Functional regulation of C/EBPβ through post-translational acetylation

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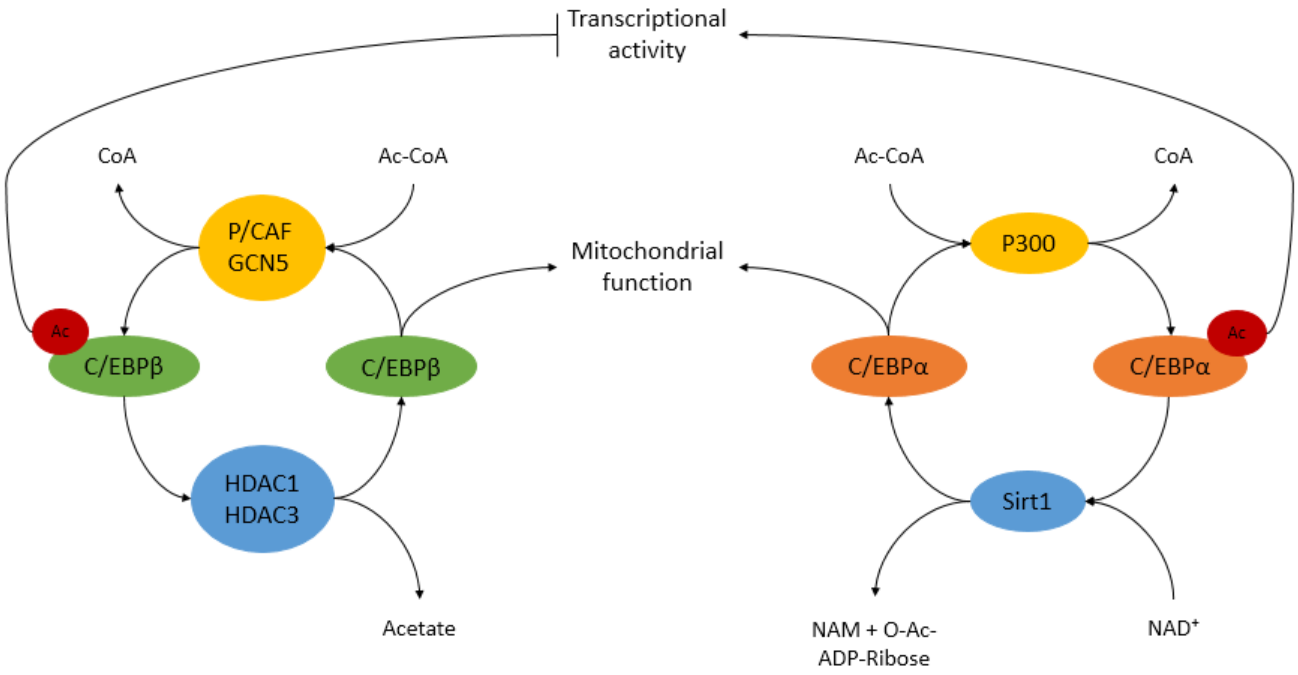

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ABSTRACT

The process of ageing is often accompanied by cancer and metabolic diseases. Members of the CCAAT/enhancer binding protein (C/EBP) family are transcription factors that form an important link between these pathologies, because of their involvement in many cellular processes, such as differentiation, proliferation and metabolism. Lysine (K) acetylation of transcription factors is a potential mechanism of adjusting transcription factor activity and thereby controlling gene regulation. In contrast to C/EBPα acetylation, not much is known about acetylation of C/EBPβ. Therefore, the aim of this project was to identify how C/EBPβ acetylation is regulated and whether acetylation affects C/EBPβ function.

HEK-293T cells were transfected with C/EBPβ isoforms in the presence or absence of various lysine acetyltransferases (KATs) or histone deacetylases (HDACs). Immunoprecipitation of C/EBPβ followed by western blot analysis using an antibody specific to acetylated lysines was performed to analyse levels of acetylation. Furthermore, acetylation (KQ) or deacetylation (KR) mutations of C/EBPβ were generated and used to examine differences in transcriptional activity and mitochondrial function.

The performed experiments showed that all isoforms of C/EBPβ are acetylated when overexpressed. The concomitant overexpression of P/CAF and GCN5 increased acetylation of C/EBPβ, while HDAC1 and HDAC3 decreased acetylation of C/EBPβ. The acetylation mimicking mutation of K239 impaired transcriptional activity of the C/EBPβ isoform LAP, indicating that acetylation of C/EBPβ at K239 decreases the transcriptional activity of LAP. Furthermore, the deacetylation mimicking mutation of K216/217 increased the mitochondrial oxygen consumption rate suggesting that hypoacetylation at K216/217 enhances the function of LAP to stimulate mitochondrial respiration.

Although the data obtained suggest that the C/EBPβ function is regulated by acetylation, more research is needed to strengthen the results and to identify the underlying molecular mechanisms.

KEYWORDS: C/EBP, lysine acetylation, deacetylation, KAT, HDAC

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ABBREVIATION LIST

CBP (KAT3A)	CREB-binding protein
C/EBP	CCAAT/enhancer binding protein
dsDNA	Double-stranded DNA
EV	Empty vector
GCN5 (KAT2A)	General control of amino acid synthesis protein 5
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
IP	Immunoprecipitation
K	Lysine
KAT	Lysine acetyltransferase
KR	Lysine to Arginine
KQ	Lysine to Glutamate
P300 (KAT3B)	E1A-binding protein, 300 kDa
P/CAF (KAT2B)	P300/CBP associated factor
PTM	Post translational modification
Q	Glutamate
R	Arginine
Sirt	Sirtuin
ssDNA	Single-stranded DNA
TF	Transcription factor
TIP60 (KAT5)	Tat-interactive protein, 60 kDa
WB	Western Blot
wt	Wild type

INTRODUCTION

Ageing and C/EBPs

A frequently studied subject in the field of biomedical sciences is ageing. The process of ageing is associated with cancer (de Magalhães, 2013) and metabolic diseases (Labbadia, 2015; Ghachem, 2019) amongst others. An important link between ageing, cancer and metabolism is formed by the CCAAT/enhancer binding protein (C/EBP) family (Schäfer, 2018; Kimura, 2012; Zidek, 2015). C/EBPs are transcription factors that play a role in various cellular processes, such as differentiation, proliferation, metabolism and inflammation (Ramji, 2002). The C/EBP family consists of six different members named after the first six letters of the Greek alphabet (α through ζ). C/EBPα and C/EBPδ are predominantly found in adipose tissue, intestine and lungs. C/EBPα and C/EBPβ mainly reside in the liver, but like C/EBPδ, are also expressed in adipose tissue, intestine and lungs. C/EBPε can be found in myeloid and lymphoid cells, while C/EBPγ and ζ are expressed ubiquitously (Lekstrom-Himes, 1998). Although the different C/EBPs differ in localisation, together they form a complex system of gene regulation (Tsukada, 2011).

Special about C/EBPα and C/EBPβ homeostasis is their regulation through differential translation initiation. The gene sequences of C/EBPα and C/EBPβ contain two and three translation initiation sites respectively. Initiation of translation at these different sites establishes one isoform per site. P42 and P30 are the isoforms resulting from differential initiation of C/EBPα, while LAP*, LAP and LIP are C/EBPβ isoforms (fig. 1). The longer isoforms P42, LAP* and LAP generally have activating effects, while the shorter isoforms P30 and LIP generally have inhibiting effects on gene regulation (Nerlov, 2010). Regulation of the expression of these protein isoforms has been identified as an important component of the process of ageing. Recently, it has been demonstrated that reduced expression of one specific isoform of C/EBPβ has beneficial effects not only on lifespan, but also on health span in mice (Müller C, 2018).

Regulation through Acetylation

While regulation of expression of C/EBPs plays and important role in ageing, C/EBPs in turn have important effects on expression of other genes. An important process that regulates the function of C/EBPs and their regulation of gene expression is acetylation. Acetylation is a post-translational modification (PTM) by which a hydrogen atom of a protein is substituted by an acetyl group (fig. 2). Lysine acetylation is a particularly important process in the regulation of TFs. In this process, acetyl coenzyme-A donates an acetyl group to be transferred to a lysine residue by Lysine acetyltransferases (KATs). This can be reversed by histone deacetylases (HDACs), which are categorised into separate classes (I through IV). Class III HDACs (sirtuins) are special, since this group requires NAD^+ as cofactor, which means their activity is influenced by nutrients (Menzies, 2016). Acetylation of histone tails can activate gene expression directly by rendering the chromatin into a more open conformation (Koprinarova, 2016). Furthermore, acetylation of TFs such as C/EBPs, can regulate gene expression indirectly through changing their DNA binding capacity, protein stability, enzymatic activity, protein-protein interactions or subcellular localization. Here, the effect of acetylation depends on which lysine residue is acetylated (Bararia, 2016; Zaini, 2018).

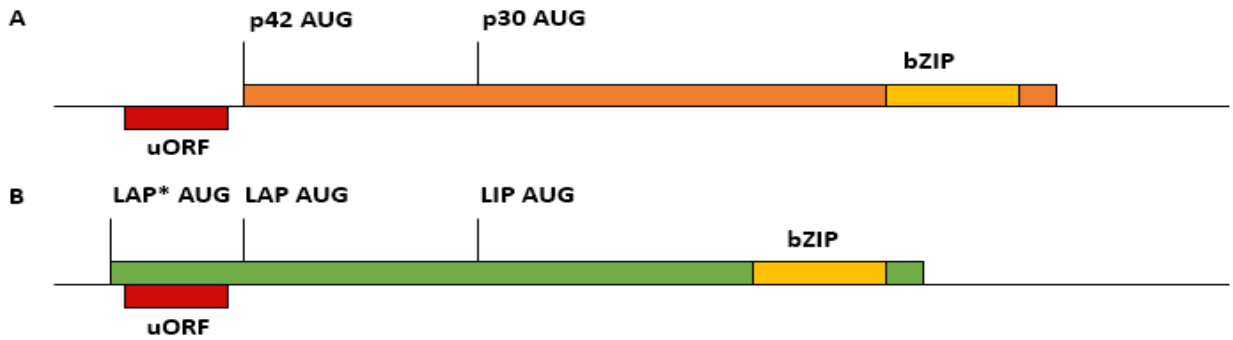

Figure 1. Differential translation initiation of C/EBPα and C/EBPβ

A schematic representation of C/EBPα and C/EBPβ mRNA. Indicated is the relative position of the upstream open reading frames (uORF) to the various translation initiation sites (AUG) that encode the different isoforms of C/EBPα (P42 and P30, A) and C/EBPβ (LAP*, LAP and LIP, B). Also included are the basic leucine zipper domains (bZIP).

