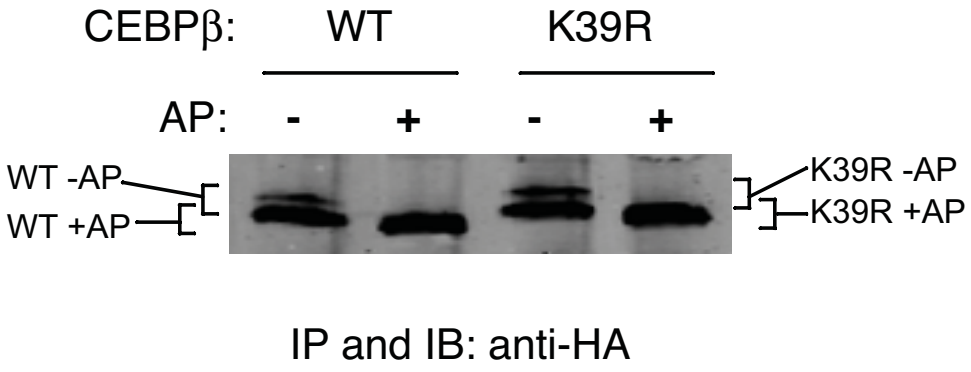
Appendix A.



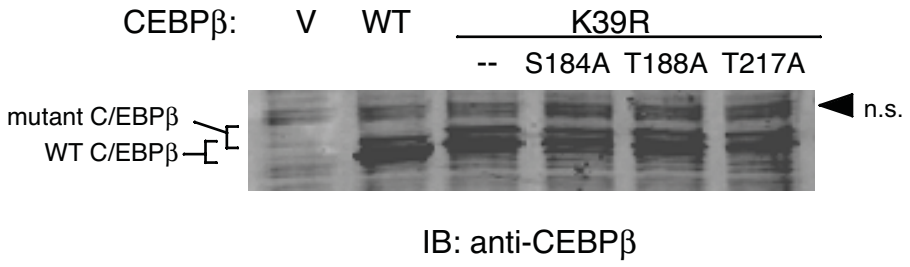
Appendix A. Stable expression of K39 mutants of C/EBPβ in 3T3-L1 cells does not prevent expression of adipogenic marker proteins. To examine whether K39R C/EBPβ would inhibit adipose differentiation, 3T3-L1 cells stably expressing pLNCX2 vector (V), or WT, K39R, or K39Q C/EBPβ were induced to differentiate using MDI medium. Top panel shows expression of C/EBPβ (anti-HA). On day 3, expression of C/EBPα, PPARγ, aP2 were slightly increased with mutant, as well as WT, C/EBPβ relative to V, indicating that K39R does not impair expression of adipogenic marker proteins. Samples taken from other days of adipose differentiation also indicate that K39R mutants did not prevent expression of adipogenic proteins (data not shown).

Appendix B.



Appendix B. Alkaline phosphatase treatment of both WT and K39R C/EBPβ increases electrophoretic mobility. To examine whether K39R caused alteration in phosphorylation state of C/EBPβ, plasmids for WT or K39R HA-C/EBPβ were expressed in 293T cells. C/EBPβ was immunoprecipitated with anti-HA, then treated with alkaline phosphatase (AP, 40 U) or H_2O control treatment for 1 hr at 37°C while on the beads, and then C/EBPβ was eluted from beads using SDS protein dye. Immunoprecipitates were then used for immunoblotting with anti-HA. In the absence of AP treatment, K39R exhibits a slight upward shift relative to WT (lane 3 vs 1). AP treatment resulted in faster migration of K39R C/EBPβ (lane 4), suggesting that phosphorylation of K39R slowed its migration relative to WT C/EBPβ.

Appendix C.



Appendix C. Slower migration of K39R C/EBPβ is not altered by its combined mutation with phosphorylatable residues S184A, T188A, or T217A. To determine whether residues are phosphorylated differentially in WT and K39R C/EBPβ, plasmids for WT or K39R C/EBPβ, alone, or in combination with phosphorylation mutants S184A, T188A, T217A, were expressed in 293T cells to examine migration of mutant C/EBPβ relative to WT C/EBPβ. Lysates were used for immunoblotting with anti-C/EBPβ. All combination mutants migrated similarly to K39R C/EBPβ alone, indicating that phosphorylation at the sites tested does not induce the upward shift observed with K39R C/EBPβ. n.s. =nonspecific band.