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Post-transcriptional control of C/EBPα and C/EBPβ proteins

Zaini, Mohamad Amr

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CHAPTER II

C/EBPα and C/EBPβ transcription factors: Their post-transcriptional control and roles in various tissues

Mohamad Amr Zaini

European Research Institute for the Biology of Ageing (ERIBA), University Medical Center Groningen, the Netherlands.

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Abstract

The CCAAT/enhancer binding proteins (C/EBP) α and β are members of a family of transcription factors consisting of six proteins, C/EBPα to C/EBPζ. They play decisive roles in many biological processes including cellular proliferation and differentiation, immune response, inflammatory processes, energy metabolism, hematopoiesis and adipogenesis. C/EBP proteins contain activation domains at the N-terminus and a basic leucine zipper (bZIP) domain at the C-terminus that consists of a DNA-contacting basic part followed by a leucine rich dimerization motif. All C/EBPs show structural homology and high conservation in their bZIP domains. They share 90% of the amino acid sequence homology in this domain[1], which enables them to form homo- or heterodimers and to interact with the same cognate DNA recognition site. On the other hand, C/EBPs are less conserved in their activation domains, and consequently they vary in their trans-activation potential and function. Within the family of C/EBPs, C/EBPα and C/EBPβ share unique biological and regulatory features that will be the topic of this review.

C/EBPα and C/EBPβ expression

The expression of both C/EBPα and C/EBPβ is highly regulated throughout differentiation stages as well as in response to physiological and pathological changes. In general, C/EBP transcription factors are expressed in a large variety of tissues. C/EBPα is expressed at high levels in the differentiated liver and adipose tissues but at lower levels in hematopoietic cells, lung, intestine, adrenal gland, placenta, and skin[2,3]. C/EBPβ is detected in almost every tissue with high expression in liver, adipose tissue, lung, spleen, intestine, kidney, pancreas, monocytes and granulocytes[1,4].

Structures, DNA binding and dimerization

C/EBPα is the founding member of the C/EBP family. It was purified in 1986 from rat liver as a CCAAT box DNA motif binding protein. The cloning of its cDNA also led to the identification of C/EBPβ and other C/EBPs[4-9]. Currently, *CEBPA* and *CEBPB* genes from many different vertebrate species have been cloned and characterized[1].

C/EBPα and C/EBPβ proteins bind to the DNA as dimers. The dimerization domains consist of α-helices with leucine repeats that form a coiled coil by hydrophobic and electrostatic interactions with the repeats of the dimer partner. The leucine zipper region is conserved between all C/EBPs allowing for homo- and hetero-dimerization. The leucine zipper positions the C-terminal DNA-contacting basic α-helices in such a way that they can bind to the palindromic recognition sequence in the DNA major groove[10] **(Fig. 1)**. The optimal recognition sequence for C/EBPs is the palindromic sequence A/GTTGCGC/TAAC/T with possible variation in three bp in one of the half sites[10-14]. In addition, C/EBPα and C/EBPβ proteins can form heterodimers with other transcription factors. Several reports have shown an interaction between C/EBPβ and the activating transcription factor 4 (ATF-4), which belongs to the CREB/ATF family of transcription factors. The C/EBPβ-ATF4 heterodimer does not bind to the normal C/EBP binding site but it does bind to a subclass of asymmetric cAMP response elements exemplified by those in the phosphoenolpyruvate carboxykinase (PEPCK) and proenkephalin genes that are not recognized by C/EBPβ homodimer[15]. Cohen *et al* showed that the C/EBPβ/ATF4 heterodimer has a unique DNA binding specificity, which regulates expression of set

of genes that may prime human mesenchymal stem cells (hMSCs) into a preadipocyte state[16]. Furthermore, it has been shown that this heterodimer is important for the stimulation of osteoblast differentiation by enhancing Runx2 activity[17]. ATF4 also dimerizes with C/EBPα and inhibits C/EBPα-mediated transcriptional activation of myeloid-specific genes[18]. C/EBPβ dimerizes with the p50 subunit of NFκB and activates transcription of inflammatory cytokines[19,20]. In vitro C/EBPβ bZIP dimerization with Fos and Jun from the AP-1 family of transcription factors decreases its transcriptional activity and DNA binding ability [21].

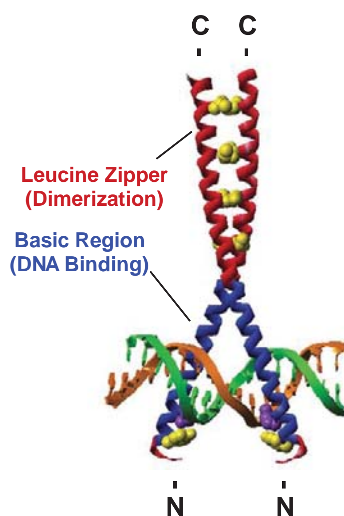

Figure 1. Structure of the C/EBP basic region domain bound to DNA. Two identical chains of C/EBP proteins are represented. Only the leucine-zipper domains and the DNA-binding regions are shown here. Two α-helixes dimerize through the leucine zipper domains. Each monomer binds to one-half of a palindromic recognition sequence. The structure was modified from reference[11].

Translation control

A unique feature of C/EBPα and C/EBPβ biology is that three protein isoforms (extended, full length and truncated isoforms) are translated from a single mRNA by using three successive translation initiation sites. The protein isoforms for C/EBPβ are called, C/EBPβ-LAP* (also called LAP1), C/EBP-LAP (also called LAP2) (Liver Activator Protein) and C/EBPβ-LIP (Liver Inhibitory Protein), and for C/EBPα they are called Extended-C/EBPα, C/EBPα-p42 and C/EBPα-p30. Both extended and full-length isoforms are translated from a proximal start codon, while the N-terminally truncated isoform is translated form a distal AUG codon[22-24] **(Fig. 2)**. The extended C/EBPα is produced from a non-canonical start codon (GUG in human and CUG in rodents), and the C/EBPβ-LAP*-AUG start codon lacks the consensus Kozak sequence, which results in both cases in a less efficient translation initiation and thus in low expression of these two isoforms[25]. The translation of the full-length isoforms

is the result of leaky scanning over the first and weak initiation site for the extended isoform and normal translation initiation at the initiation codon. The translation of different C/EBP isoforms is a result of the non-optimal strength of the Kozak sequence of the proximal translation initiation sites in combination with a cis-regulatory upstream open reading frame (uORF) in the 5' untranslated regions (5' UTRs) of the C/EBPα and C/EBPβ mRNAs. This uORF is evolutionarily conserved between species in both C/EBPα and C/EBPβ **(Fig. 3)**. The translation of the truncated isoform requires the primary translation of the uORF. After termination of uORF translation and because of its small size, the small subunit of the ribosome remains attached to the mRNA and is able to resume scanning. Thus, translation re-initiation at the distal AUG codon can occur[23,25,26].