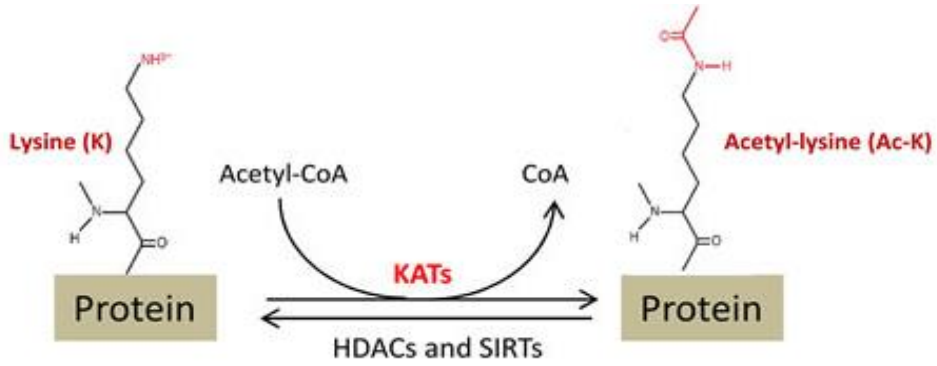

Figure 2. Schematic representation of lysine acetylation
The post translational modification (PTM) lysine acetylation is tightly controlled by various enzymes. Acetyl transferases (KATs) are able to place a lysine residue onto proteins, which can be reversed by histone deacetylases (HDACs) and sirtuin deacetylases (Di Martile, 2016).

Acetylation of C/EBPα, C/EBPβ and C/EBPε

C/EBPα, C/EBPβ and C/EBPε have been identified as targets for lysine acetylation. C/EBPε acetylation modulates DNA-binding abilities which impacts neutrophil differentiation and is a crucial component in this process (Bartels, 2015). C/EBPα according to the study of Zaini et al, is acetylated at multiple lysines by E1A-binding protein, 300 kDa (P300) and deacetylated by sirtuin 1 (Sirt1). This study demonstrated that acetylation at lysines at position 159 and 298 (K159 and K298) increases transcriptional activity, without altering DNA-binding ability. On the other hand, deacetylation has been associated with induced expression of mitochondria genes (Zaini, 2018). Another study indicated general control of amino acid synthesis protein 5 (GCN5, KAT2A) as a regulator of C/EBPα by acetylating K298, K302 and K326. In this study, DNA-binding ability is reduced by acetylation, which impairs granulocytic differentiation (Bararia, 2016). A proposed mechanism is that C/EBPα mediates adaption of energy homeostasis to changes in nutrient supply, controlled by Sirt1, while GCN5 acts as a negative regulator of C/EBPα function in myeloid differentiation. The inhibiting or activating effects of acetylation of C/EBPα could be regulated by interaction with different specific TFs and co-factors. While C/EBPα acetylation has been widely examined, less is known about C/EBPβ acetylation.

So far, it has been demonstrated that C/EBPβ is acetylated in vivo at K39, K117 and K215/216 by P300 and p300/CREB-binding protein associated factor (P/CAF). Deacetylation of K39 has been indicated to block C/EBP-mediated transcriptional activation, which is enhanced by acetylation of K39 (Ceseña, 2006). On the other hand, deacetylation is required for activating transcription of the Id-1 gene, because acetylation reportedly alters DNA-binding activity of C/EBPβ (Xu, 2003). These findings suggest that acetylation of C/EBPβ, like C/EBPα acetylation, has both inhibiting and activating effects. This could indicate that C/EBPβ function is similarly regulated through acetylation compared to C/EBPα. However, it is not yet known whether the K159 and K289 lysine residues of C/EBPα which are conserved in C/EBPβ as K134 and K293 are also acetylated. These lysines are located in protein domains that are also strongly conserved between C/EBPα and C/EBPβ, which makes it likely that regulation of protein function through acetylation is similar between both C/EBPs.

The aim of this study is to identify how lysine acetylation of C/EBPβ is regulated and how C/EBPβ function is affected by acetylation. By examining the levels of lysine acetylation of the C/EBPβ isoform LAP in response to the overexpression of different KATs and HDACs or in response to different glucose concentrations, we wanted to identify the mechanism behind the regulation of C/EBPβ acetylation. Furthermore, analysis of the transcriptional activity and mitochondrial function of acetylation mutants of C/EBPβ LAP should elucidate the effects of the acetylation status on C/EBPβ function. Therefore, this project might bring new insights into the regulation of the C/EBPβ activity, which could have implications also for the C/EBPβ function in the ageing process.

RESULTS

All C/EBPβ isoforms are acetylated in HEK-293T cells, independently of glucose availability

There are numerous lysines in the C/EBPβ isoforms that are conserved across vertebrate species, which could be potential targets for acetylation (sfig 1). To identify whether C/EBPβ is acetylated, HEK-293T cells were transfected with eukaryotic expression vectors for the different C/EBPβ isoforms LAP*, LAP

or LIP that were either tagged with a FLAG epitope or untagged. Preliminary testing of the success of transfection by western blotting demonstrated that both FLAG-tagged and non-tagged C/EBPβ isoforms were successfully overexpressed (fig. 3A). Analysis of C/EBPβ lysine acetylation using immunoprecipitation (IP) with an FLAG specific antibody followed by Western Blotting (WB) with an antibody recognizing acetylated lysines demonstrated that FLAG-tagged C/EBPβ LAP*, LAP and LIP are all acetylated, while transfection with an empty vector (EV (pcDNA3)) as a negative control showed no specific acetylation (fig. 3C). Furthermore, another negative control consisting of lysis buffer and beads only, identified bands present in all samples to be inherent to IP in this lysis buffer. C/EBPβ IP of non-tagged isoforms also showed acetylation of all three isoforms (fig. 3B). Doubling

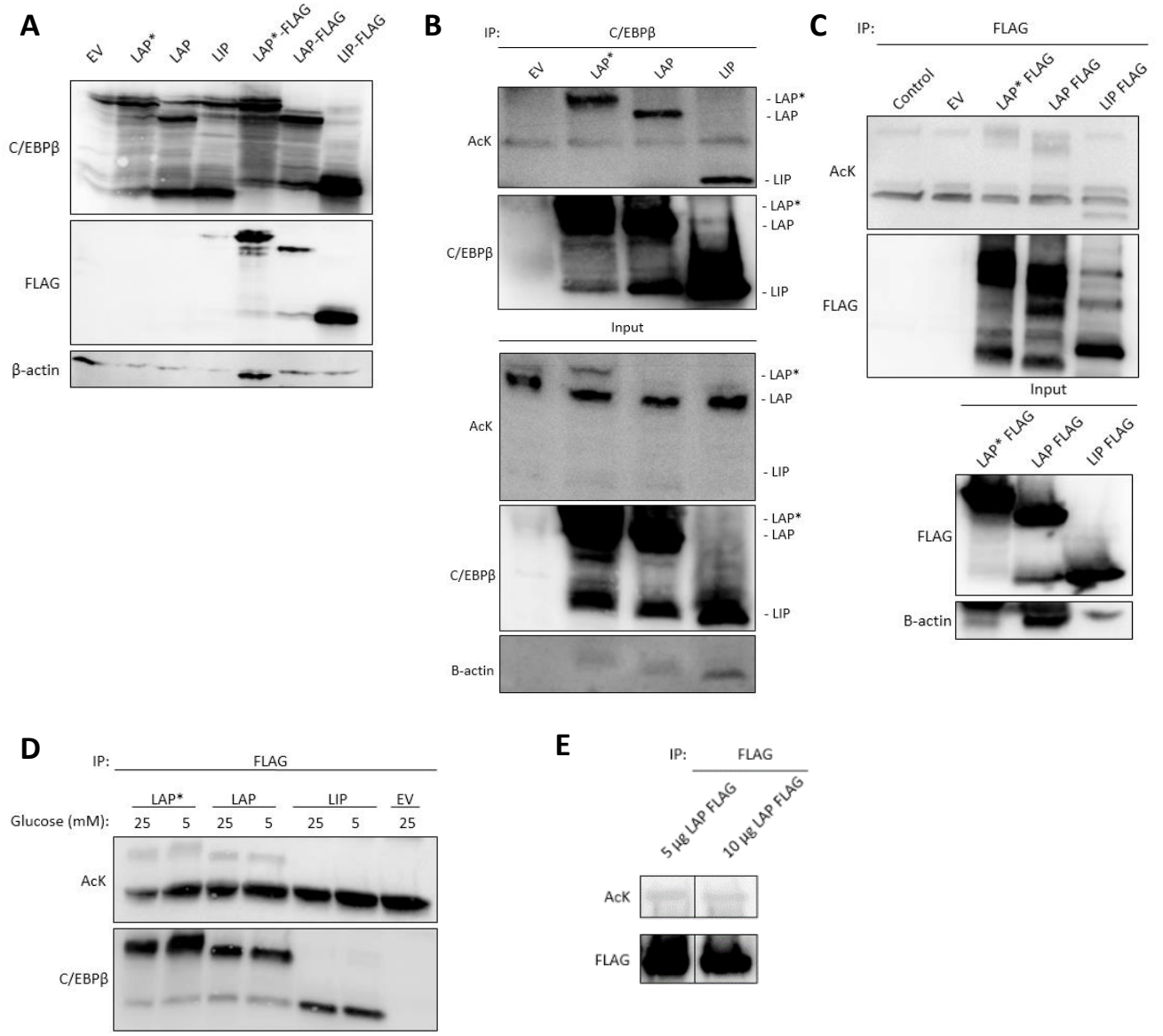

Figure 3. All C/EBPβ isoforms are acetylated when overexpressed in HEK-293T cells

(A) Western Blot analysis of cell lysates of HEK-293T cells transfected with non-FLAG-tagged and FLAG-tagged C/EBPβ isoforms LAP*, LAP and LIP. Antibody staining as indicated. Detection of β-Actin served as loading control of the input. **(B)** Western Blot analysis of immunoprecipitated (IP) C/EBPβ and total lysates (Input) of HEK293T cells transfected with C/EBPβ isoforms LAP*, LAP and LIP without FLAG-tag. Stained with antibodies as indicated. Detection of β-Actin served as loading control of the input. **(C)** Western Blot analysis of immunoprecipitated (IP) FLAG and total lysates (Input) of HEK293T cells transfected with FLAG-tagged C/EBPβ isoforms LAP*, LAP and LIP. Antibody staining as indicated. Results are representative for 3 experiments. Detection of β-Actin served as loading control of the input. **(D)** Western Blot analysis of immunoprecipitated (IP) FLAG and total lysates (Input) of HEK293T cells transfected with FLAG-tagged C/EBPβ isoforms LAP*, LAP and LIP under high (25 mM) or low (5 mM) glucose conditions. Antibody straining as indicated. Detection of β-Actin served as loading control of the input. **(E)** Western Blot analysis of immunoprecipitated (IP) FLAG of HEK293T cells transfected with 5 µg or 10 µg LAP FLAG-tagged construct. Antibody staining as indicated.

the amount of LAP C/EBPβ expression vector did not seem to alter the level of acetylation, indicating that expression of C/EBPβ does not increase when more expression construct is transfected (fig. 3E). To study the effects of nutrient availability on acetylation, HEK-293T cells transfected with C/EBPβ isoforms were cultured in medium containing either high (25 mM) or low (5 mM) glucose concentrations. Glucose concentration did not appear to affect the levels of acetylation of either of the isoforms (fig. 3D). Taken together, these results show that LAP*, LAP and LIP C/EBPβ are acetylated at lysine residues when overexpressed. This acetylation does not depend on the glucose availability.

