Transcriptional Regulation of Adipocyte Differentiation: A Central Role for CCAAT/Enhancer-binding Protein (C/EBP) β

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A detailed understanding of the processes controlling adipogenesis is instrumental in the fight against the obesity epidemic. Adipogenesis is controlled by a transcriptional cascade composed of a large number of transcriptional factors, among which CCAAT/enhancer-binding protein (C/EBP) β plays an essential role. During 3T3-L1 adipocyte differentiation, C/EBP β is induced early to transactivate the expression of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), two master transcription factors for terminal adipocyte differentiation. Studies in recent years have revealed many new target genes of C/EBP β, implicating its participation in many other processes during adipogenesis, such as mitotic clonal expansion, epigenetic regulation, unfolded protein response, and autophagy. Moreover, the function of C/EBP β is highly regulated by post-translational modifications, which are crucial for the proper activation of the adipogenic program. Advances toward elucidation of the function and roles of the post-translational modification of C/EBP β during adipogenesis will greatly improve our understanding of the molecular mechanisms governing adipogenesis.

Adipose tissue is not only a key depot for energy storage but is also involved in the dynamic regulation of metabolism (1). The upsurge of adipose tissue mass plays a central role in obesity-related complications such as type 2 diabetes, hypertension, hyperlipidemia, and arteriosclerosis (2). Both the increase of adipocyte size (hypertrophy) and the increase of adipocyte number (hyperplasia) are major contributors to the development of obesity (3). Thus, a tight control of adipocyte development and function is critical in maintaining whole body energy homeostasis, and a full understanding of the mechanisms regulating adipose formation would provide precious information on the way to control of obesity.

Much of our knowledge of adipocyte differentiation has been obtained by studying adipocyte cell culture models. The 3T3-L1 cell line is one of the best studied cellular models (4). Upon the treatment with differentiation inducers (a combination of 3-isobutyl-1-methylxanthine, dexamethasone, and insulin), growth-arrested 3T3-L1 preadipocytes re-enter the cell cycle, a process referred to as mitotic clonal expansion (MCE), which contributes to the hyperplasia of adipocytes. The adipogenic gene expression program is initiated during and after 2–3 rounds of MCE, ultimately leading to terminal adipocyte differentiation (5).

The adipogenic program requires a cascade of multiple transcription factors (6), among which is CCAAT/enhancer-binding protein β (C/EBPβ), an important transcriptional factor belonging to the leucine zipper family. Knockdown of C/EBP β in 3T3-L1 preadipocytes blocks adipogenesis (7, 8), whereas its overexpression is sufficient to induce 3T3-L1 adipocyte differentiation without the hormonal inducers normally required (9). The functional importance of C/EBP β during adipocyte development has also been demonstrated in vivo. Disruption of the C/EBP β gene in mice caused decreased fat mass because of impaired development of adipose tissue (10). Thus, C/EBP β plays a crucial role during adipocyte differentiation.

As an important early factor of adipogenesis, C/EBP β is induced rapidly after the addition of adipogenic stimuli and is responsible for inducing the expression of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), two master adipogenic transcription factors, by binding to their promoters (11). In this way, C/EBP β promotes terminal adipocyte differentiation. Besides the abovementioned function, a number of studies have illuminated additional roles of C/EBP β during adipogenesis, through its transcriptional regulation of many new target genes. Furthermore, the function of C/EBP β is elaborately regulated by post-translational modifications (PTMs), including phosphorylation, acetylation, methylation, O-GlcNAcylation, ubiquitination, and SUMOlation. Herein, the new functions and PTMs of C/EBP β during adipogenesis will be reviewed.