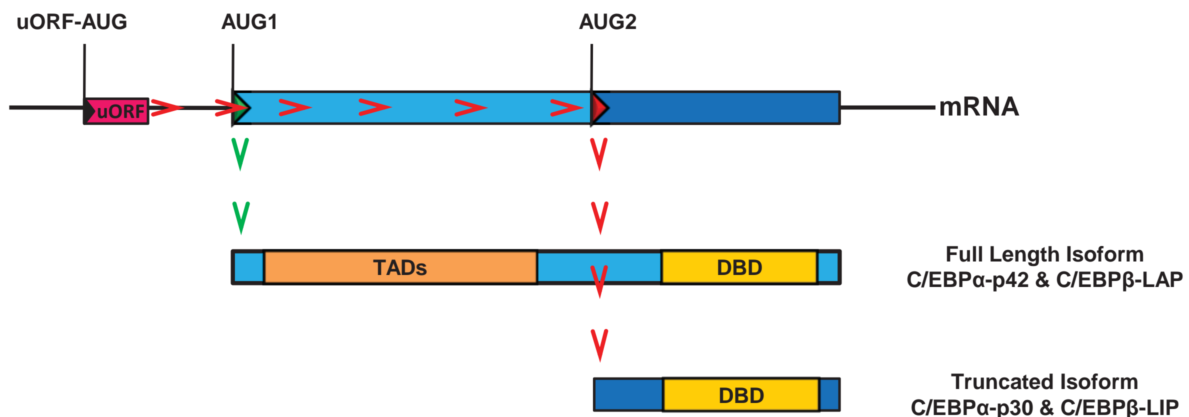

Figure 2. Two isoforms are translated from one mRNA by using different translation initiation sites. The full length isoforms are translated by normal translation initiation at AUG1. Translation into the truncated isoforms is initiated at AUG2 through a re-initiation event that requires the prior translation of the uORF *cis*-regulatory element. For the clarity of the translation mechanism, the extended isoforms were not mentioned in this figure. TADs: Transactivation domains, DBD: DNA binding domain, LZIP: Leucine zipper domain.

This coupling between the translation of the truncated isoforms and the uORF was demonstrated by mutation of the uORF translation initiation site into either a stronger or weaker Kosak sequence, which resulted in a shift in the isoform ratio toward increased or decreased truncated isoforms expression respectively[23]. The removal of the uORF stop codon completely abolished C/EBP-LIP or C/EBPα-p30 expression which means that the translation of the truncated isoforms requires translation termination at the uORF stop codon to produce post-termination ribosomes that are able to resume scanning and re-initiate translation.

The truncated C/EBPα-p30 and C/EBPβ-LIP isoforms share the same sequence and structure with their respective full-length C/EBPα-p42 and C/EBPβ-LAP but they lack the N-terminal transactivation domains **(Fig. 2)**. Since the truncated isoforms contain the DNA binding domain, they act as inhibitors of the extended and full-length isoforms by competitive binding to the same recognition sequences[27]. Therefore, the ratio between the truncated and full-length isoforms is of great importance for the biological functions of C/EBPα and C/EBPβ proteins.

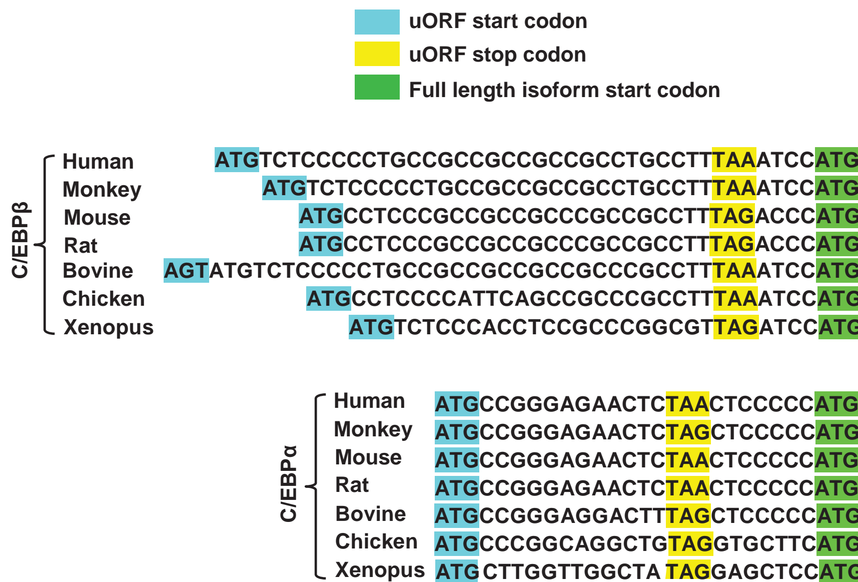

Figure 3. Representation of C/EBPα and C/EBPβ uORF sequences showing that the uORF ends few nucleotides upstream of the full length isoform translation start site. This distance is conserved between different species.

The translation of C/EBP truncated isoforms is regulated via the mTORC1-4EBP1/2-eIF4E signalling pathway in a uORF dependent manner **(Fig. 4)**. Pharmacological inhibition of mTORC1 by rapamycin resulted in a shift in the C/EBP isoform ratio toward less truncated isoforms while over-expression of eIF4E shifted the isoform ratio toward more truncated isoforms[26,28,29]. The exact molecular mechanism by which mTORC1-4E-BP1/2-eIF4E signalling regulates LIP expression is not known so far.