Overexpression of P/CAF and GCN5 increases acetylation of C/EBPβ in HEK-293T cells

Now that C/EBP was shown to be acetylated in HEK293T cells, the next step was to determine how this process is facilitated. Since KATs are able to transfer acyl groups from acetyl co-enzyme A to lysine residues in DNA, the ability of different KATs to acetylate C/EBPβ was investigated. Different KATs (P300, P/CAF, GCN5, TIP60 and CBP) were co-transfected with FLAG-tagged C/EBPβ LAP in HEK-293T cells. To confirm successful co-transfection, cell lysates were analysed using Western Blotting. Whereas overexpression of P/CAF and GCN5 (which have a FLAG-tag) could be confirmed by detection with an FLAG-specific antibody and p300 and CBP (which have an HA-tag) could be confirmed by detection with an HA-specific antibody, overexpression of TIP60 (which has no tag) could not be detected with an TIP60-specific antibody (fig. 4A). This could either be explained by incompetence of the overexpression vector construct, by inefficient transfection or by inadequate detection sensitivity of the used TIP60 antibody. Next, levels of acetylation of C/EBPβ in the absence and presence of KATs were analysed. FLAG immunoprecipitation was performed on cells co-transfected with FLAG-tagged C/EBPβ LAP and one of the KATs. Western Blot analysis demonstrated that overexpression of C/EBPβ LAP in combination with either P/CAF or GCN5 increased acetylation compared to co-overexpression of C/EBPβ LAP and an empty vector (EV). Co-overexpression of C/EBPβ LAP and p300, TIP60 or CBP did not clearly alter lysine acetylation compared to co-overexpression of LAP C/EBPβ and empty vector. However, p300 slightly increased lysine acetylation in total cell lysates (fig. 4B). To assess whether the intrinsic KAT function facilitates acetylation of C/EBPβ, the KAT domain was deleted in both P300 and P/CAF (P300 mut, P/CAF ΔHAT). Again, the increase of lysine acetylation of LAP

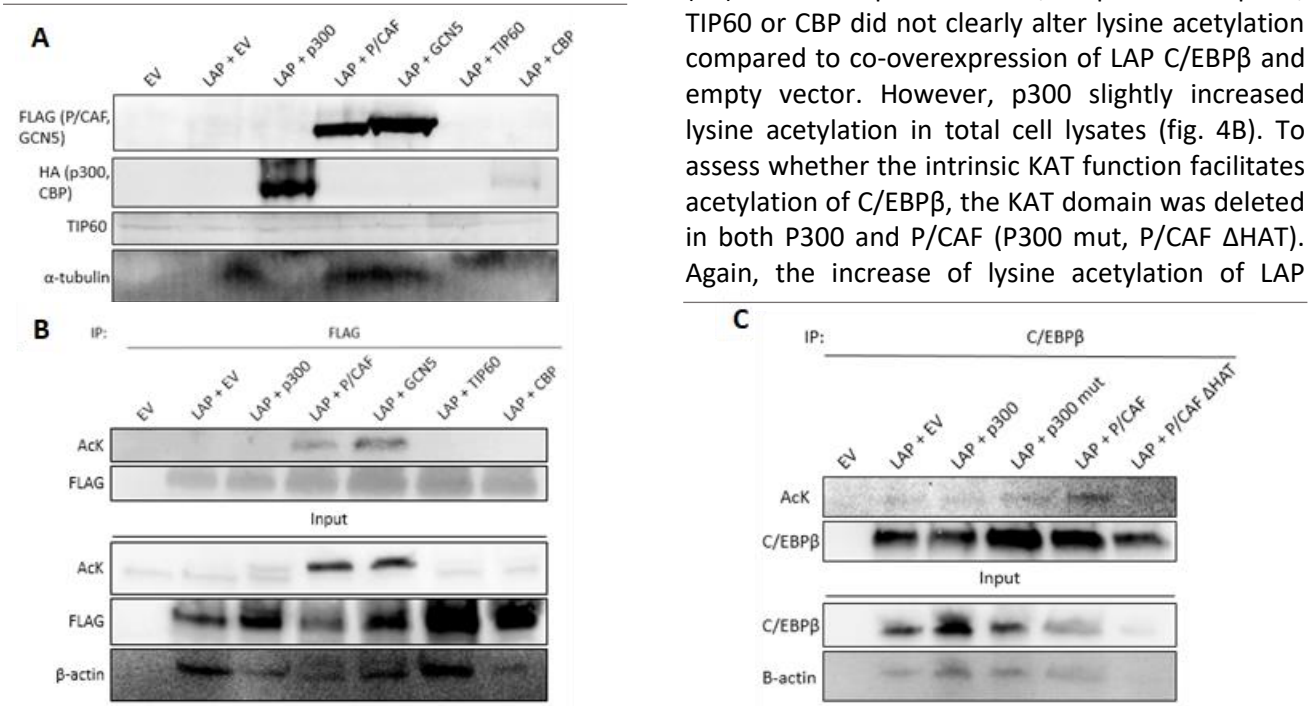

Figure 4. Overexpression of P/CAF and GCN5 increases acetylation of LAP C/EBPβ

(A) Western Blot analysis of HEK293T cells transfected with lysine acetyltransferases (KATs) p300, P/CAF, GCN5, TIP60 or CBP in combination with C/EBPβ LAP as indicated. Transfection with an empty vector (EV) or co-transfection with C/EBPβ LAP and an empty vector were used as control. Membranes were stained with antibodies as indicated. Detection of α-Tubulin served as loading control of the input. **(B)** Western Blot analysis of immunoprecipitated (IP) FLAG and total lysates (Input) of HEK293T cells co-transfected with C/EBPβ LAP and one of the acetyltransferases (p300, P/CAF, GCN5, TIP60 or CBP) as indicated. Empty vector (EV) alone or in combination with LAP C/EBPβ transfection were used as a control. Antibody staining as indicated. Detection of β-Actin served as loading control of the input. Results are representative for two experiments. **(C)** Western Blot analysis of immunoprecipitated (IP) C/EBPβ and total lysate (Input) of HEK-293T cells co-transfected with C/EBPβ LAP and P300-HA, P300 mut, P/CAF-FLAG, P/CAF ΔHAT or EV. Antibody staining as indicated. Detection of β-Actin served as loading control of the input.

C/EBPβ due to overexpression of P300 was minimal. Acetylation levels of C/EBPβ LAP where not decreased upon deletion of P300's KAT domain. P/CAF overexpression did increase acetylation, which was dependent on the enzymatic activity of P/CAF since a P/CAF mutant with a deletion of the KAT domain did not increase LAP acetylation when compared to overexpression of wt-LAP C/EBPβ alone (fig. 4C). An inactive mutant of GCN5 was not available and could therefore not be tested. Taken together, these results indicate that GCN5 and P/CAF facilitate acetylation of C/EBPβ. P300 might be involved in acetylation of C/EBPβ, although acetylation is only slightly enhanced. No firm statements can be made about TIP60 and CBP due to lacking or low overexpression.

C/EBPβ can be deacetylated by various classes of deacetylases

Next, it was examined how deacetylation of C/EBPβ is facilitated. For this purpose, HEK-293T cells were transfected with two classes of lysine deacetylases, sirtuins (class III HDACs) and histone deacetylases (HDACs). IP was performed to pull down FLAG-tagged C/EBPβ LAP proteins. At first sight, Sirt1 did not seem to distinctly decrease acetylation of C/EBPβ when overexpressed compared to when not overexpressed. However, P/CAF induced acetylation of C/EBPβ LAP was greatly reduced by Sirt1. (fig. 5A). It should, however, be mentioned that staining with various antibodies of both IP and input, suggests that protein content is not similar for all samples which makes the interpretation of the Sirt1 effect more difficult. Studying the role of HDACs in

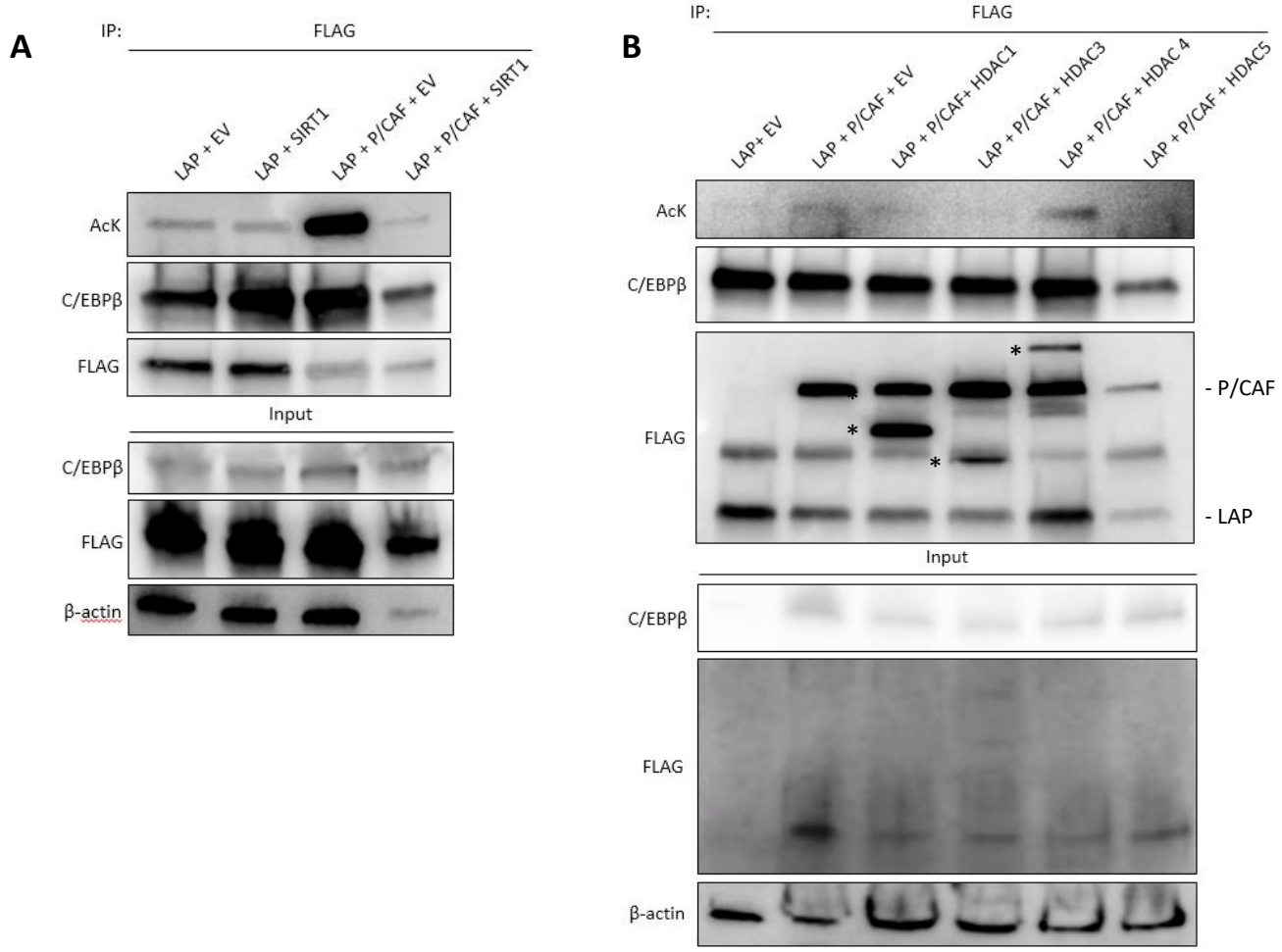

Figure 5. C/EBPβ can be deacetylated by various classes of deacetylases

(A) Western Blot analysis of immunoprecipitated (IP) C/EBPβ LAP FLAG and total lysates (Input) of HEK293T cells co-transfected with C/EBPβ LAP and Sirt1 with or without P/CAF as indicated. Co-transfection with C/EBPβ LAP and empty vector (EV) or C/EBPβ LAP, P/CAF and empty vector (EV) was used as a control. Membranes were stained with antibodies as indicated. **(B)** Western Blot analysis of immunoprecipitated (IP) FLAG and total lysates (Input) of HEK293T cells transfected with C/EBPβ LAP in combination with P/CAF and one deacteylases (HDAC1, HDAC3, HDAC4 or HDAC5 (*)) as indicated. C/EBPβ LAP in combination with empty vector (with or without P/CAF) was used as a control. Antibody staining as indicated. Detection of β-Actin served as loading control of the input.