Role and Mechanism of C/EBP β in Mitotic Clonal Expansion

Despite some controversy (12), multiple studies indicate that MCE is a necessary step for the terminal adipocyte differentiation of 3T3-L1 preadipocytes. The extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 and cyclin-dependent kinase inhibitor roscovitine, which inhibit the cell cycle at different points, block MCE as well as adipogenesis (5, 13). The

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2 The abbreviations used are: MCE, mitotic clonal expansion; C/EBP β, CCAAT/enhancer-binding protein β; C/EBPα, CCAAT enhancer binding protein α; IRE1α, inositol-requiring enzyme 1α; PKM2, M2 isoform of pyruvate kinase; PIA51, protein inhibitor of activated STAT1; PTM, post-translational modification; PPARγ, peroxisome proliferator-activated receptor γ; SUMO, small ubiquitin-like modifier; UPR, unfolded protein response; XBP1, X-box binding protein 1; PRMT4/CARM1, protein arginine methyltransferase 4; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein.
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DNA synthesis inhibitor aphidicolin and the anti-proliferation reagent rapamycin also block MCE and 3T3-L1 preadipocyte differentiation (14, 15). Moreover, knockdown of histone acetyltransferase binding to ORC1 (HBO1), a positive regulator for the initiation of DNA replication, impairs the ability of 3T3-L1 preadipocytes to differentiate into mature adipocytes by inhibiting DNA replication and MCE (16). It is hypothesized that DNA replication during MCE increases the accessibility of promoter or enhancer elements to factors required for transcription of genes involved in the initiation of differentiation (17).

Several lines of evidence have shown that C/EBPβ is involved in MCE. When subjected to the same differentiation protocol as 3T3-L1 preadipocytes, a subset of mouse embryo fibroblasts undergoes MCE and terminal differentiation into adipocytes. Mouse embryo fibroblasts from C/EBPβ−/− mice, however, neither undergo MCE nor differentiate into adipocytes (5). Furthermore, knockdown of C/EBPβ by siRNA in 3T3-L1 preadipocytes prevents MCE as well as adipocyte differentiation (7).

Additionally, overexpression of a dominant-negative C/EBPβ (A-C/EBP) that blocks C/EBPβ DNA binding activity by dimerizing through its leucine zipper (18) also disrupts MCE and adipogenesis in 3T3-L1 cells (19). Intriguingly, C/EBPβ takes part in the proliferation of certain other cell types such as lobuloalveolar cells, osteoblasts, and keratinocytes (20–22), further supporting an important role of C/EBPβ in cell proliferation.

To understand how C/EBPβ promotes MCE, a promoter-wide ChIP-on-chip analysis combined with gene expression microarrays was performed to identify the potential target genes of C/EBPβ at the early stage of 3T3-L1 adipocyte differentiation (8). Four cell cycle genes (Cdc45l, Mcm3, Gins1, and Cdc25c) and the chromatin assembly gene histone H4 were identified as C/EBPβ target genes. Mcm3 is a component of MCM2–7 (mini-chromosome maintenance proteins 2–7) complex, whereas Gins1 is a subunit of GINS (go-ichi-ni-san) complex. Cdc45l, MCM2–7, and GINS form a large complex referred to as CMG, which is involved in the regulation of eukaryotic chromosomal DNA replication (23). Cdc25c is a phospho-tyrosine phosphatase that contributes to S-phase and eukaryotic chromosomal DNA replication (23). Cdc25c is a required step for adipocyte differentiation. G9a is an important euchromatic methyltransferase that is responsible for the majority of H3K9me2 in the cells (30). Recent evidence suggests that G9a-mediated H3K9me2 mainly associates with transcriptional silencing (31). G9a plays important roles in various biological processes and has been shown to be a repressor of adipogenesis (27). In 3T3-L1 cells, a transient induction of G9a by C/EBPβ was detected during MCE (32). Then, G9a inhibited PPARγ and C/EBPα expression through H3K9 dimethylation of their promoters. Hence, C/EBPβ up-regulates G9a that delays the transactivation of PPARγ and C/EBPα so as to guarantee MCE, providing another line of evidence for the participation of C/EBPβ in epigenetic regulation.

The embryonic M2 isoform of pyruvate kinase (PKM2) has attracted much attention because of its critical role in aerobic glycosis of tumor cells, namely the Warburg effect (33). Instead of PKM1, tumor cells commonly express PKM2, which may contribute to the metabolism shift from oxidative phosphorylation to aerobic glycosis and tumorigenesis (34). Besides, PKM2 has also been reported to promote tumor growth via regulating cell cycle progression and oncogene expression (35, 36). Of interest, PKM2 expression is elevated during the early stage of 3T3-L1 adipogenesis, and knockdown of PKM2 compromises MCE (37). Further studies, however, are needed to investigate the mechanism of PKM2 in MCE. Importantly, PKM2 is identified as a target gene of C/EBPβ during MCE (37). Consequently, transactivation of PKM2 by C/EBPβ contributes to facilitating MCE. Collectively, these findings (Fig. 1) provide new clues to understanding the action of C/EBPβ in the proliferation of certain specific cell types.

