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REVIEWS

Review

Emerging Role of C/EBPβ and Epigenetic DNA Methylation in Ageing

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Changes in epigenetic DNA methylation are the most promising predictor of biological age and lifespan in humans, but whether methylation changes affect ageing is unresolved. Here, we discuss converging data, which indicate that one mode by which aberrant DNA methylation can affect ageing is via CCAAT/enhancer binding protein beta (C/EBPβ). This basic leucine-zipper (bZIP) transcription factor is controlled by the lifespan regulator mechanistic/mammalian target of rapamycin complex 1 (mTORC1) and plays an important role in energy homeostasis and adipose tissue differentiation. Emerging evidence indicates that access of C/EBPβ proteins to cognate binding sites is regulated by DNA demethylation via ten-eleven translocation (TET) methylcytosine dioxygenases and their adaptor proteins growth arrest and DNA damage-inducible protein 45 alpha (GADD45α) and inhibitor of growth 1 (ING1). We discuss the emerging causal nexus between C/EBPβ, energy metabolism, and DNA demethylation in organismal ageing.

Epigenetic DNA methylation: Driver or Bystander of Ageing?

A multitude of molecular alterations accompany organismal ageing [1,2]. Among them, epigenetic alterations have a great influence on ageing, including histone modifications, exchange of canonical histones by histone variants, and the noncoding RNA expression pattern [3]. One of the best documented changes during ageing involves alterations in DNA methylation (Box 1). However, whether methylation changes can affect ageing is unclear. Recent evidence indicates that access of the C/EBPβ transcription factor to cognate binding sites is regulated by DNA demethylation and that impaired demethylation of C/EBPβ sites can lead to premature ageing [4]. This **basic leucine zipper (bZIP)** protein (see Glossary) plays an important role in controlling energy homeostasis and is regulated by **mechanistic/mammalian target of rapamycin complex 1 (mTORC1)**, a major regulator of health and lifespan. Here, we provide an integrated view on DNA methylation and ageing together with the role C/EBPβ may play in metabolism and longevity.

Mammalian DNA methylation is an epigenetic mark that occurs mostly on CpG dinucleotides. Globally, DNA methylation and CpG density show a bimodal distribution. CpG islands (CGIs), GC-rich regions with high densities of CpGs found near promoter regions, are generally unmethylated. In contrast, most regions with low CpG density are heavily methylated in normal tissues, notably at repetitive DNA, where the bulk of genomic DNA methylation resides. Besides unmethylated and heavily methylated regions, a third category exists, namely, lowly methylated regions (LMRs), which are non-CGI loci associated with regulatory elements such as **transcription factor (TF)** binding sites. TF binding is necessary and sufficient to create LMRs [5].

The genome-wide pattern of DNA methylation is tissue and cell state specific. It is the result of the action of cytosine DNA methyltransferases, of which there are three in mammals, DNMT1, DNMT3A, and DNMT3B. However, methylation patterns are also shaped by specific pruning of methyl marks, both by active as well as passive DNA demethylation [6]. Active DNA methylation proceeds by oxidation of methyl groups via the TET family of methylcytosine dioxygenases [7]. TET enzymes oxidize 5mC sequentially to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Thymine DNA glycosylase (TDG) removes 5fC and 5caC to restore unmethylated cytosine.

DNA methylation is mostly associated with gene silencing, although the correlation of methylation with gene activity is often loose [6]. For example, despite millions of genomic sites that become unmethylated in embryonic stem cells lacking *Dnmt1*, including at least 6100 promoters, only a few

Highlights

Ageing is closely associated with and influenced by energy metabolism, and C/EBPβ is emerging as a key regulator of energy metabolism and longevity.

DNA hypermethylation in GADD45α/ING1 mutant mice is associated with progeria due to a failure of TET-dioxygenase mediated demethylation of C/EBPβ binding sites.

Accordingly, GADD45α/ING1 mutant mice phenocopy major symptoms of C/EBPβ mutant mice, indicating that a GADD45α–ING1–C/EBPβ axis regulates energy metabolism and ageing.

mTORC1 controls the translation of Cebpb-mRNA into two isoforms, the transcriptional activator liver-enriched activating protein (LAP), and inhibitor liver-enriched inhibitory protein (LIP). C/EBPβ supermice, in which the inhibitory LIP is inactivated display healthier ageing and prolonged life span.

The results indicate a causal nexus between C/EBPβ, energy metabolism, and DNA demethylation in organismal ageing.