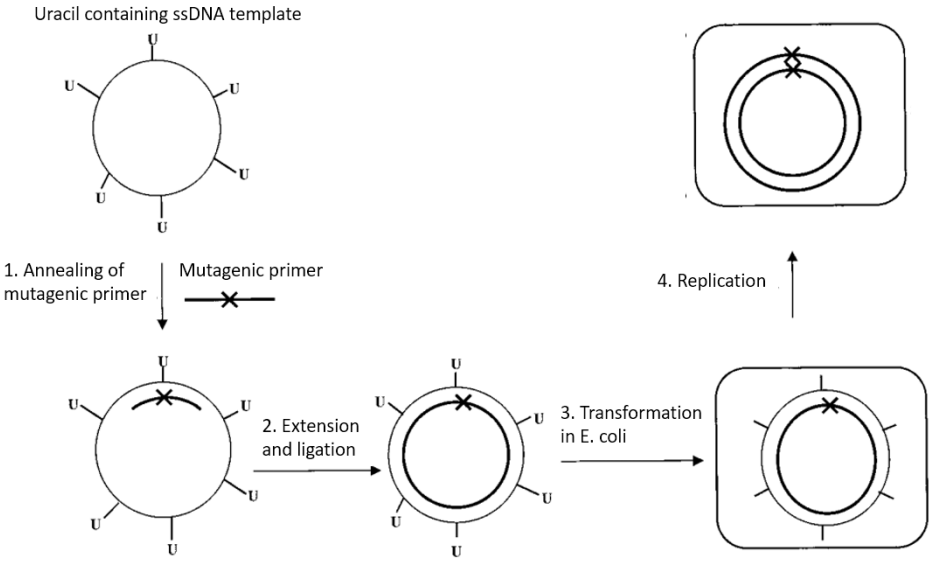

Figure 6. Schematic representation of the Kunkel method

A mutagenic primer is annealed to an uracil containing ssDNA circular template that was prepared through phage infection of an UTPase and uracil deglycosylase negative E.coli strain containing a phagemid with the sequence to be mutated. Addition of a mix containing dNTPs, polymerase and ligase enzymes, ensures extension and ligation of the primer and ssDNA to dsDNA conversion. The dsDNA construct is then transformed in an UTPase and uracil deglycosylase positive E. coli strain for replication.

acetylation of C/EBPβ LAP gave more conclusive results. C/EBPβ. HDAC1 and HDAC3 clearly diminish the P/CAF induced acetylation of C/EBPβ LAP (fig 5B). The effect of HDAC5 on LAP C/EBPβ acetylation was less clear. Although anti-AcK staining indicated less acetylation of C/EBPβ in response to HDAC5 overexpression, overall expression of LAP C/EBPβ and other FLAG-tagged proteins was also less (fig 5B). This makes the interpretation of the effects of HDAC5 less conclusive. Taken together, HDAC1 and HDAC3 contribute to deacetylation of LAP C/EBPβ, while deacetylation of C/EBPβ by Sirt1 and HDAC5 is less evident. HDAC4 does not deacetylate LAP C/EBPβ.

Preparation of acetylation and deacetylation mimicking DNA constructs.

After examining proteins involved in the regulation of C/EBPβ acetylation, the next step was to determine the effects of acetylation and deacetylation on transcriptional activity and mitochondrial function of C/EBPβ. For this purpose, DNA constructs were created to mimic either acetylation or deacetylation of specific lysine sites. Acetylation was mimicked by replacing lysine with glutamine (Q), while deacetylation was mimicked by replacing lysine with arginine (R). The Kunkel method was used to prepare constructs of each mutation (Kunkel, 1991). A schematic representation of this process can be seen in figure 6. The mutations were originally constructed using different uracil containing rat C/EBPβ ssDNA templates. From these successfully mutated constructs each mutation was introduced into the C/EBPβ LAP pcDNA3 expression vector by cloning (see materials and methods). Due to time limitations, not all mutants could be prepared. Table 1 represents which mutations were successfully prepared and which mutations were subsequently successfully cloned into pcDNA3. Here, amino acid and nucleotide sequences are also indicated.

Acetylation of lysine 239 reduces transcriptional activity

Acetylation of lysine 159/298 has been demonstrated to enhance transactivation of C/EBPα (Zaini, 2018). Therefore, the relation between acetylation status of individual lysine residues in C/EBPβ and its transactivation capacity was attempted to be identified. For this purpose, lysine (K) to glutamine (Q) or Lysine (L) to arginine (R) mutations were generated at several sites to mimic acetylation or deacetylation, respectively. Previous studies have already identified K39, K117 and K216/217 as possible acetylation sites in C/EBPβ (Ceseña, 2006, Xu, 2003). K134 and K239 of C/EBPβ are the homologous lysine residues of K159 and K298 in C/EBPα. These lysines where therefore chosen to be studied. A luciferase assay, using a reporter containing a C/EBP binding site

Table 1. List of mutations and the accompanying amino acid and nucleotide sequences. Successful preparations are also indicated.

Mutation		Amino acid sequence	Nucleotide Sequence	Mutation successfully constructed?	Successfully cloned into pcDNA3?
K39Q	*34*	LAYGAQAARAA	*gcccaagcg*		
K39R	*34*	LAYGARAARAA	*gcccgagcg*		
K134Q	*129*	PPAALQAEPGF	*ctccaagcc*	✓	
K134R	*129*	PPAALRAEPGF	*ctccgagcc*	✓	
K117Q	*112*	GRAGAQAAPPA	*gcgcaggcc*	✓	
K117R	*112*	GRAGARAAPPA	*gcccgagcc*	✓	
K216/217Q	*211*	AKAKAQQAVDKL	*gcccagcaggcg*	✓	✓
K216/217R	*211*	AKAKARRAVDKL	*gcccgagcgagcg*	✓	✓
K239Q	*234*	NIAVRQSRDKA	*cgccaagagc*	✓	✓
K239R	*234*	NIAVRRSRDKA	*cgccgagagc*	✓	✓

demonstrated strongly increased transcriptional activity upon co-transfection with a wt C/EBPβ LAP expression construct compared to the co-transfection with the empty vector. This LAP mediated reporter activation was not altered by the deacetylation mimicking (L to R) mutations. The acetylation mimicking mutation K216/217Q did also not significantly affect transcriptional activity of LAP. The K239Q mutation, on the other hand, significantly decreased transcriptional activity compared to wt-LAP (fig. 7). Taken together, these results indicate that the acetylated state of some, but not all lysine residues, decreases transcriptional activity of C/EBPβ.

Deacetylation of C/EBPβ increases mitochondrial function.

Deacetylation of C/EBPα has been shown to induce mitochondrial respiration through the activation of genes coding for different mitochondrial genes (Zaini, 2018). We wanted to test whether deacetylation of C/EBPβ would have similar effects on mitochondrial function. Therefore, we used the seahorse flux analyser to determine the oxygen consumption rate (OCR) as a measure for mitochondrial respiration using Hepa 1-6 mouse hepatoma cells transfected with C/EBPβ LAP wt and the different acetylation mutants. The results of this experiment suggest that deacetylated C/EBPβ might increase mitochondrial respiration similar to C/EBPα. This effect can most clearly be seen in case of overexpression of the double deacetylation mimicking mutant,–K216/217R. Although the basal oxygen consumption rate (OCR) was not significantly altered upon transfection of a C/EBPβ LAP K216/217R construct (fig. 8A), the maximal OCR was significantly increased (fig. 8B). When compared to EV and to K216/217Q, overexpression of the K216/217R double mutant clearly increased maximal OCR in all four time points of the measurement (fig 8C, 8D). In contrast the K239R mutant did not significantly affect neither basal nor maximal OCR (fig 8A, 8B and sfig. 3B). On the other hand, the effects of the acetylation mimicking mutants on mitochondrial respiration were less

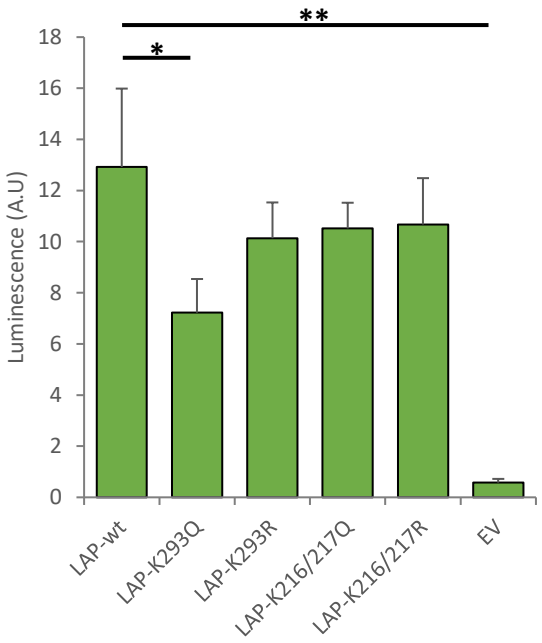

Figure 7. Acetylation of K239 reduces transcriptional activity
Bar graph of luciferase assay of HEK-293T cells transfected with a C/EBP responsive luciferase reporter, Renilla expression vector for normalisation and C/EBPβ LAP-WT or one of the C/EBPβ LAP mutatants (K to Q or K to R). Transfection with empty vector (EV) was used as control. Individual bar represent 3 replicates. Statistical differences were analysed using a t.test. Error bars represent ±SD. *p < 0.05, **p < 0.01