Role of C/EBPβ in Terminal Adipocyte Differentiation

C/EBPβ is an important factor to initiate the transcriptional cascades that culminate in the expression of two essential adipogenic factors, PPARγ and C/EBPα (38). Apart from its well established role in activating the expression of PPARγ and
C/EBPβ, studies in recent years have brought to light a number of new targets of C/EBPβ, which extends our knowledge of its role in terminal adipocyte differentiation.

Unfolded protein response (UPR) is a complex signaling cascade activated by the perturbations in endoplasmic reticulum homeostasis to coordinate multiple signaling pathways and control a variety of physiologies (39). Among the three branches of UPR, the inositol-requiring enzyme 1 (IRE1)/X-box binding protein 1 (XBP1) pathway, which plays a crucial role in glucose and lipid metabolism as well as in insulin function, is the most conserved branch (40). Because dramatic transformations take place during the differentiation from preadipocytes to mature adipocytes, it is hypothesized that adipocytes might exhibit increased level of UPR so as to relieve the stress burden on the endoplasmic reticulum imposed by the increased biosynthesis of protein and lipids (41). A recent study demonstrates that adipogenesis is associated with the increase of UPR and that the IRE1α-XBP1 pathway is indispensable for adipogenesis (42). Knockdown of IRE1α or XBP1 in 3T3-L1 cells significantly inhibits adipogenesis, and XBP1 could directly transactivate the expression of C/EBPα, a master gene of adipogenesis, to promote adipocyte differentiation. Intriguingly, C/EBPβ is responsible for the induction of XBP1 by binding to its proximal promoter region (42). Thus, through regulating the expression of XBP1, C/EBPβ participates in the activation of UPR, a required process for adipogenesis.

Autophagy is a cellular process that delivers cytosolic components to lysosomes for degradation (43). It is involved in a variety of physiological and pathophysiological processes, such as nutrient starvation, immune responses, tumor suppression, cell death, and so on (44). Recent studies have demonstrated that autophagy is required for cell differentiation of certain cell types, including adipocyte differentiation (45, 46). Autophagy was induced during adipogenesis, promoting the degradation of Klf2 and Klf3, two negative regulators of adipocyte differentiation, which is mediated by the adaptor protein p62/SQSTM1 (47). In 3T3-L1 cells, C/EBPβ has been identified as an activator of autophagy through the transactivation of Atg4b, an important autophagy gene that exposes glycine from LC3 precursor at its C terminus to form LC3-I and is essential for autophagosome formation (47). Of interest, C/EBPβ has been shown to regulate circadian autophagy rhythm in the liver (48). These findings highlight an important role of C/EBPβ in controlling the program of autophagy gene expression during some biological processes, including adipogenesis.