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Box 1. Epigenetic DNA Methylation Clocks

Statistical analysis of publicly available human DNA methylation data led to the proposition of signature methylated CpGs, designated 'epigenetic clocks', whose methylation level tightly correlates with biological age. Hannum's clock is applicable to whole blood and interrogates 71 CGs. Horvath's clock is a multitissue predictor and interrogates 353 CGs [65]. Hannum's and Horvath's clocks have correlation coefficients >0.9 with chronological age and average errors below 5 years. Horvath's clock is the most widely used and it permits comparing the biological age of different tissues from the same individual. Epigenetic age according to Horvath's clock is close to zero for embryonic cells and shows biphasic behavior, ticking fast during childhood, and from ~20 years of age the clock continues at a slower constant rate. These epigenetic clocks predict biological age and inform about positive or negative deviation of biological from chronological age, as well as predicting time to death. For example, DNA from tumour tissue shows accelerated biological age compared with chronological age of the patient. HIV infection, known to induce premature biological aging, also accelerates the epigenetic clock [66]. Conversely, super-centenarians may have decelerated epigenetic age [66]. *In vitro*, DNA methylation age increases with cell passage number while reprogramming can reverse DNA methylation age [65,67]. Recently improved multitissue clocks for the first time also reveal age acceleration in DNA from Hutchinson Gilford progeria syndrome patients [68] and strongly predict time to death and time to heart disease [69].

hundred genes are actually de-repressed [8]. Instead of acute gene activity DNA, methylation may rather confer a memory or propensity to activate a gene upon cellular differentiation or environmental stimuli [9].

DNA methylation changes associated with ageing, including global hypo- and local hypermethylation, have been known for decades. This trend is similarly seen in cancer cells [10]. The genome-wide drift in ageing cells towards global DNA hypomethylation occurs mostly in repetitive regions where the bulk of methylation lies. This hypomethylation is thought to be responsible for reactivation of **retrotransposons** and is one of the mechanisms that may be promoting cancer [11]. Regions undergoing local hypermethylation are more complex. For example, in ageing mice, **Polycomb target genes** and certain tumor suppressor genes are enriched among the hypermethylated genes [12]. DNA methylation changes with ageing are so stereotypical, that epigenetic clocks have been devised to predict biological age and lifespan (Box 1). The most widely used, the Horvath clock, utilizes a few hundred diagnostic CpGs and is applicable to multiple tissues. However, the number of sites needed for age prediction in blood DNA has been reduced to just three CpGs [13] or even one [14], but they perform less accurately.

Specific regions undergoing methylation changes across different biological samples are commonly referred to as differentially methylated regions (DMRs) and are regarded as possible functional regions involved in gene transcriptional regulation. Hence, there was a lot of hope that DMRs associated with ageing, including the CpGs monitored in epigenetic clocks, would be informative about mechanisms of ageing. However, characterization of ageing DMRs from epigenetic clocks or other studies has not yielded much mechanistic insight [15,16]. For example, between three epigenetic methylation clocks devised for mice [17–19], little overlap is found [15].

There is a propensity of clock CpGs to be found near genes involved in development and differentiation [16] and this appears consistent with the tendency of hypermethylated ageing DMRs to locate at **bivalent chromatin domain promoters** harboring Polycomb target genes [20–22]. Since Polycomb and bivalent promoters frequently regulate developmental control genes, age-related hypermethylation ultimately may reflect changes in tissue homeostasis and cell differentiation [16]. Other than this rather general conclusion, there is no cell biological process, including expression level of genes nearby clock CpGs or ageing DMRs, which show consistent correlation with the methylation level. Hence a key question remains: is DNA methylation a driver or a passenger of the ageing process?

Ageing by Reduced Access to C/EBPβ Binding Sites

Recent evidence for a causative role of DNA hypermethylation in ageing comes from a mouse model that is a homozygous mutant for two stress response genes, *Gadd45a* and *Ing1*, and displays a

Glossary

Autophagy: cellular mechanism eliminating and recycling unnecessary or defective cell components.
Basic leucine zipper (bZIP): superfamily of transcription factors; the leucine zipper mediates the required dimerization for DNA binding specified by the basic region at duplex DNA sequences, as homodimers or heterodimers.
Bivalent chromatin domains: regions of regulatory chromatin bound by histones modified by repressive and activating marks.
Calorie/dietary restriction (CR): reduced calorie intake baring malnutrition, which alters metabolic function and is associated with increased lifespan in various organisms.
Cellular senescence: phenomenon whereby normal cells cease to divide after a limited number of cell divisions and display an array of altered molecular features.
ChIPseq: method combining chromatin immunoprecipitation with massively parallel DNA sequencing in order to map global binding sites for any protein of interest.
DNA-damage response: network of cellular pathways that sense, signal, and repair DNA lesions.
Lipogenesis and lipolysis: metabolic process whereby fat is generated or degraded, for metabolic energy storage and release, respectively.
Mammalian or mechanistic target of rapamycin complex 1 (mTORC1): protein complex composed of mTOR itself, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (MLST8), proline-rich Akt substrate 40kD (PRAS40), and DEP domain-containing mTOR-interacting protein (DEPTOR). mTORC1 functions as a nutrient/energy/redox sensor and controls various processes including protein synthesis.
Memory:naïve T-cell: ratio between memory T cells that act in response to re-exposure to antigens and naïve T cells that form the pool of T cells that are activated in response to unknown antigens. The ratio increases during ageing and is a measure of immunological ageing.