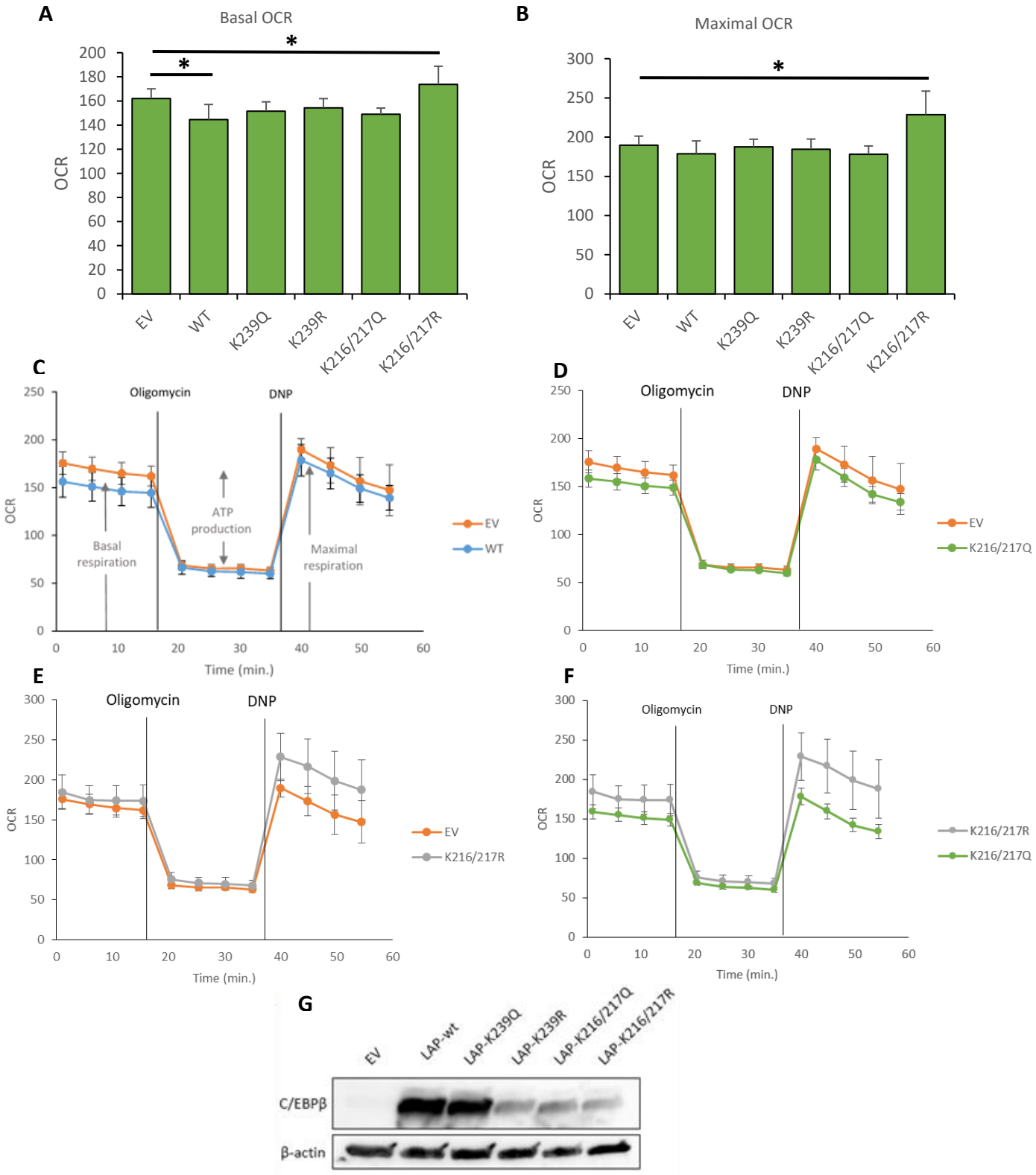

Figure 8. Deacetylation of C/EBPβ increases mitochondrial function

(A) Basal oxygen consumption rate (OCR) of HEPA1-6 cells transfected with C-EBPβ LAP-wt or one of the acetylation/deacetylation mimicking mutants. **(B)** Maximal oxygen consumption rate (OCR) HEPA1-6 cells transfected with C-EBPβ LAP-wt or one of the acetylation/deacetylation mimicking mutants. **(C-E)** Oxygen consumption rate (OCR) of HEPA1-6 cells transfected with C/EBPβ LAP-wt (C), -K216/217Q (D) or –K216/217R (E) as compared to HEPA1-6 cells overexpressing an empty vector (EV). Values are means ± SD, (n = 6). **(F)** Oxygen consumption rate (OCR) of HEPA1-6 cells transfected with C/EBPβ LAP-K216/217R as compared to HEPA1-6 cells overexpressing C/EBPβ LAP-K216/217Q. Values are means ± SD, (n = 6). **(G)** Western blot analysis of HEPA1-6 cells transfected with C/EBPβ LAP -K239Q, -K239R, -K216/217Q or –K216/217R. Transfection with EV and C/EBPβ LAP-wt served as controls. Antibody staining as indicated. Detection of β-Actin served as loading control of the input.

convincing. Basal OCR was significantly reduced by K216/216Q overexpression compared to the empty vector control, but this reduction was rather mild and not evident in all measured time points (fig. 8A, 8F). Moreover, maximal OCR was not effected by K239Q nor K216/217Q overexpression (fig. 8B). Although not significant, there was a trend present where LAP wt and K216/217Q overexpression lead to decreased basal and maximal OCR (fig. 8E, 8F). K239Q overexpression similarly slightly but not significantly decreased basal OCR, but did not affect maximal OCR (fig 8A, 8B, sfig. 3A). Western blot analysis showed differential levels of overexpression; both wt and K239Q had higher protein expression levels, compared to the other constructs, while the β-actin levels were similar (fig. 8G). This makes the interpretation of the results more complicated. Taken together, deacetylation of C/EBPβ at lysines 216/217 increases mitochondrial function, while acetylation rather seems to decrease mitochondrial function.

DISCUSSION

This study demonstrates that the C/EBPβ isoforms LAP*, LAP and LIP are all acetylated when overexpressed in HEK-293T cells. The acetylation levels are seemingly independent of glucose availability. We found that the regulation of C/EBPβ acetylation is controlled by different enzymes. Our results demonstrate that P/CAF and GCN5 facilitate acetylation, while HDAC1 and HDAC3 facilitate deacetylation of C/EBPβ. In addition the class III deacetylase SIRT1 might also be involved in C/EBPβ deacetylation although in this case our data are less clear and have to be repeated. Furthermore, we could show that the acetylation mimicking mutation K293Q significantly decreases the transcriptional activity of C/EBPβ LAP and that the acetylation mimicking mutation of K216/217 significantly reduces basal OCR. The deacetylation mimicking mutation of K216/217 significantly increases the mitochondrial function of C/EBPβ LAP. Although not significant, an acetylation mimicking mutation of K293 might decrease the mitochondrial function of C/EBPβ LAP trend-wise. These results illustrate that both transcriptional activity and mitochondrial respiration are impacted by the acetylation status of C/EBPβ.

Acetylation of C/EBPβ

Previous studies have identified K39, K117 and K216/217 as sites of acetylation in C/EBPβ in HEK-293T cells and Ba/F3 cells (Ceseña, 2006; Xu, 2003). Therefore, these sites were chosen to be mutated in this study. In addition, we wanted to compare acetylation of C/EBPβ to that of C/EBPα. For this reason, we have also included K134 and K293, the C/EBPβ lysine residues that are homologous to the most important acetylation sites in C/EBPα, K159 and K298. Because we wanted to be able to compare our findings of C/EBPβ to recent findings of C/EBPα, we have used to same cell types as Zaini, et al. In the present study, we aimed to investigate the effects of the different lysine mutations on total acetylation of LAP C/EBPβ in HEK-239T cells by performing IP. From this experiment we wanted to see if one of the lysine residues is predominantly acetylated. We also wanted to determine whether acetylation of one of the lysine sites stimulates acetylation of subsequent sites as seen for K298 in C/EBPα. Although this experiment was performed, the resulting WB showed no bands at all, suggesting that the experiment failed. Since there was no time left to repeat the experiment, no statements can be made about acetylation levels in the acetylation mutants and the influence on other acetylation sites. Besides repetition of this experiment, future experiments should also combine several R mutants to determine which sites mainly contribute to acetylation of C/EBPβ. Mass spectrometry would be a good method to discover which lysines are acetylated in each cell line.

While the studies mentioned before (Ceseña, 2006; Xu, 2003) have already identified C/EBPβ to be acetylated, data are limited to acetylation of the longer isoforms LAP* and LAP. The study presented here demonstrates that all three isoforms, including the shorter isoform LIP, are acetylated when overexpressed in HEK-293T cells. However, quantitative comparisons cannot be made, since expression of house-keeping gene β-actin in the input controls was variable between samples of both IPs using either the FLAG or the C/EBPβ specific antibody. Nonetheless, both experiments are excellent indicators of acetylation of C/EBPβ. The use of anti-FLAG beads seemed to be more effective since IP using protein A beads was not always successful. When possible, the preferred method was therefore an IP with use of anti-FLAG beads. Attempts to detect acetylation of

endogenous C/EBPβ isoforms in Hepa1-6 mouse hepatoma cells and Fao rat hepatocarcinoma cells failed. This was probably due to low expression levels of endogenous C/EBPβ.

Regulators of C/EBPβ acetylation/deacetylation

The profound structural commonalities of the DNA binding and dimerization domain of C/EBPα and C/EBPβ lead to the presumption that acetylation in this domain might be regulated similarly. C/EBPα has been shown to be acetylated mainly by p300, but our study indicates a more important role for P/CAF and GCN5 in the acetylation of C/EBPβ. In the experiments presented here, FLAG-tagged LAP C/EBPβ was co-transfected with differently tagged KATs. It could be argued that IP using anti-FLAG beads artificially increases acetylation of P/CAF and GCN5 (which are also FLAG-tagged), because both proteins are brought into close proximity. Therefore, an IP using the C/EBPβ specific antibody would be less biased. As mentioned before, IP using the C/EBPβ specific antibody together with protein A beads was not always successful. Also in this case, protein A beads were not effective to pull down C/EBPβ after overexpression of the five different KATs. A smaller experiment with protein A beads was successful, but it only contained wt and mutated P/CAF and P300 and not the other KATs. From this experiment we can clearly conclude that P/CAF increases acetylation of C/EBPβ LAP more strongly than P300 and that the catalytic function of P/CAF is required for acetylation of C/EBPβ LAP.

Unfortunately, overexpression of TIP60 could not be confirmed by anti-TIP60 staining. Therefore, it cannot be concluded that lack of acetylation under TIP60 overexpression is due the inability of TIP60 to acetylate C/EBPβ. It could also be that transfection with TIP60 was unsuccessful. If the transfection of TIP60 was successful but the antibody detection did not work in the western blotting it would be still unclear whether TIP60 can acetylate C/EBPβ or not because TIP60 needs to be phosphorylated in order to carry out its KAT function. Phosphorylation of TIP60 correlates with the G2/M phase of the cell cycle (Lemercier, 2003) indicating that TIP60 only exhibits KAT functions when cells are dividing. Since not all cells divide at the same time, it could be hypothesised that the amount of cells in G2/M phase (and with that in a C/EBPβ acetylated state) is below the detection threshold. However, the most likely argument is that the transfection with TIP60 simply did not work.

Since our findings strongly indicate that P/CAF acetylates C/EBPβ, P/CAF was co-transfected with different deacetylases to increase the initial acetylation signal. P/CAF-induced acetylation was clearly reduced upon overexpression of HDAC1 and HDAC3. However, overexpression of HDAC5 could not be detected by WB due to either a lower protein content of this sample or due to unsuccessful transfection of the HDAC5 construct. Excluding HDAC5, all samples were loaded equally. This indicates that the impaired acetylation of C/EBPβ by HDAC1 and HDAC3 might actually be true.

Effects of sirtuins on C/EBPβ acetylation were also attempted to be examined multiple times. However, acetylation of endogenous C/EBPβ could not be detected in the control sample without sirtuin co-transfection which prevented the examination any LAP C/EBPβ deacetylation mediated by different sirtuins. In a smaller experiment, acetylation of C/EBPβ in the control could be detected. This experiment, however, only included Sirt1 and not the other sirtuins. At first sight no clear differences in basal acetylation level were visible after Sirt1 overexpression. However, the differences in the levels of LAP C/EBPβ should be taken into account. Under overexpression of Sirt1, more LAP C/EBPβ was immunoprecipitated as compared to EV overexpression. So, when the acetylation levels are similar, it should be concluded that relative levels of acetylation are decreased under Sirt1 overexpression. Similarly, co-overexpression of P/CAF and Sirt1 showed varying levels of LAP C/EBPβ, but still the relative levels of acetylation are decreased. Thus, HDAC1 and HDAC3 clearly mediate deacetylation of C/EBPβ in HEK-293T cells. Sirt1 probably also mediates C/EBPβ deacetylation, but to be able to draw firm conclusions, the experiment needs to be repeated.