FIGURE 1. Multiple roles of C/EBPβ during adipogenesis. Besides its well-known function in the direct transactivation of C/EBPα and PPARγ, many new roles of C/EBPβ during adipogenesis have been revealed in the past decade. At the early stage of 3T3-L1 adipocyte differentiation, C/EBPβ transactivates the expression of multiple cell cycle-related genes to facilitate MCE, a required step for terminal adipocyte differentiation. A novel feed forward mechanism involving C/EBPβ and Kdm4b in the regulation of MCE is illustrated. Moreover, C/EBPβ transiently transactivates the expression of G9a, which delays the expression of C/EBPα and PPARγ, two anti-proliferation factors, so as to ensure MCE. The transactivation of Kdm4b (a histone demethylase) and G9a (a histone methyltransferase) by C/EBPβ provides evidence for the epigenetic control of MCE by C/EBPβ. At the late stage of 3T3-L1 adipocyte differentiation, C/EBPβ is involved in the activation of UPR and autophagy, through the transactivation of Xbp1 and Atg4b, respectively. In addition, C/EBPβ activates the expression of some other transcriptional factors and inhibits the expression of Wnt10b, an anti-adipogenic factor. Together, these effects ultimately lead to the activation or up-regulation of C/EBPβ and PPARγ, thereby promoting terminal adipocyte differentiation. Black solid lines with arrowheads or blunt ends indicate transcriptional regulation of gene expression. Black dashed lines with arrowheads indicate promotion of activity. A black dashed line with a blunt end indicates inhibition of protein stability. Blue dashed lines with arrowheads indicate promotion of biological processes.
Many other target genes of C/EBPβ have been reported and shown to be important for terminal adipocyte differentiation. For instance, C/EBPβ transactivates the expression of Klf5, sterol-responsive element-binding protein 1c (SREBP1c), and early B-cell factor 1 (Ebf1). Klf5 is a key transcription factor for adipogenesis through promoting PPARγ expression (49). SREBP1c is an important pro-adipogenic transcriptional factor that regulates the expression of many lipid metabolism genes and contributes to the generation of endogenous PPARγ ligands (50). Ebf1 promotes adipogenesis by activating PPARγ transcription (51). On the other hand, C/EBPβ is involved in suppression of Wnt/β-catenin signaling through transcriptional inhibition of the expression of Wnt10b, a major Wnt ligand that inhibits adipogenesis (52). Taken together, these findings shed light on the multiple roles of C/EBPβ in terminal adipogenic differentiation (Fig. 1).

**Post-translational Modifications (PTMs) of C/EBPβ during Adipogenesis**

Because of the important role of C/EBPβ in triggering the adipogenic program, it is necessary to gain mechanistic insights into the regulation of C/EBPβ so as to better understand the process controlling adipogenesis. The regulation of C/EBPβ during adipogenesis occurs at multiple levels, including transcriptional regulation, translational regulation, and PTM. Studies on the PTMs of C/EBPβ, including phosphorylation, O-GlcNAcylation, acetylation, methylation, ubiquitination, and SUMOylation, have progressed a lot in recent years, and this progress will be discussed in detail here (Fig. 2). Some of the studies that did not investigate cells during adipogenesis will also be discussed, which might help us better understand the PTMs of C/EBPβ.

Regulation of signaling transduction depends not only on the identity of phospho-sites but also on when phosphorylation events occur. Studies have shown that sequential phosphorylation of C/EBPβ is critical for 3T3-L1 adipocyte differentiation (53). C/EBPβ is expressed rapidly after adipogenic induction (2–12 h) and phosphorylated on Thr-188 by MAPK after 2–12 h of adipogenic induction followed by GSK3β-mediated phosphorylation on Ser-184 or Thr-179 (13). At the onset of S-phase, GSK3β translocates into the nucleus and C/EBPβ is phosphorylated by GSK3β on Ser-184 or Thr-179 (53). Phosphorylation

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**FIGURE 2. The PTMs of C/EBPβ during adipogenesis.**