Other translation initiation factors besides eIF4E also play a role in the regulation of the C/EBP isoform ratio especially in the translation re-initiation event of the truncated isoforms. Eukaryotic initiation factor 2 (eIF2) is an important factor regulating the re-initiation potential of the post-termination small ribosomal subunits that have finished translating the uORF. eIF2 reloads the ribosomal complex with the initiator tRNA (Met-$tRNA_i^{Met}$) in a GTP-loaded ternary complex which is a pre-requisite for translation re-initiation at the truncated isoform start codon. The post-

termination small ribosomal subunit loaded with Met-$tRNA_i^{Met}$ continues scanning the mRNA until reaching the C/EBP truncated isoform AUG-codon. Then, the large ribosomal subunit joins and GTP is hydrolysed to release the eIF2-GDP complex and to re-initiate translation. The activity of eIF2 is restored by the guanine-nucleotide exchange factor eIF-2B, a process that is inhibited by eIF2α-kinases that phosphorylate the eIF2α-subunit of eIF2-GDP and thereby limit C/EBPα-p30 and C/EBPβ-LIP translation re-initiation. Inhibition of the eIF2α kinase PKR or over-expression of its dominant negative mutant (PKRΔ6) or of a non-phosphorylatable mutant of eIF2α resulted in higher efficiency in Met-$tRNA_i^{Met}$ loading and as a consequence in a shift in the C/EBP isoform ratio toward higher truncated isoform expression[23] **(Fig. 4)**.

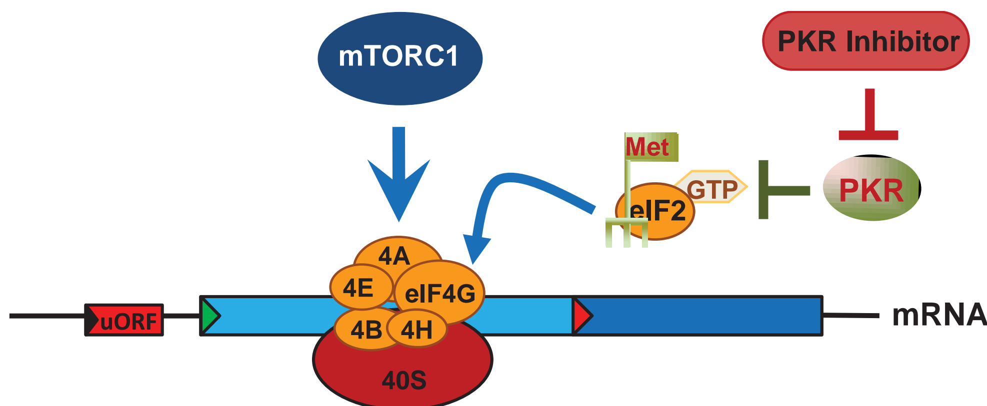

Figure 4. Regulation of translation re-initiation at the C/EBPα or C/EBPβ mRNA. For proceeding of scanning along the mRNA after uORF translation termination, the translation machinery requires two important factors. (1) mTORC1 activity to maintain the phosphorylation of protein factors that make up the initiation complex. (2) Initiator tRNA reloading by the ternary complex. PKR (eIF2-kinase) inhibit this reloading and inhibiting this kinase results in increased tRNA loading. Thus, mTORC1 activity and proper tRNA reloading support translation re-initiation of C/EBPα and C/EBPβ mRNAs.

Post-translational modifications

Post-translational modifications (PTMs) including methylation, phosphorylation, acetylation, O-GlcNAcylation, ubiquitination and sumoylation play an important role in regulating the function of C/EBPα and C/EBPβ.

Phosphorylation

C/EBPα is found to be phosphorylated at multiple sites, which regulates its function in different tissues. C/EBPα phosphorylation at S21 is mediated by extracellular signal–regulated kinases (ERK1/2) and prevents C/EBPα from inducing granulocyte differentiation[30,31]. On the other hand, it has been found that phosphorylated C/EBPα at S248 has higher binding affinity to the promoter of the granulocyte-colony stimulating factor receptor (G-CSFR), which results in improved granulopoiesis[32]. In hepatocytes, the phosphorylation at S193 stimulates the binding of C/EBPα to the cyclin dependent kinase-2 (cdk-2) and to the ATPase subunit of the SWI/SNF chromatin remodeling complex which inhibits hepatocyte proliferation[33,34]. In addition, the phosphorylation of C/EBPα at S193 by cyclin dependent kinase-4 (cdk-4) stimulates the C/EBPα/p300 complex formation[35,36] and increases steatosis in mice[37,38]. It was also observed that phosphorylation of C/EBPα by insulin regulated glycogen synthase kinase 3 (GSK3) at T222, T226 and S230 stimulates adipogenesis[39,40]. Similar to C/EBPα, the function of C/EBPβ is regulated by phosphorylation at multiple sites. It has been shown that in the early stage of 3T3-L1 adipocyte differentiation C/EBPβ is phosphorylated sequentially, first by MAPK on Thr188. This phosphorylation is important but not sufficient for C/EBPβ to acquire DNA binding activity. Later when the cells enter the S-phase, the activity of MAPK declines but the C/EBPβ phosphorylation on Thr188 is maintained by the high activity of CDK2/Cyclin A and C/EBPβ is further phosphorylated at Ser184 and Thr179 by GSK3 which is localized in the nucleus at this stage. It has been suggested that phosphorylation of Thr188 functions as a priming step for the secondary phosphorylation on Ser184 and Thr179 and this dual phosphorylation is important for C/EBPβ dimerization through its C-terminal bZIP domain, its DNA binding and transcriptional regulatory activity[41-43]. Further studies showed that phosphorylation of C/EBPβ on Thr188 by MAPK or CDK2/Cyclin A improves its stability by preventing its calpain-dependent degradation[44] **(Fig. 5)**.