Nutrient dependent regulation of C/EBPβ acetylation

Since enzymatic activity of metabolic enzymes such as Sirt1 is regulated by nutrients, we wanted to identify if glucose concentrations affect enzyme-mediated acetylation of C/EBPβ. Low glucose levels activate Sirt1 by increasing the NAD+/NADH ratio (Houtkooper, 2012). Acetyltransferases on the other hand, are more active under high glucose levels, due to the substrate acetyl-CoA being more abundant (Albaugh, 2011). C/EBPα had already been

demonstrated to be deacetylated by Sirt1 under low glucose conditions and that acetylation of C/EBPα mediates the effects of Sirt1 on mitochondrial function (Zaini, 2018). From the results of our study is not clear if glucose concentrations affect C/EBPβ acetylation or not. Unfortunately, lysine acetylation of endogenous C/EBPβ could not be detected using Hepa 1-6 and Fao cells growing under either high or low glucose concentration. A western blot with Hepa 1-6 and Fao cells indicated that at least the expression levels of endogenous levels of C/EBPβ are not effected by different glucose levels (sfig. 2). However, in HEK-293T cells overexpressing C/EBPβ isoforms, the reduction in glucose concentrations did not seem to affect the C/EBPβ acetylation levels. It is possible that glucose dependency is cell-type specific. Hepa 1-6 and Fao cells, being cells from a metabolic organ, the liver, might be more affected by changes in glucose concentration than embryonic kidney cells. Therefore, we cannot conclude from our experiments if C/EBPβ is deacetylated upon the reduction in glucose levels similar to C/EBPα. Repeating this experiment in Hepa 1-6 and Fao cells is required. Such a nutrient dependent regulation of C/EBPβ acetylation would be very interesting, because both C/EBPβ and the nutrient regulated Sirt1 are known to be involved in the regulation of health- and lifespan (Müller, 2018; Mitchell, 2019). Therefore the potential nutrient-regulated deacetylation of C/EBPβ by Sirt1 could play an important role for health- and lifespan regulation.

Effect of acetylation on the transcriptional activity of C/EBPβ

Acetylation and deacetylation mimicking mutants of different lysine residues were used to investigate the effects of acetylation on the transactivation function of C/EBPβ LAP. A luciferase reporter assay identified the acetylation mimicking mutation of lysine 239 (K239Q) to inhibit the transcriptional activity of LAP, while the deacetylation mimicking mutations had no effect on transcriptional activity. In C/EBPα, the acetylation of K159, the homologous lysine residue to C/EBPβ K134 and K298, the homologous lysine residue to C/EBPβ K239, both increased the transcriptional activity. Unfortunately, effects of C/EBPβ K134 acetylation status could not be tested, because subcloning into the expression vector was not finished yet. As mentioned before, acetylation of proteins can affect gene expression on different levels; DNA binding, protein stability, protein-protein interactions or subcellular localization. From this experiment, it cannot be concluded how the K239Q mutation decreases transcriptional activity. Since the K239 lies within the DNA binding domain it is possible that acetylation affects the DNA binding potential of C/EBPβ. However, the homologous lysine residue in C/EBPα did not inhibit the DNA binding (Zaini 2018). Additional experiments have to clarify the underlying mechanism of the effect of K239 acetylation in C/EBPβ.

Acetylation of C/EBPβ K216/217 has been shown to inhibit DNA-binding *in vitro* (Xu, 2003). In our reporter assay no effects were seen with the K216/217Q acetylation mimicking mutation compared to the wt LAP construct. This puts the inhibition of DNA binding by acetylation of these lysine residues into debate, because LAP C/EBPβ needs to bind DNA to be able to transactivate. The DNA binding activity of this mutant and of the K239Q mutant could be tested using bandshift experiments. In addition, the subcellular localization of the K216/217Q mutant should be examined by immunofluorescence experiments since the lysines 216/217 are located in one of the two nuclear localization signals of C/EBPβ and thus the subcellular localization of C/EBPβ could be affected.

Effect of acetylation on the mitochondrial function of C/EBPβ

Mitochondrial respiration was examined by measuring oxygen consumption rates (OCR) of Hepa 1-6 liver cells overexpressing the different acetylation or deacetylation mimicking mutants using a Seahorse Flux analyser. In C/EBPα, expression of the deacetylation mimicking mutants (KR mutants) resulted in an increased oxygen consumption compared to the expression of a wt C/EBPα construct. Previous research has demonstrated that oxygen consumption of cells is increased by LAP C/EBPβ in mouse embryonal fibroblasts (Zidek, 2015). In this study, although not significant, LAP C/EBP overexpression rather inhibits OCR compared to an EV. Both K239Q and L216/217Q mutants seem to have similar effects as the wt LAP construct. The K239R and K216/217R mutant on the other hand seem to increase basal OCR again to

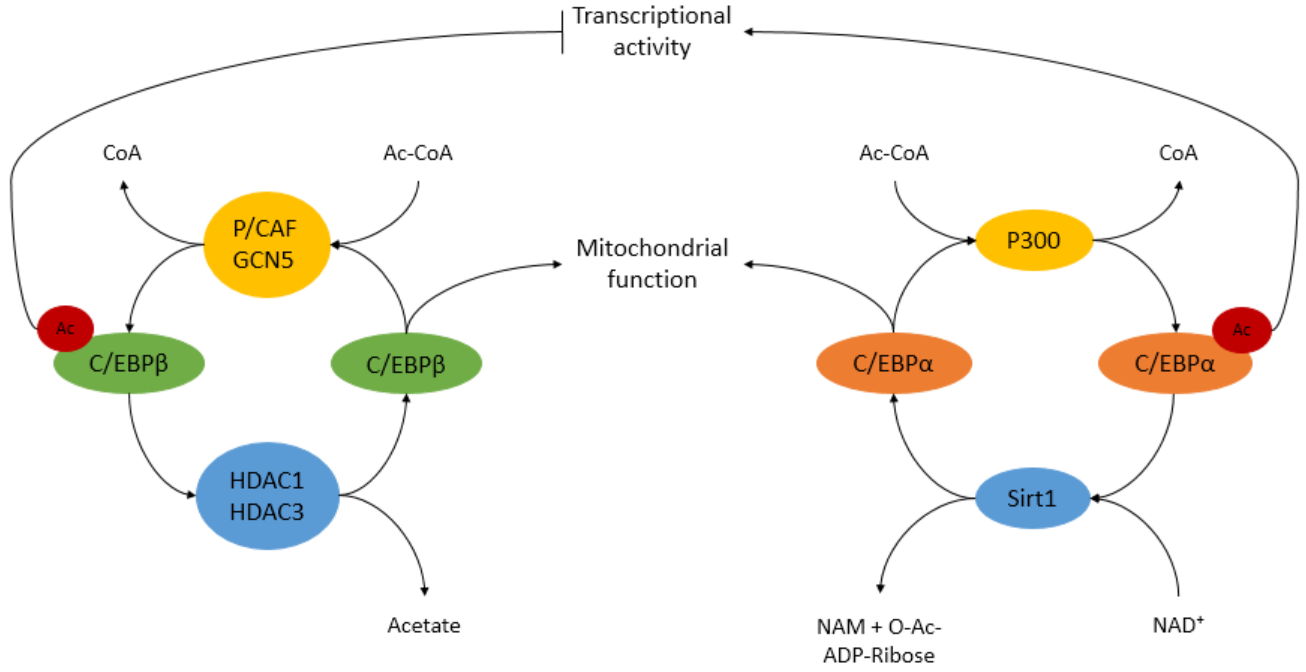

Figure 9. Proposed mechanisms of C/EBPα and C/EBPβ acetylation. C/EBPβ is mainly acetylated by P/CAF and GCN5 and deacetylated by HDAC1 and HDAC3. C/EBPα is mainly acetylated by P300 and deacetylated by Sirt1. Acetylation of C/EBPβ impairs transcriptional activity, while acetylation of C/EBPα promotes transcriptional activity. Deacetylation of both C/EBPα and C/EBPβ enhances mitochondrial function

levels seen in EV overexpression. It could be hypothesised that acetylation of wt C/EBPβ LAP is already high in the Hepa 1-6 cells and therefore cannot stimulate mitochondrial respiration. This could be also the reason why the Q mutants do not further inhibit acetylation. R mutants could than interfere with the inhibitory function of acetylated wt- C/EBPβ LAP. An important follow up experiment would be to check for acetylation of transfected wt C/EBPβ LAP compared to mutants in Hepa 1-6 cells. Interestingly, expression of the K216/217R deacetylation mimicking mutant significantly increased the maximal OCR when compared to either empty vector, wt LAP or the K216/217Q acetylation mimicking mutant. This could mean that like with C/EBPα, the deacetylation of C/EBPβ stimulates mitochondrial respiration. However, this experiment has to be repeated to verify this finding.

Outlook and summary

In this study only the effects of acetylation of C/EBPβ LAP had been investigated. Acetylation of the other C/EBPβ isoforms could potentially have different outcomes which might be interesting to test in the future. Another experiment to be performed is a genome-wide transcriptome analysis to examine differential activation or repression of genes between acetylation and deacetylation mimicking mutants. A reporter assay can only mimic a subset of target gene promoters, while the actual regulation of target genes is much more complex due to interactions between different TFs and cofactors, the interaction between promoters and enhancers and the embedding of the promoters and enhancers in the chromatin. This cannot be completely mimicked with a reporter assay, which is only the first step of the analysis.

Taken together, this project suggests that C/EBPβ is mainly acetylated by P/CAF and GCN5. HDAC1 and HDAC3 deacetylate C/EBPβ. Acetylation of C/EBPβ inhibits transcriptional activity of C/EBPβ, while it enhances the transcriptional activity of C/EBPα. This suggests that C/EBPα and C/EBPβ are regulated differently. One result shared between C/EBPα and C/EBPβ is that hypoacetylation enhances their mitochondrial function (fig. 9) Due to time limitations many experiments could not be repeated. Results from these missing experiments would have helped to answer the question more conclusively if C/EBPα and C/EBPβ are regulated similarly through acetylation/deacetylation.

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MATERIALS & METHODS

DNA constructs

DNA constructs of both FLAG-tagged and non-tagged C/EBPβ isoforms were generated in-house previously. Source of acetyltransferases and deacetylases can be found in table 3. DNA constructs containing K to Q or K to R mutations were generated by site directed mutagenesis according to the Kunkel method (Kunkel, 1991). Lysines that were mutated to either glutamine (Q) or arginine (R) are highlighted in supplementary figure 1. Uracil containing ssDNA was prepared using the E. coli strain CJ236 containing phagemid DNA and a helper phagemid. Rat C/EBPβ-wt pGEM-7Zf (+) single-stranded (ss) DNA was used as a template for generating C/EBPβ K239Q and K239R mutants. Rat C/EBPβ~uORF-pSG5 ssDNA was used as a template for generating C/EBPβ K216/217Q, K216/217R, K134Q, K134R, K117Q and K117R mutations. Rat C/EBPβ ΔuORF ATG ss uDNA was used as a template for the generation of the K39Q and K39R mutations. Primers containing the desired mutations were phosphorylated and annealed to the ss uDNA template. Addition of a mix containing dNTPs, T4 DNA polymerase and T4 DNA ligase enzymes, ensured extension of the primers and ssDNA to dsDNA conversion.