A. Phosphorylation. C/EBPβ is phosphorylated on Thr-188 by MAPK (2–12 h after adipogenic induction) and by CDK2 (12–24 h after adipogenic induction), followed by GSK3β-mediated phosphorylation on Ser-184 or Thr-179. This dual phosphorylation induces conformational changes in C/EBPβ, which activates its DNA binding and facilitates adipogenesis. B. O-GlcNAcylation. The modification of O-GlcNAc on Ser-180 and Ser-181 of C/EBPβ prevents its phosphorylation on Thr-188, Ser-184, and Thr-179, thus suppressing its DNA binding activity. OGA, β-N-acetylglucosaminidase; OGT, β-N-acetylglucosaminyltransferase. C. Acetylation. In general, acetylation of C/EBPβ increases its transcriptional activity to promote adipogenesis. D. Methylation. PRMT4/CARM1 dimethylates C/EBPβ on Arg-3, which interferes with the interaction between C/EBPβ and SWI/SNF and inhibits adipogenesis. MAPK/CDK2-mediated phosphorylation on Thr-188 could block PRMT4/CARM1-mediated dimethylation of C/EBPβ on Arg-3. E. SUMOylation and ubiquitination. PIAS1-mediated SUMOylation of C/EBPβ on Lys-133 promotes its ubiquitination and proteasomal degradation, thereby suppressing adipogenesis. SUMO-specific protease SENP2 reverses the SUMOylation of C/EBPβ to promote adipogenesis. The cross-talks between different types of PTMs are indicated by red solid lines with arrowheads or blunt ends. The black solid lines with arrowheads at both ends indicate protein interaction.
of Thr-188 appears to prime C/EBPβ for the subsequent phosphorylation on Ser-184 or Thr-179. Studies indicate that this dual phosphorylation induces a conformational change in C/EBPβ that allows dimerization through its C-terminal leucine zipper domain. Dimerization brings the adjacent basic regions into position to hold the C/EBP regulatory elements of its target genes in a "scissor-like" grip (54). All these actions of the dual phosphorylated C/EBPβ facilitate its DNA binding and transcriptional regulatory activities. Recent studies showed that phosphorylation also contributes to the stability of C/EBPβ. Both ex vivo and in vitro experiments indicated that phosphorylation on Thr-188 by MAPK or CDK2/cyclin A protected C/EBPβ from calpain-mediated proteolysis (55).

Protein O-GlcNAc glycosylation is a widespread PTM for both nuclear protein and cytoplasmic protein. It is different from the classical glycosylation of secreted proteins and membrane protein, but is similar to phosphorylation on some level. Both O-GlcNAcylaton and phosphorylation can take place on serine and threonine residues. It has been demonstrated that C/EBPβ could be modified by O-GlcNAcylaton on Ser-180 and Ser-181, which are very close to its phosphorylation sites (Thr-188, Ser-184, Thr-179) (56). Studies have proved that the O-GlcNAcylaton of these two sites suppressed the phosphorylation on the adjacent sites, thereby delaying 3T3-L1 adipocyte differentiation (56). Thus, O-GlcNAcylaton of C/EBPβ modulates its phosphorylation and transcription activity through the adjacent sites-mediated competition.

Protein acetylation contributes to the protein interaction with DNA and/or other proteins, like co-activators and other transcriptional regulators. C/EBPβ has a plurality of acetylation sites, whose acetylation can modulate its function. Cesena et al. (57) discovered that the nuclear co-activator p300 possesses acetyltransferase activity and modifies C/EBPβ on multiple lysine residues. The acetylation on Lys-39 of C/EBPβ is critical for its transcriptional activity. Furthermore, Lys-39 deacetylation mediated by HDAC1 down-regulates its activity during adipogenesis (58). Wiper-Bergeron et al. (59) reported that, in the process of glucocorticoid-induced preadipocyte differentiation, acetylase GCN5 and p300/CBP-associated factor (PCAF) mediate C/EBPβ acetylation on Lys-98, Lys-101, and Lys-102, and this acetylation acts as a molecular switch repressing the interaction of C/EBPβ with HDAC1 and reducing the affinity between C/EBPβ and the corepressor mSin3α. In some cases, however, HDAC1 can strengthen the function of C/EBPβ. Xu et al. (60) reported that acetylation on Lys-215 and Lys-216 decreases the binding activity of C/EBPβ to the promoter of ID1 (inhibitor of DNA-binding protein), and HDAC1-mediated deacetylation can activate this transcription.

Methylation modifies not only DNA and histone, but also some transcription factors. Pless et al. (61) found that Lys-39 of C/EBPβ could be modified by histone methyltransferase G9a and that this modification could inhibit its transcriptional activity. Moreover, the interaction of C/EBPβ with G9a could be inhibited by C/EBPβ phosphorylation (61). Kowenz-Leutz et al. (62) showed the relationship between C/EBPβ phosphorylation and arginine methylation. Protein arginine methyltransferase 4 (PRMT4/CARM1) interacts with C/EBPβ and di-methylates it on Arg-3. The phosphorylation of C/EBPβ by Ras/PRMT4/CARM1 inhibits the methylation on Arg-3. The Arg-3 methylation constrains the interaction between C/EBPβ and SWI/SNF and inhibits adipocyte differentiation. Consequently, C/EBPβ phosphorylation by Ras signaling pathway and arginine methylation reciprocally regulates the interaction between C/EBPβ and epigenetic complexes during adipocyte differentiation.