Acetylation

Many transcription factors are subjected to lysine acetylation that may affect their DNA binding ability, stability, cellular localization or protein interaction[45]. Recently Bararia et al showed that C/EBPα is acetylated by GCN5 acetyl transferase at multiple

residues including K298 and K302, which impairs its DNA binding ability and thereby inhibits its transcriptional activity. Acetylated C/EBPα is found at higher levels in human myeloid leukemia cell lines and acute amyloid leukemia (AML) samples and at lower levels in differentiated granulocytes. C/EBPα lost its granulopoietic function when acetylated and mutations mimicking C/EBPα acetylation failed to induce granulocytic differentiation in cell lines and primary hematopoietic cells[46]. Similar to C/EBPα, the function of C/EBPβ is modulated by acetylation. Cesena et al showed that C/EBPβ is acetylated by the p300 acetyltransferase at multiple lysines. The main acetylation site is K39. It is located in the transactivation domain and regulates the transcriptional activity of C/EBPβ[47]. K39-C/EBPβ is deacetylated by the HDAC1 histone deacetylase and a K39R C/EBPβ deacetylation mimicking mutant failed to activate key adipocyte target genes[48]. Furthermore, C/EBPβ was found to be acetylated at K98, K101 and K102 by GCN5 and p300/CBP associated factor (PCAF) in differentiating preadipocytes in response to glucocorticoid treatment. This acetylation represses C/EBPβ interaction with HDAC1 resulting in induced C/EBPβ transactivation efficiency and higher transcription of its target genes like C/EBPα and PPARγ[49]. However it was shown that C/EBPβ and HDAC1 interaction does not always have a repressive effect on C/EBPβ. Xu et al showed that the deacetylation of K215/K216 C/EBPβ by HDAC1 enhanced its activity toward the *Id1* (inhibitor of DNA binding protein) promoter[50]. Thus, it is clear that depending on the promoter context and/or the lysine residue, acetylation can either stimulate or inhibit the transcriptional activity of C/EBPβ **(Fig. 5)**.

Sumoylation and Ubiquitination

Sumoylation of transcription factors is an important mechanism of regulating many cellular processes. Addition of the Small Ubiquitin-like Modifier protein (SUMO) may compete with other post translational modifications like acetylation or ubiquitination for specific lysine residues in the target protein and it acts as a space regulator that affects protein-protein interactions. Both C/EBPα and C/EBPβ are found to be sumoylated at a conserved motif that consists of five amino acids (I/V/L)KXEP termed inhibitory domain motif[51]. Sumoylation of C/EBPα prevents its interaction with BRG1 (a core subunit of the SWI/SNF chromatin remodeling complex) and by

that the induction of proliferation arrest and the transactivation of the liver specific albumin gene[52]. Accordingly, the level of (K159) sumoylated C/EBPα decreases during hepatocyte differentiation[52,53]. C/EBPβ is sumoylated at K173 resulting in inhibition of its transcriptional activity on the cyclin D1 promoter[54] and the release of its inhibitory effect on c-Myc transcription in murine T cells[55]. In addition it has been suggested that sumoylation regulates C/EBPβ function by affecting its cellular localization[55]. PIAS1 (a SUMO E3 ligase) was reported to interact and sumoylate C/EBPβ at K133 leading to increased ubiquitination and degradation[56]. In addition to sumoylation, C/EBPα and C/EBPβ are regulated by ubiquitination. C/EBPα was found to be targeted for ubiquitin-mediated proteasome degradation by the E3 ubiquitin ligase E6AP which negatively regulates C/EBPα function in granulopoiesis and adipogenesis[57,58]. In AML, the Tribl1-COP1 mediated ubiquitination and subsequent degradation of C/EBPα accelerates the development of AML[59]. Trivedi et al showed that active JNK increases C/EBPα protein stability by inhibiting its ubiquitination, which has a major impact on C/EBPα transcriptional activity and DNA binding ability in both acute promyelocytic NB4 cells and acute myelomonocytic HL60 cells[60]. The degradation of C/EBPα by the ubiquitin proteasome machinery supports the development of liver cancer in older mice. Mechanistically, the gankyrin protein, whose expression is elevated during tumor formation, associates with S193 phosphorylated C/EBPα and targets it for ubiquitin proteasome system (UPS) degradation[35]. Furthermore, it has been shown that the E3 ubiquitin ligase Nrdp1 (Neuregulin Receptor Degradation Protein 1) interacts and ubiquitinates C/EBPβ via Lys-63-linked ubiquitination. C/EBPβ transcriptional activation of the Arg1 reporter gene was enhanced by this ubiquitination in the presence of IL-4 stimulation indicating that ubiquitination of C/EBPβ may contribute to a nonproteolytic pathway that up-regulates Arg1 expression and promotes M2 macrophage polarization[61] **(Fig. 5)**.

Methylation and GlcNAcylation

In addition to DNA and histones, transcription factors can be methylated on the side chain nitrogen of arginine (R) and lysine (K) residues. Both C/EBPα and C/EBPβ are found to be extensively K and R methylated in their N-termini[62]. C/EBPβ is methylated at K39 by the G9a histone methyltransferase and this modification

abrogated the transactivation potential of C/EBPβ. Mutated C/EBPβ with K39 exchanged to alanine (A) was resistant to G9a methylation induced inhibitory effect[63]. Kowenz-Leutz et al found that C/EBPβ is methylated at a highly conserved arginine residue in position 3 (R3) located in the N-terminal transactivation domain by the protein arginine methyltransferase 4 (PRMT4/CARM1). This methylation event inhibits the interaction of C/EBPβ with the SWI/SNF chromatin remodeling complex and the mediator complex and was abrogated through phosphorylation of C/EBPβ by the Ras/MAPK signaling pathway[64]. In addition to methylation, C/EBPβ is modified by O-GlcNAcylation on Ser180/181. Interestingly this modification impairs the phosphorylation of the neighboring sites Thr179, Ser184 and Thr188 and results in delayed 3T3L1 adipocyte differentiation[65]. Thus, C/EBPβ is an example of transcription factor whose interaction with the epigenetic gene regulatory protein complexes is regulated by a crosstalk between different post-translational modifications.