The primers used for site directed mutagenesis are shown in supplementary table 1. E. coli (TOP10F) were then transformed with the mutated plasmids and plated on LB-agar plates containing ampicillin (100µg/ml). Ampicillin resistant clones were picked and plasmid DNA was amplified using a mini prep protocol. The plasmid DNA was then screened for successful mutation by restriction digest (see supplementary table 2 for changed restriction sites due to the introduced mutation). For further cloning, mutations were introduced into specific C/EBPβ isoforms by ligation into the pcDNA3 vector, cut with *EcoRI* and treated with phosphatase. K239Q, K239R (3' insert), wt-LAP* and wt-LAP C/EBPβ (5' insert) double-stranded (ds) DNA were digested with *EcoRI* and *BstBI* and run on a 1% agarose gel. Fragments of the constructs (LAP*: 455 bp, LAP: 388 bp, K239Q/R: 976 bp) were isolated. Fragments of LAP* in combination with K239Q or K239R and fragments of LAP in combination with K239Q or K239R were ligated into pcDNA3. K216/217Q, K216/217R (3' insert), wt-LAP* and wt-LAP C/EBPβ (5' insert) dsDNA were digested with *EcoRI* and *BstBI* and run on a 1% agarose gel. Fragments of the constructs (LAP*: 455 bp, LAP 388 bp, K134Q/R: 967 bp) were isolated. Fragments of LAP* in combination with K216/217Q or K216/217R and fragments of LAP in combination with K216/217Q or K216/217R were ligated into pcDNA3. K134Q, K134R, K117Q, K117R (3' insert), wt-LAP* and wt-LAP C/EBPβ (5' insert) dsDNA were digested with *EcoRI* and *EarI* and run on a 1% agarose gel. Fragments of the constructs (LAP*: 250 bp, LAP 200 bp, K134Q/R: 1104 bp K117Q/R: 1104 bp) were isolated. Fragments of LAP* in combination with either of the 3' inserts and fragments of LAP C/EBPβ in combination with either of the 3' inserts were ligated into pcDNA3. Ligated plasmids were transformed into E. coli (TOP10F) and plated on LB-agar plates containing ampicillin (100µg/ml). Ampicillin resistant clones were picked and plasmid DNA was amplified using a mini prep protocol. The plasmid DNA was then screened for successful cloning by restriction digest using EcoRI. To ensure correct orientation of the inserts, an orientation digest (*BamHI, NheI*) was performed.

Cells and transfection

HEK-293T, Hepa 1-6 and Fao cells were cultured in DMEM supplemented with 10% FBS (TICO Europe), penicillin/streptomycin and Hepes buffer at 37 °C and 5% CO2. HEK-293T cells were seeded in 10 cm dishes at a density of 3.8 x 10^6 cells/dish 24 hours prior to transfection. 5 µg of expression vector was used for single transfection while 4 µg or 3 µg of expression vector was used for co-transfection (with 2 or 3 constructs respectively, unless otherwise indicated). For transfection, the calcium phosphate method was used. Approximately 16 hours after transfection cells were washed twice with 1x PBS. Deacetylase inhibitors Trichostatin A (TSA, 10 µM, Sigma-Aldrich) and Nicotinamide (NAM, 5 mM, Sigma-Aldrich) were added 16 hours before harvesting. Cells were harvested in PBS and prepared for IP. Hepa 1-6 and Fao cells were seeded at 1/8 and 1/6 respectively of one full maxi plate. 24 hours after seeding, cells were washed twice with PBS and culturing medium was replaced with DMEM containing either high glucose (25 mM) or low glucose (5mM) concentrations, supplemented with deacetylase inhibitors TSA (10 µM) and NAM (5 mM). After incubating 16 hours in the specific glucose medium under normal culturing conditions, cells were harvested and prepared for IP.

Immunoprecipitation (IP)

Cells were lysed by incubation in Lysis Buffer (50mM Tris/HCl pH 7.4, 300mM NaCl, 1mM EDTA, 1% Triton X-100 supplemented with 1 mM phenylmethyl sulfonyl fluoride (PMSF), protease inhibitor cocktail (Roche), and the deacetylase inhibitors TSA 1 mM and NAM 5mM) for 30 minutes on ice. Binding of antibodies (C/EBPβ (C19), Santa Cruz Technologies) to protein A magnetic beads (Novex) was facilitated by incubation for approximately 1 hour at RT under constant rotation. For binding of proteins to the anti-FLAG-M2 (Sigma-Aldrich) or to C/EBPβ specific antibody bound to protein A magnetic beads, lysates and beads were incubated for 3 hours at 4 °C under constant rotation. After precipitation, beads were washed 5 times with wash buffer (50mM Tris/HCl pH 7.4, 300mM NaCl, 1mM EDTA, 0.1% Triton X-100 supplemented with 1 mM PMSF, protease inhibitor cocktail (Roche), and the deacetylase inhibitors TSA 1 mM and NAM 5mM). To complete the washing procedure, beads were washed once with PBS. To dissociate proteins from the beads, samples were dissolved in 2x SDS loading buffer with low β-mercaptoethanol (2x SDS loading buffer without β-mercaptoethanol was used for transfections with C/EBPβ LIP) and heated at 95 °C for 3 minutes. Hereafter, protein content was analysed using Western Blotting.

Western Blotting (WB)

Proteins were separated on a precast 10 well gel using 1x SDS running buffer (2.5 mM Tris; 19.2 mM Glycine; 0.01% SDS) and then transferred onto a PVDF membrane using Trans-Blot Turbo RTA Midi PVDF Transfer Kit. The Trans-Blot Turbo System was used to transfer proteins from the gel to the PVDF membrane (all BioRad). Membranes were blocked in 5% milk in TBS-T (Tris-buffered saline + tween) for approximately 1 hour. Washing of membranes was performed using 1x TBS-T. Membranes were incubated with primary antibodies (listed in table 2) overnight at 4 °C under constant agitation. Used dilutions and product details can be found in table 2. Incubation with secondary antibodies mouse or rabbit HRP-linked whole antibody (both Amersham Life Technologies) lasted 1 hour at RT under constant agitation. ECL (PerkinElmer) was added to chemiluminescently visualise bands using the ImageQuant LAS 4000 Mini (GE Health Care). ImageQuant TL (GE Health Care) was used to record the imaged membranes.

Luciferase Assay

For the luciferase assay, 2.5 x 10^4 HEK-293T cells/well were seeded in a 96-well plate. 24 hours after seeding, cells were transfected in triplicates using FuGENE (Promega) with a mixture of Opti-MEM containing the M6D firefly luciferase reporter (Sterneck, et al. 1992), Renilla expression vector (for monitoring transfection efficiencies) and C/EBPβ expression vectors as indicated. After 48 hours, firefly luciferase and Renilla luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega) according to protocol. A GloMax-Multi Detection System (Promega) was used to measure luminescence. The Dual-GLo preset protocol of the GloMax system was used for the correct settings for the measurements.

Measurement of OCR

For measuring the oxygen consumption rate (OCR), Hepa 1-6 cells were seeded at a density of 5.0 x10^5 cells/well of a 6 well-plate. Cells were transfected with 5 µg expression vector (EV, wt-LAP C/EBPβ or mutated LAP C/EBPβ) 24 hours after seeding using FuGENE. The next day, cells were counted six times to ensure precise seeding of equal amount cells. Cells were then seeded at a density of 2.5 x 10^5 cells/well of a Seahorse 96-well plate. 16 hours later, OCR were measured using a Seahorse XF96 Extracellular Flux analyser (Seahorse Bioscience) by injection of Oligomycin A (25 µM), Dinitrophenol (500 µM), Rotenone (20 µM), Antimycin A (40 µM) and Bromopyruvate (1 mM) on different time points. OCR of each transfection was measured in eightfold.

Statistical Analysis

Excel (Microsoft Office) was used to perform statistical analysis. A two tailed independent t.test was used to compare different groups. When $p < 0.05$, differences between groups were considered to be significant. Values of groups represent the mean of all samples of that group. Error bars represent the calculated standard deviation (SDEV) of each group.

Table 2. List of used antibodies. * Antibody used for IP, ** secondary antibody (all other listed antibodies are primary antibodies)

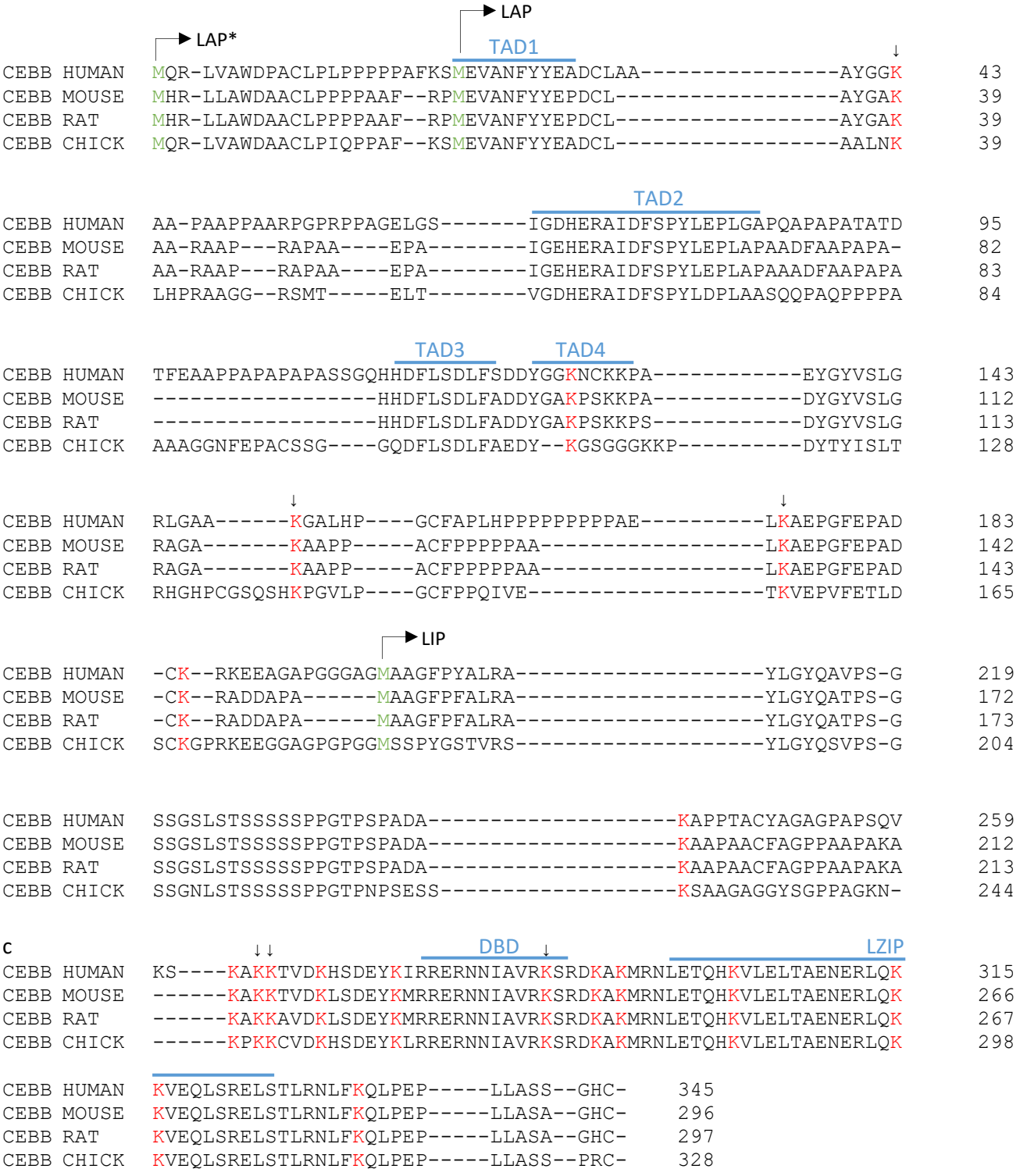
Antibody	*Dilution*	*Manufacturer*	*Catalogue number/manufacturer*
Mouse Monoclonal anti-acetyl-Lysine, clone 4G12	1:500	Millipore	# 05-515
Rabbit Monoclonal [E299] anti-C/EBPβ	1:1000	Abcam	#ab32358
Rabbit Polyclonal anti-C/EBPβ (C19)*	1 µg	Santa Cruz Biotechnology	#sc-150
Mouse Monoclonal anti-FLAG-M2	1:1000	Sigma-Aldrich	#F3165
HA	1:1000	Convace	MMS-101R
TIP60	1:1000	Novus Biologicals	NBP2-20647
Mouse anti-β-actin (cl4)	1:10000	MP Biomedicals	#691001
Mouse anti-α Tubulin (TU-02)	1:10000	Santa Cruz Biotechnology	#sc-80355
Sheep ECL anti-Mouse IgG, HRP-linked whole AB**	1:5000	Amersham Life Technologies	#NA931-1ML
Donkey ECL anti-Rabbit IgG HRP-linked whole AB**	1:5000	Amersham Life Technologies	#NA934-1ML