The lysine residue is not only the substrate of acetylation and methylation but is also modified by ubiquitin. Through the sequential action of E1-activating enzyme, E2-binding enzyme, and E3 ligase, the ubiquitin polymers are connected to the target proteins. Hattori et al. (63) found that the C/EBP family proteins are degraded by the ubiquitin-proteasome pathway. In the process of C/EBP protein ubiquitination, ubiquitin ligases or modifying enzymes specifically recognize the monomer form of C/EBP proteins, so as to remove the transcriptionally inactive monomer of C/EBP proteins and maintain a basal level of C/EBP proteins in cells. The homologous dimerization of C/EBPβ or the heterologous dimerization of C/EBPβ with other C/EBP family proteins can make the protein itself stable (63).

With in-depth study of ubiquitination, the small ubiquitin-like modifications (SUMOlation), have attracted more and more attention. SUMOylation is a reversible PTM that regulates the protein subcellular localization, nucleocytoplasmic transport, protein stability, and interaction, by the conjugation of the small ubiquitin related modifier (SUMO) to target proteins (64). Kim et al. (65) reported that C/EBP family proteins, including C/EBPa, C/EBPβ, C/EBPδ, and C/EBPε, are modified by SUMO. There is a conserved motif containing 5 amino acids ((I/V/L)KXE/L) in C/EBP family proteins, and the lysine residue in this motif is specific to SUMO modification (65). C/EBPβ is a SUMO target, and SUMO modification controls its transcriptional activity. Eaton and Sealy (66) found that SUMO is conjugated to Lys-173 residue of C/EBPβ, and blocking SUMOlation on Lys-173 by Lys to Ala mutation relieves its repression on cyclin D1 promoter. Subramanian et al. (67) found that SUMO modification in the synergy control motifs of multiple C/EBP molecules could limit their transcriptional activity. Berberich-Siebelt et al. (68) also found that SUMO could be conjugated to the lysine residue of C/EBPβ in the conserved motif Ile/Val-Lys-X-Glu of the central regulatory domain, which weakened the inhibitory effect of C/EBPβ on c-Myc in murine T cells. Interestingly, this SUMOlation promoted the location of C/EBPβ around the centrosome of heterochromatin, which suggests that SUMO could regulate C/EBPβ function by changing its subnuclear localization (68). It was recently reported that PIAS1, a SUMO E3 ligase, could interact with C/EBPβ and SUMOylate it on Lys-133, leading to increased ubiquitination and degradation of C/EBPβ (69). Consequently, PIAS1 is a negative regulator in adipogenesis by promoting the SUMOlation and degradation of C/EBPβ. Conversely, the SUMO-specific protease Sentrin/SUMO-specific protease 2 (SENP2) plays a critical role in promoting adipogenesis by de-SUMOlation and stabilization of C/EBPβ (70).

In summary, the modification of C/EBPβ, involving the cross-talks of different types of PTMs, finely tunes its function.
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Considering the fact that the current studies on C/EBP PTMs are performed in vitro, knock-in mice expressing PTM-related C/EBP mutants are needed to dissect the physiological relevance of C/EBP PTMs in regard to adipose tissue development.

Conclusion

Much progress has been made in the past decade in defining the role of C/EBPβ during adipogenesis. The expression and activity of C/EBPβ play a profound role in modulating a wide array of target genes that are important in facilitating adipogenesis. Also, the identification and characterization of C/EBPβ target genes have provided critical information for understanding the function of C/EBPβ in adipogenesis. Meanwhile, the PTM controlling C/EBPβ function has been intensively explored. It should be noted, however, that some studies on the role and regulation of C/EBPβ are mainly based on murine cell models in vitro, which heightens the need for further verifying these findings in vivo and translating them from mouse to human. As our knowledge of the multifaceted nature of C/EBPβ during adipogenesis increases, it is believed that C/EBPβ and factors regulating its function will provide potential targets for the treatment of obesity-related disorders.

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