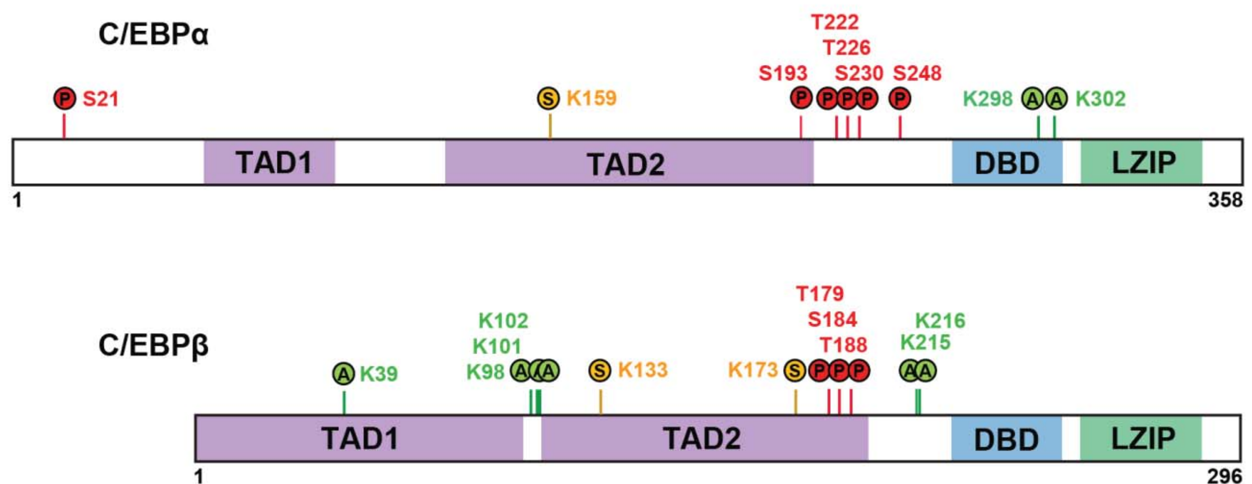

Figure 5. Map of the structural features and post-translational modifications of both C/EBPα and C/EBPβ proteins. The transactivation domains (TAD), DNA binding domain (DBD) and leucine zipper domain (LZIP) are shown. Phosphorylation, acetylation and sumoylation sites are indicated by red P, green A and yellow S respectively.

C/EBPα and C/EBPβ functions

Adipocytes

Adipose tissue is an important site for lipid storage, energy homeostasis, and whole-body insulin sensitivity. The majority of cells present in the adipose tissue are adipocytes but it also contains other cell types like preadipocytes, endothelial cells, macrophages and other immune cells[66]. C/EBPs and PPARγ are the key transcriptional regulators in stimulating the transcriptional events leading to adipocytes differentiation and also controlling the fully differentiated adipocytes[67]. C/EBPα and C/EBPβ seem to have different roles in the differentiation of adipocytes. The role of the adipocytes differentiation factors are investigated mainly by studies on the 3T3-L1 preadipocyte cell line. These cells differentiate upon treatment with a cocktail of hormonal factors including a cAMP elevator, a glucocorticoid and insulin. By this treatment the expression of C/EBPβ and C/EBPδ is transiently increased and the growth arrested pre-adipocytes enter a proliferation phase called mitotic clonal expansion phase[68]. In the next phase, C/EBPβ and C/EBPδ directly induce the expression of PPARγ and C/EBPα which in their turn drive the preadipoctes to exit the cell cycle and induce the transcription of the adipose genes that characterize the terminally differentiated adipocytes[69-71].

Ectopic expression of C/EBPα and C/EBPβ induced differentiation in 3T3-L1 without the treatment with the adipogenic hormones[72]. The deletion of both C/EBPβ and C/EBPδ blocks adipogenesis and mouse embryonic fibroblast lacking these two proteins failed to express any adipocyte markers including C/EBPα or PPARγ after the hormonal stimulation[72]. Stimulating the differentiation process in 3T3-L1 cells by hormonal treatment failed when C/EBPα antisense-RNA was expressed[73]. To support and retain a differentiated adipocyte phenotype C/EBPα and PPARγ stimulate each other's expression in a positive feedback loop[74,75].

Myeloid cells

Myeloid cell differentiation (myelopoiesis) is the process in which the myeloid cell progenitors differentiate into myeloid cells such as granulocytes (eosinophilic, basophilic and neutrophilic) and monocytes **(Fig. 6)**. In the hematopoietic system, C/EBPα is most prominently expressed in myeloid progenitor cells and its expression

is subsequently downregulated as the cells differentiate[76]. C/EBPα plays an important role in directing myelopoiesis after its initiation by PU1 and RUNX1 in cooperation with other myeloid transcription factors and by upregulating granulocyte colony stimulating factor (G-CSF) and interlukin-6 (IL-6)[77-79]. The deletion of the *CEBPA* gene resulted in a selective block at the common myeloid progenitor (CMP) to granulocyte-macrophage progenitor (GMP) transition. It subsequently reduced the formation of granulocytes and monocytes[80] correlating with the absence of G-CSF and IL-6 whose expression in C/EBPα deficient progenitors rescues granulopoiesis[78,79]. C/EBPα is not required after the GMP stage for granulocyte differentiation as normal granulopoieses was obtained in vitro upon C/EBPα conditional deletion in GMPs[80]. While C/EBPα functions as a master transcriptional regulator of the steady-state granulopoiesis, C/EBPβ functions as a master regulator of the emergency granulopoiesis which is a haematopoietic response program characterized by a strikingly increased de novo production of neutrophils from myeloid progenitors in the bone marrow to counteract neutrophil depletion upon uncontrolled microbial infection. During emergency granulopoiesis, C/EBPβ expression is stimulated by phosphorylated STAT3 (pSTAT3) which gets translocated to the nucleus via G-CSFR-JAK signaling. High C/EBPβ levels stimulate the transcription of *Myc* and also inhibits the transcriptional repression of C/EBPα on *Myc* expression by replacing C/EBPα on the *Myc* promoter through competition for binding. By that, the proliferative effect of C/EBPβ overrules the anti-proliferative effect of C/EBPα resulting in increased myeloid progenitor cell proliferation and neutrophil generation[81]. C/EBPβ is highly expressed in myelomonocytic cells and macrophages and it is involved in the regulation of several monocytic differentiation genes which are important for the morphology, proliferation and antimicrobial capacity of macrophages[82]. C/EBPβ deficient mice were able to generate macrophages-like cells but these cells have reduced functional potential and they failed to be activated in response to the active stimuli like LPS or bacteria. C/EBPβ deficient mice were more prone to infections due to the defect in the macrophages activity and the resulting escape of the phagocytosed bacteria from the phagosome to the cytoplasm[83-85].