Table 3. List of details of used products and reagents.

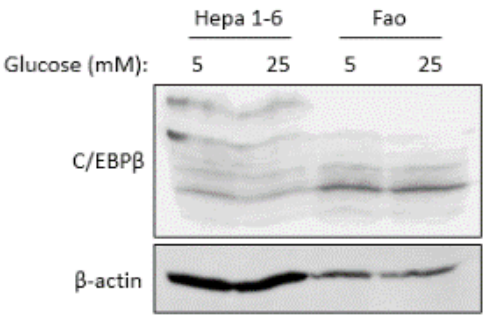
Product/reagent	*Manufacturer*	*Catalogue number*
HEK-293T	ATCC	#CRL-3216
Hepa 1-6	ATCC	#CRL-1830
Fao	Sigma	#89042701
DMEM	Gibco	#41966052
Opti-MEM	Gibco	#31985070
FBS	TICO Europe	#70630-500ML
0,05% Trypsin EDTA (1x)	Gibco	#25300054
Penicillin/Streptomycin	Gibco	#15140122
Hepes Buffer Solution (1M)	Gibco	#15630080
Sodium Pyruvate 100 mM (100x)	Gibco	#11360070
FuGENE	Promega	#E231A
TSA	Sigma-Aldrich	#T8552
NAM	Sigma-Aldrich	#47865U
1x Running Buffer	In-house	-
1x Turbo Transfer Buffer	BioRad	#10026938
Protease inhibitors cocktail (mini)	Roche	#04693159001
Protease inhibitors cocktail	Roche	#04693132001
Western Lightning Plus-ECL	PerkinElmer	#NEL104001EA
QIAEX II Gel Extraction Kit	QIAGEN	#20051
PureYield Plasmid Midiprep System	Promega	# A2492
Dual-Glo Luciferase Assay System	Promega	#E2940
Trans-Blot® Turbo™ RTA Midi PVDF Transfer Kit	BioRad	#170-4273
Mini Protean TGX gels	BioRad	#4561094
Glomax-Multi Detection System	Promega	-
ImageQuant LAS 4000 Mini	GE Health Care	-
Trans-Blot Turbo System	BioRad	# 170-4150
Seahorse XF96 Extracellular Flux analyzer	Seahorse Bioscience	-
Excel	Microsoft Office	-
PowerPoint	Microsoft Office	-
ImageQuant TL	GE Health Care	-
Wave 2.6.1	Agilent	-

SUPPLEMENTAL INFORMATION

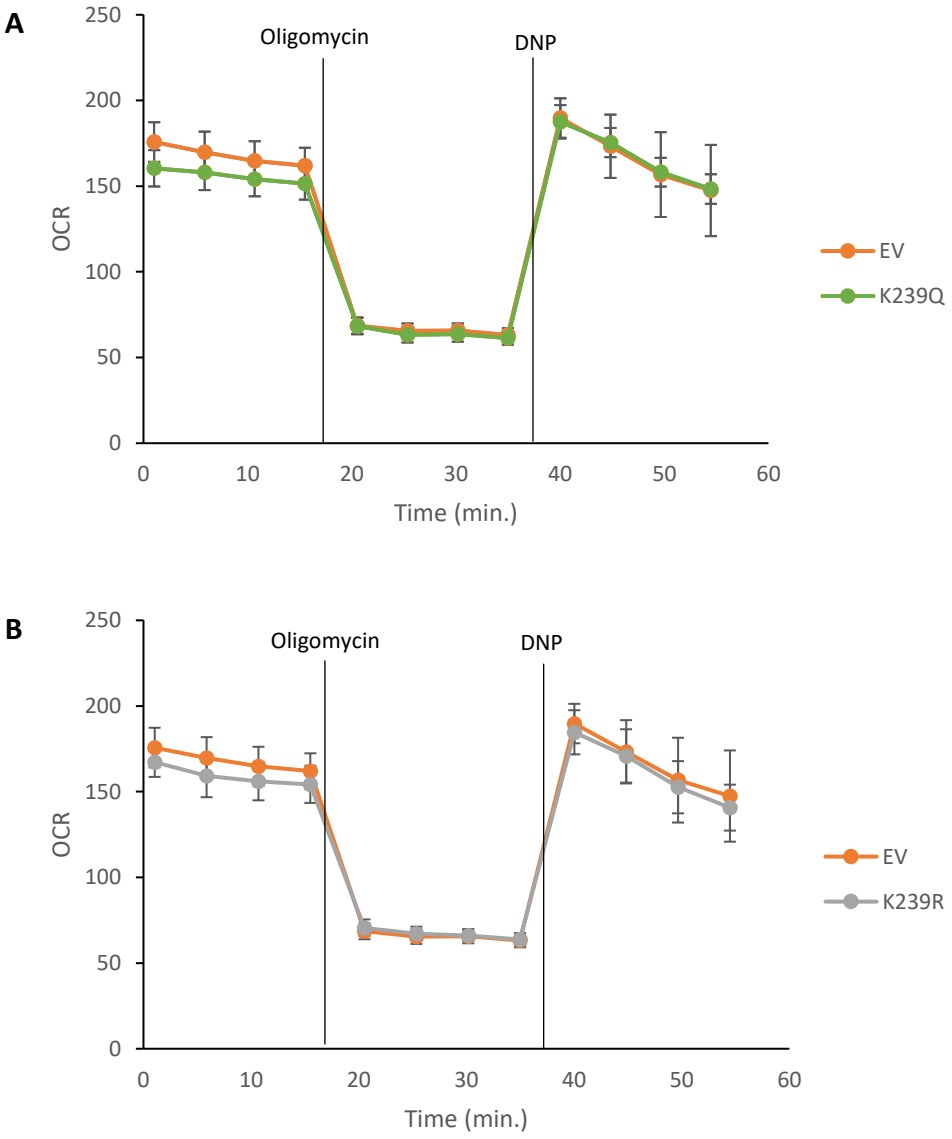
Alignment of C/EBPβ vertebrate orthologs



Supplementary figure 1.
Alignment of vertebrate C/EBPβ orthologs. Highlighted in red are the lysines conserved across the four orthologs. K39, K117, K134, K216/217 and K293 were attempted to be mutated in this study (↓). Translation initiation sites are indicated as hooked arrows followed by one of the isoforms (LAP*, LAP and LIP). TAD: transactivation domain, DBD: DNA binding domain, LZIP: leucine zipper domain.



Supplementary figure 2.
Western Blot analysis of cell lysates of Hepa 1-6 and Fao cells cultured in medium containing low (5 mM) or high (25 mM) glucose concentrations. Antibody staining as indicated.



Supplementary figure 3.
A, B. Oxygen consumption rate (OCR) of HEPA1-6 cells overexpressing K239Q (A) or K239R (B) as compared to HEPA1-6 cells overexpressing an empty vector (EV). Values are means ± SD, (n = 6)

Supplementary table 1. Forward (5' to 3') sequences of primers used for site directed mutagenesis (Kunkel method).

Mutation	Primer Sequence (5' to 3')
K39Q	*CCTGGCCTACGGGGCCCAAGCGGCCCGCGCCGCGC*
K39R	*CCTGGCCTACGGGGCCCGGGCGGCCCGCGCCGCGC*
K117Q	TCGGCCGCGCGGGCGCGCAGGCCGCACCGCCCGC
K117R	CGGCCGCGCGGGCGCCCGGGCCGCACCGCCCGCC
K134Q	*GCCTCCCGCCGCACTCCAGGCCGAGCCGGGCTTCG*
K134R	*GCCTCCCGCCGCACTCCGGGCCGAGCCGGGCTTCG*
K216/217Q	*CGCCAAGGCCAAGGCCCAGCAGGCGGTGGACAAAGCTGAG*
K216/217R	*CGCCAAGGCCAAGGCCCGGCGGGCGGTGGACAAAGCTGAG*
K239Q	*CAACATCGCGGTGCGCCAGAGCCGCGACAAGGCCAAG*
K239R	*CAACATCGCGGTGCGCCGGAGCCGCGACAAGGCCAAG*

Supplementary table 2. Restriction enzyme site changes due to KQ or KR mutations. Changes in the nucleotide sequence are also listed.

Mutation	Nucleotide sequence	Restriction enzyme site changes
K39Q	*gcc<u>aaggcg</u> -> gcc<u>caagcg</u>*	ApaI site generated
K39R	*gcc<u>aaggcg</u> -> gcc<u>cggcg</u>*	ApaI site generated
K134Q	*ctc<u>aaggcc</u> -> ctc<u>caagcc</u>*	NarI site destroyed, BssHII site generated
K134R	*ctc<u>aaggcc</u> -> ctc<u>cggcc</u>*	SmaI site generated
K117Q	*gcc<u>aaggcc</u> -> gcg<u>caggcc</u>*	SmlI site destroyed
K117R	*gcc<u>aaggcc</u> -> gcc<u>cggcc</u>*	SmlI site destroyed
K216/217Q	*gcc<u>aagaaggcg</u> -> gcc<u>cagcaggcg</u>*	XcmI site destroyed
K216/217R	*gcc<u>aagaaggcg</u> -> gcc<u>cggcggcg</u>*	XcmI site destroyed
K239Q	*cgc<u>aagagc</u> -> cgc<u>cagagc</u>*	FspI site destroyed
K239R	*cgc<u>aagagc</u> -> cgc<u>cggagc</u>*	FspI site destroyed

n