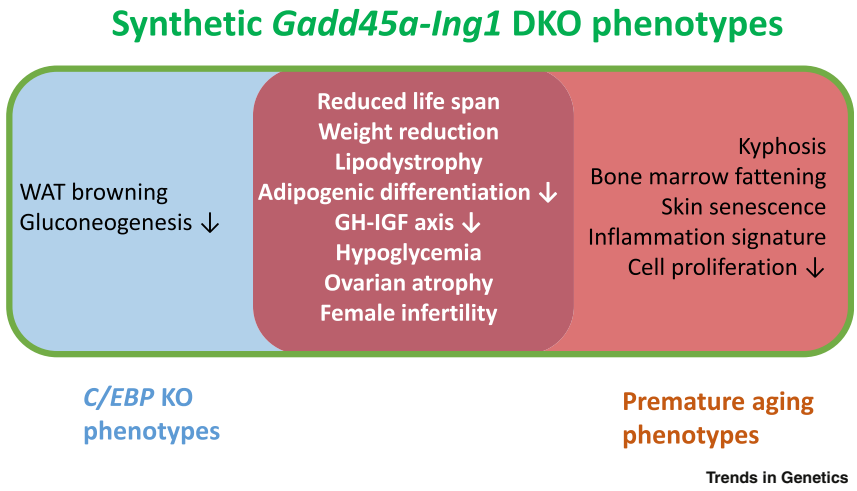

Figure 1. Congruence of Progeric Phenotypes in *Cebpb* and *Gadd45a/Ing1* Mutant Mice.
Overlap of synthetic phenotypes of *Gadd45a/Ing1* double knockout (DKO) mice with phenotypes exhibited by mice mutant for *Cebpb* and progeroid mice, respectively. Abbreviations: C/EBP, CCAAT/enhancer binding protein; GH, growth hormone; IGF, insulin-like growth factor; KO, knockout; WAT, white adipose tissue. Redrawn after [4].

premature ageing phenotype (hereafter referred to as double knockout; DKO) [4]. GADD45α and ING1 cooperate as adapter proteins in TET/TDG-mediated DNA demethylation, targeting the demethylating enzymes to specific genomic loci [4,23–26] Consequently, DKO mouse embryonic fibroblasts (MEFs) display mostly hypermethylated DMRs [4]. Both the hypermethylated DMRs and GADD45α binding sites are located next to **superenhancers** that also have signatures of C/EBPβ binding sites. Indeed DKO cells show reduced binding of C/EBPβ to cognate sites. Consistently, DKO mice phenocopy symptoms of mutants of *Cebpb* or the related *Cebpa* gene; for example, with defects in adipogenesis, fat browning, increased catabolism, and female infertility. *Ing1* appears to be the driver of these phenotypes, as for example, single *Ing1* mutant MEFs already display mild adipogenesis defects, which are exacerbated in DKOs. **ChIPseq** reveals that Ing1 protein in MEFs binds to promoters, which show a gene ontology signature for signaling by the insulin receptor, a top prolongevity gene, and motifs for E2F TFs. E2Fs are prominent regulators of metabolism, including adipogenesis and insulin signaling [27], and they are well established downstream mediators of C/EBPβ and C/EBPα during cell differentiation [28]. Moreover, binding sites of E2F1 show a strong enrichment among ageing-associated genes [29].

Most of the *Cebp* mutant-like symptoms observed in *Gadd45a/Ing1* DKO mice are also characteristic for aged or **progeroid** mice (Figure 1), indicating that the premature aging of the DKO mice, in fact, reflects impaired C/EBP function. This suggests a model where GADD45α–ING1 promote enhancer demethylation to permit C/EBPβ binding and transactivation of target genes, which maintains energy homeostasis and prevents organismal ageing. While these results indicate that aberrant DNA methylation can drive premature ageing, it remains to be shown if the hypermethylated CpGs identified in the DKO mice also show corresponding changes in physiologically aged mice and whether they overlap with CpGs defined in mouse epigenetic clocks. Curiously, DNA methylation actually enhances C/EBPβ binding to canonical TTGC|GCAA sites *in vitro* [30], while *in vivo* unmethylated sites are preferred [31], possibly due to interference by methyl-CpG-binding domain (MBD) proteins.