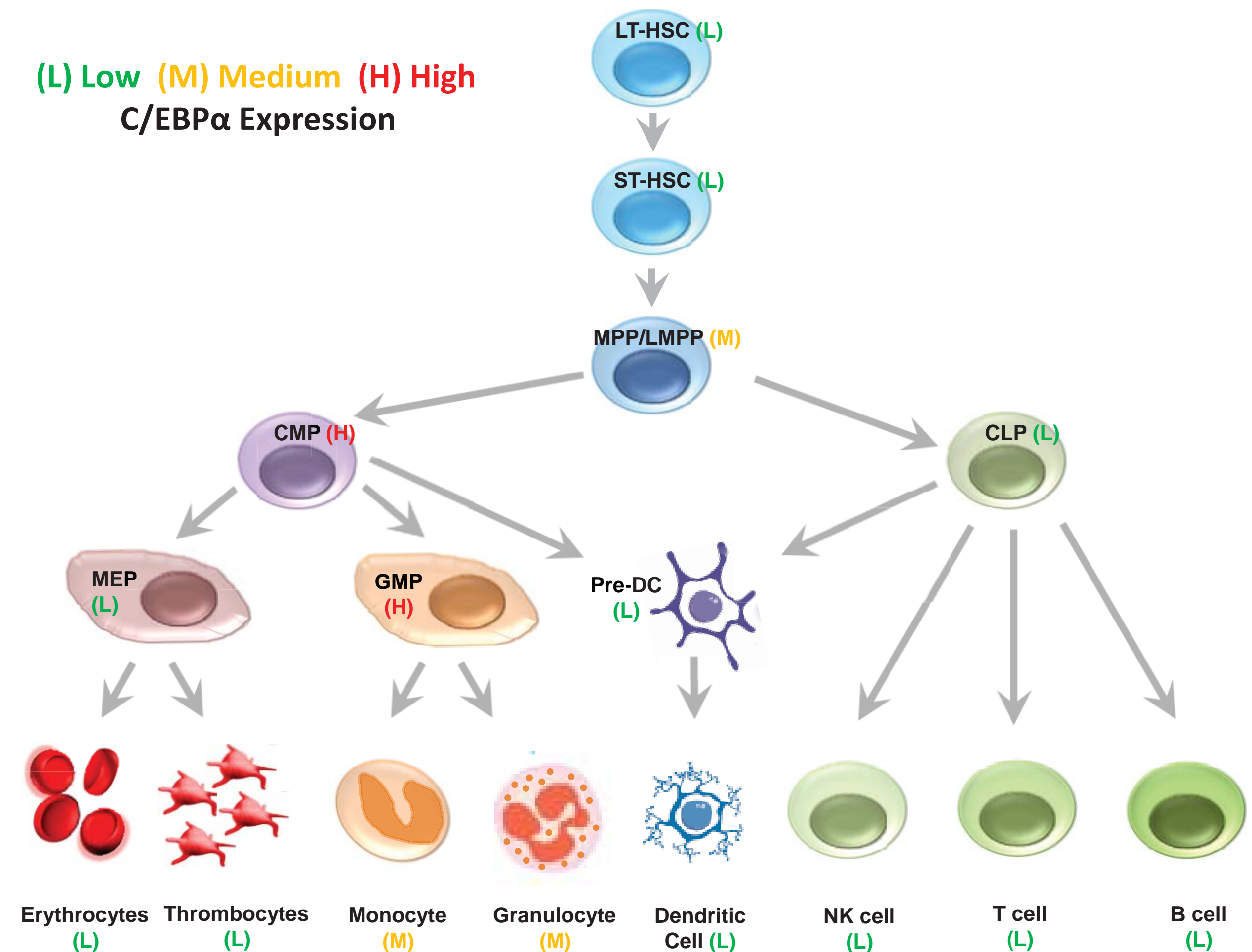

Figure 6. The hematopoietic pyramid showing the expression of C/EBPα. LT-HSC and ST-HSC: Long and Short Term Hematopoietic Stem Cell. MPP: Multipotential Progenitor. LMPP: Late Multipotential Progenitors. CMP: Common Myeloid Progenitor. CLP: Common Lymphoid Progenitor. MEP: Megakaryocyte Erythroid Progenitor. GMP: Granulocyte Macrophage Progenitor. Pre-DC: Pre-Dendritic cell. The Figure is modified from reference[76].

Metabolism

C/EBPα is involved in the expression of many genes that regulate different metabolic processes in liver and adipose tissue. Therefore, McKnight et al have described C/EBPα as the central regulator of energy metabolism[86]. This turned to be right not only for C/EBPα but also for C/EBPβ. C/EBPα deficient mice were phenotypically normal at birth with normal body weight and no obvious abnormalities. However, these mice died soon few hours after birth due to hypoglycemia resulting from insufficient levels of glycogen in the liver as a result of impaired expression of glycogen synthase. In addition, C/EBPα knock-out mice had reduced expression of gluconeogenic enzymes like phosphoenolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase and glucose-6-phosphatase[87]. C/EBPα deficient mice also had high

levels of ammonia in their blood comparing to control mice due to the failure in expressing ornithine-cycle enzymes[87]. Conditional knockout of the *CEBPA* gene specifically in the liver resulted in severe liver derangements associated with insufficient expression levels of PEPCK, glycogen synthase and many other liver specific genes involved particularly in gluconeogenesis, glycogen synthesis and bilirubin clearance[88]. This indicates that C/EBPα is essential for the initial expression of PEPCK and glycogen synthase at birth and also for the maintenance of their expression in adult liver. C/EBPβ deficient mice have two different phenotypes[89-91]. Half of the mice homozygous for *CEBPB* deletion survived to adulthood (phenotype A), and the other half died at birth (phenotype B). Similarly to C/EBPα knockout phenotype, the C/EBPβ deficient mice with phenotype B had malfunctioning glucose homeostasis leading to death two hours after birth because of hypoglycemia[90,91]. These mice were unable to perform gluconeogenesis, which was confirmed by undetectable levels of PEPCK. But unlike the C/EBPα knockout, C/EBPβ deficient mice with this phenotype synthesize and store glycogen but are unable to effectively mobilize it[89,91]. However, C/EBPβ knockout mice with phenotype A survived to adulthood displaying several altered metabolic features[89,90]. The liver of these mice had insufficient levels of cAMP and upon 18 hours fasting they suffered from hypoglycemia due to hepatic glycogenolysis failure. They also had lower levels of plasma lipids due to defects in adipose tissue lipolysis and a decreased number of adipocytes resulting in significant lower white adipose tissue mass[89,90]. In addition, deletion of the *CEBPB* gene protected mice from high fat diet-induced obesity and fatty liver. C/EBPβ deficient mice fed with high fat diet for 12 weeks had lower levels of blood triglycerides, free fatty acids and cholesterol and reduced body fat comparing to WT mice. They also had less hepatic triglyceride accumulation and decreased expression of lipogenic and fatty acid synthesis genes in the liver. Furthermore, C/EBPβ deficient mice fed with high fat diet had higher CO_2 production and increased levels of β-oxidation genes in their brown adipose tissue and increased levels of the uncoupling proteins 1 and 3 (UCP1 and 3) in their muscles suggesting increased energy expenditure[92]. Since the ratio between C/EBPβ-LAP and C/EBPβ-LIP is important for regulating C/EBPβ transcription factor function[26], Zidek et al studied the effect of C/EBPβ-LIP depletion in knock-in mice that carry a mutation in the uORF on systemic metabolism[26]. The ΔuORF mice showed no overt

developmental defects or premature death[93]. These mice display metabolic improvements that resemble those found upon calorie restriction (CR), including improved glucose tolerance and insulin sensitivity, decreased total fat mass and increased energy expenditure and physical activity. Furthermore, ΔuORF mice showed higher expression levels of β-oxidation genes in the liver and also increased expression levels of genes involved in lipogeneses, lipolysis in the white adipose tissue (WAT). Since C/EBPβ-LIP expression is regulated by mTORC1 signaling[26] and since inhibition of mTORC1 is seen as a major downstream effect of caloric restriction the CR-like phenotype of the C/EBPβ$^{\Delta uORF}$ animal model indicates that the healthy metabolic profile obtained by CR could be partly caused by the reduction of mTORC1-mediated C/EBP-LIP expression **(Fig. 7)**.

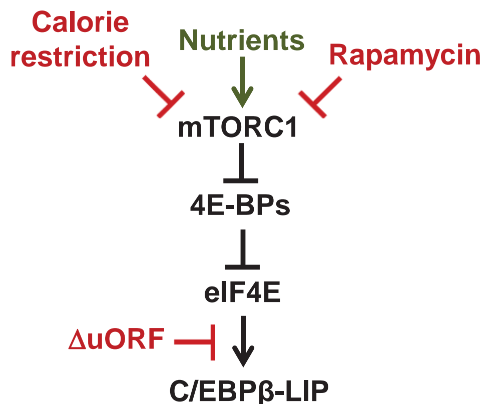

Figure 7. Diagram showing that mTORC1 regulates C/EBPβ-LIP expression *via* the 4E-BPs/eIF4E axis. Deleting the uORF (required for mTORC1-stimulated C/EBPβ-LIP translation) in the C/EBPβ-mRNA in mice resulted in an improved metabolic phenotype with features also found under calorie restriction.

Inflammation

C/EBPβ was originally identified by its ability to regulate genes in response to IL-1 and IL-6[8,94] and since then many studies proved the role of C/EBP proteins in the inflammatory response[83]. C/EBPα and C/EBPβ are differentially modulated during inflammation. The C/EBPβ mRNA is upregulated by inflammatory stimuli like turpentine oil, bacterial lipopolysaccharide (LPS) and recombinant cytokines such as IL-1, IL-6 and TNFα, while C/EBPα is downregulated by the same conditions[95,96]. CEBP binding sites were detected in the regulatory regions of many genes involved in the inflammatory response including several cytokines, cytokine receptors and liver acute phase genes[1,83]. C/EBPα is known to be downregulated by inflammatory cytokines and its function in the inflammatory response is not well investigated. In contrast it has been shown that C/EBPβ regulates many targets during the

inflammatory response[83]. However, not all of these genes were affected in C/EBPβ deficient mice pointing toward a functional crosstalk between C/EBPβ and other C/EBPs that have normal expression in these mice[83]. This idea was supported by the finding of Yan et al who showed that C/EBPβ and C/EBP-δ heterodimers have higher inflammatory activation potential in macrophages than homodimers[97]. Furthermore, it was observed that the expression of the C/EBPβ-LIP isoform is specifically upregulated in mice treated with LPS while C/EBP-LAP expression was not affected suggesting a specific role of C/EBPβ-LIP in the inflammatory response[98]. This role was further investigated by Wethmar et al using the C/EBPβ$^{\Delta uORF}$ mice that unlike the control mice failed to express high level of C/EBPβ-LIP after LPS administration or after partial hepatoctomy (PH). The ΔuORF mice had higher IL-6 serum levels and increased expression of acute-phase response genes after PH compared with control animals suggesting that C/EBPβ-LIP serves to restrict the trans-activation of early acute-phase response genes[93]. C/EBPβ was also found to be upregulated and to control the induction of many pro-inflammatory genes in response to palmitate and high fat diet. C/EBPβ deletion completely diminished the high fat diet-induced development of inflammation. C/EBPβ knockout macrophages have increased levels of the anti-inflammatory cytokine IL-10 and decreased levels of the NLRP3 gene, which is necessary for the activation of the inflammasome[99]. The NFκB signaling pathway is the main player in regulating the inflammation in macrophages and adipose tissues. C/EBPβ knockdown in macrophages and differentiated adipocyte cell lines suppressed the binding activity of NFκB upon palmitate treatment while the over expression of C/EBPβ in these cells results in higher NFκB binding activity and pro-inflammatory cytokines gene expression suggesting a role of C/EBPβ in increasing the transactivation potential of NFκB in response to fatty acid exposure[99]. Since the induction of C/EBPs occurs after few hours of the inflammatory stimulus, it is believed that C/EBPβ and C/EBP-δ function mainly in the maintenance of the activated state of the inflammatory genes. C/EBPs might substitute the transcription factors NFκB and Stat3 that are activated faster but transiently and function as the inflammation primers. Stat3 was observed to play a role in the upregulation of both C/EBPβ and C/EBP-δ by IL-6 induction[100]. The pre-existing C/EBPβ proteins which can be activated by phosphorylation might contribute to inflammatory initial induction by interacting with NFκB[83].

Cellular proliferation

C/EBPα was first identified as an inhibitor of mitotic growth in fibroblasts and later this function was found in a wide range of cells[101]. C/EBPα is a strong inhibitor of cell proliferation and it is detected at high levels in terminally differentiated cells and at low levels in proliferating cells supporting its anti-proliferative function. Overexpression of C/EBPα in different cell lines resulted in strong inhibition of cell proliferation[2] while hepatocytes and other cells isolated from C/EBPα deficient mice had increased cell proliferation[102]. C/EBPα mediates growth arrest via different pathways by interacting with a number of proteins which are involved in cell cycle progression. Studies in developing liver and in cell culture showed that C/EBPα inhibits proliferation by inducing and stabilizing the Cyclin Dependent Kinase 2 (CDK2) inhibitor p21 and C/EBPα deficient mice had reduced level of p21[103]. In addition, C/EBPα forms a complex with E2F family members and inhibits their transcriptional activity[104]. E2F transcription factors are important for the G1-S phase progression in the cell cycle[105,106] and C/EBPα-E2F complex formation leads to the downregulation of S phase specific genes resulting in cell cycle arrest at the G1 phase[104]. Furthermore, C/EBPα was found to bind to CDK2 and CDK4 which are important kinases in cell cycle progression that induce E2F transcription factor. It was proposed that a centrally located 15 amino acid proline histidine rich (PHR) region in C/EBPα is responsible for the growth-inhibitory function of the protein in fetal liver through its ability to interact with CDK2 and CDK4, thereby inhibiting their activities[107]. C/EBPα-S193 resides within the PHR region and it has been shown that in liver tumor cells the activation of PI3K/AKT pathway blocks C/EBPα growth inhibitory activity via PP2A-mediated dephosphorylation of Ser 193 resulting in C/EBPα interaction failure with Cdks and E2F[34].

However, Porse et al showed that C/EBPα-PHR deficient mice were phenotypically similar to their control littermates and no cell cycle or developmental differences were detected[108] which questions the *in vivo* role of this domain for the anti-proliferative function of C/EBPα. In Acute Myeloid Leukemia (AML), mutations in *C/EBPA* gene were detected in almost 9% of the AML patients indicating an important role of C/EBPα in tumor suppression[109]. The most common *CEBPA* mutations result in C/EBPα-p42 ablation while the expression of C/EBPα-p30 stays preserved[110]. C/EBPα-p30 fails to interact with E2F which is sufficient for inducing

granulocyte lineage transformation[111]. Furthermore, Kirstetter et al produced a knockin mouse in which the locus of C/EBPα was modified to produce only C/EBPα-p30. These mice were still able to form GMPs but the resulting myeloid progenitors had massive self-renewal capacity and the mice developed AML and died due to combined liver and bone marrow failure. This suggests that C/EBPα-p30 is sufficient for commitment of CMPs to a myeloid fate, whereas p42 is very crucial for the proliferation control in myeloid progenitors[112]. In frame insertion mutations in the c-terminus of C/EBPα which preserve the leucine zipper domain and cause a loss of DNA binding are also a common C/EBPα mutations. The interaction of these mutated C/EBPα proteins with E2F is still to be investigated but they might have a dominant-negative effect by dimerizing with C/EBPβ and C/EBPε, which are expressed during normal granulopoiesis, and disrupting their DNA-binding function and by that blocking C/EBPβ and C/EBPε mediated pathway of E2F repression[110].

The role of C/EBPβ in cell proliferation is even more complicated. In some cases C/EBPβ play an anti-proliferative role similarly to C/EBPα. The best example of that is the mouse model in which C/EBPβ was expressed from the locus of C/EBPα which resulted in a rescue of many of C/EBPα knockout phenotypes[113,114]. In monocytic cells, C/EBPβ-LAP but not LIP reduced their cellular proliferation by affecting the retinoblastoma/E2F/Cyclin E pathway[115]. In T lymphocytes C/EBPβ-LAP represses c-Myc expression and, therefore, arrests T cells in the G1 phase of the cell cycle[55]. Furthermore, over expression of C/EBPβ-LAP in Mouse Embryonic Fibroblasts (MEFs) inhibited their proliferation by cooperating with RB:E2F and downregulating the E2F target genes[116].

On the other hand, C/EBPβ expression was detected at higher levels in many tumors in which it appears to play a crucial role in promoting cell proliferation[117-119]. C/EBPβ is important for the proliferation of hepatocytes and these cells had abnormal regenerative response when isolated from C/EBPβ deficient mice after partial hepatoctomy associated with dysregulation of cell proliferation genes[117]. The transcriptional activity of C/EBPβ is stimulated by Ras signaling[118] and it has been shown that C/EBPβ is required for Ras-mediated tumorigenesis in the skin[120,121]. Furthermore, C/EBPβ was found to activate the cyclin D1 promoter in epithelial cancer[122]. It regulates epithelial cell proliferation and differentiation in the mammary gland and C/EBPβ deficient mice had impaired differentiation and reduced

proliferation in mammary gland[123-125]. There are several reports suggesting that the C/EBPβ isoform LIP stimulates proliferation and that the ratio between C/EBPβ-LIP and LAP isoforms is decisive for proliferation control. C/EBPβ-LIP was found to be upregulated in the liver after partial hepatectomy (PH). C/EBPβ$^{\Delta uORF}$ mice that have strongly reduced LIP isoform levels and were unable to elevate C/EBPβ-LIP after PH which was associated with a delayed and less frequent cell cycle entry in the regenerating hepatocytes as compared to C/EBPβ-WT mice. Mouse Embryonic Fibroblasts (MEFs) isolated from C/EBPβ$^{\Delta uORF}$ mice similarly showed reduced expansion in an in vitro proliferation assay[93]. Enhanced C/EBPβ-LIP expression may predispose to tumourigenesis in general and knockin mice that express only C/EBPβ-LIP display enhanced tumourigenesis[126]. Furthermore, high levels of C/EBPβ-LIP were detected in Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL) cells[29], in squamous cell carcinomas (SCC)[127] and also in primary rodent mammary tumors[128]. In addition, high levels of C/EBPβ-LIP is implicated in breast cancer progression in mice[129] and humans[128,130,131] and it was linked to a loss of TGFβ-dependent cytostatic responses in metastatic cells from breast cancer patients[132].

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