Hypermethylation of C/EBPβ-dependent superenhancers in the DKO model is manifest already in naïve embryonic fibroblasts. This suggests that demethylation of C/EBPβ sites occurs during embryogenesis and poises enhancers to fire upon adequate stimuli in adult life. Thus, epigenetic ageing may in fact start during fetal life, which is supported by the finding that the DNA methylation clock

NAD$^+$: cofactor involved in several essential metabolic reactions, including redox reactions, SIRT1 and other sirtuins, and ADP-ribosylation reactions.
Oxidative phosphorylation: metabolic pathway in mitochondria used to produce adenosine triphosphate (ATP).
Polycomb proteins: family of protein complexes that remodel chromatin and promote gene silencing.
Progeria: genetic disorder of premature ageing.
Retrotransposons: abundant genomic elements that can self-amplify.
Superenhancers: large regions in the mammalian genome integrating multiple closely spaced enhancers which are collectively occupied by an array of transcription factor proteins; often associated with regulating a differentiated cell state.
Telomeres: repetitive region of chromosomal ends, which protect chromosomes from deterioration; for example, by the action of telomerase; shortened telomeres induce cellular senescence.
Transcription factors (TFs): proteins that regulate the rate of transcription from DNA into mRNA by binding to specific DNA sequences and recruiting coregulatory factors.
Upstream open reading frame (uORF): of small size that resides in the leader sequence of mRNAs and acts *cis*-regulatory on the regulation of mRNA translation.

Box 2. Transcription factors C/EBPβ and C/EBPα

C/EBPs belong to the bZIP class of TFs, including C/EBPα, -β, -γ, -δ, -ε, and -ζ, that bind specific DNA sequences as homo- and heterodimers. C/EBPα and C/EBPβ are critical regulators of energy and fat metabolism. *Cebpa* knockout mouse models suffer from impaired adipocyte function and energy homeostasis [70], and hematopoietic and lung epithelial defects [71–73]. *Cebpb* knockout mouse models most prominently suffer from macrophage dysfunction [74], lymphoproliferative disorder [75], lipodystrophy with impaired brown adipocyte differentiation and browning of WAT [76,77], female infertility [78], and mild skin hyperplasia [79]. *Cebpb* deficiency protects against high-fat-diet-induced obesity [80]; reduces adiposity, steatosis, and diabetes in the obese $Lepr^{db/db}$ mouse model [81]; and attenuates inflammation and lipid accumulation in a model for nonalcoholic steatohepatitis [82]. Both C/EBPα and C/EBPβ are important regulators of the cell cycle and cellular differentiation [28]. Cell cycle arrest induced by C/EBPα or C/EBPβ involves inactivation of E2F transcription factors that control the activation of S-phase entry genes [28]. The *Cebpba- and Cebpb*-mRNAs are translated into three protein isoforms using subsequent in-frame AUG codons. Important for this review are the C/EBPβ isoforms LAP1 and -2 and C/EBPβ-LIP [56,83]. The longer protein isoforms LAP1/2 are transcriptional activators that use their N-terminal part to recruit co-regulators. The smaller C/EBPβ–LIP protein originates from a downstream AUG codon and is therefore N-terminally truncated but retains the C-terminal bZIP DNA-binding region. It functions as competitive inhibitor of the longer isoforms through binding to the same DNA-recognition sites. In addition, more sophisticated functions may be carried out by the truncated isoforms, in particular by C/EBPα–p30 that retains part of the N-terminal domains, but also by C/EBPβ–LIP that merely consists of the bZIP domain as was suggested by a recent study [84]. The LIP:LAP ratio is translationally regulated and determines the level of active C/EBPβ [56,85].

faithfully reports chronological age of fetal retinal cells [32]. Similarly, there is evidence for DNA methylation mediating the developmental origins of adult-onset disease [33]. A fetal origin of ageing aligns with observation in wild animals that a higher quality developmental environment, for example, nutrition, can impact ageing [34]. This has been coined the 'silver spoon' effect by Grafen, referring to positive correlations between characters in the adult that are positively associated with fitness, brought about by the common underlying cause of favorable or unfavorable environmental events during development [35].

GADD45α and ING1 have previously been linked to ageing and both interact with several longevity regulators. GADD45α is a multifunctional protein involved in cell cycle regulation, apoptosis, stress response, and DNA repair [36]. As a stress response gene, *Gadd45a* is transcriptionally activated by p53 and FoxO3a. Lamin A, a protein which is mutated in Hutchinson–Gilford **progeria** interacts with Ing1 [37]; Ing1 and GADD45α both regulate **senescence** [38,39]. *HSF1* is a well-established longevity gene and GADD45α acts downstream in an HSF1–FOXO3–SOD2/CAT/GADD45α cascade implicated in stress response and lifespan extension [40]. In *Drosophila*, overexpression of *D-GADD45* increases longevity, with long-lived animals showing resistance to stress [41]. GADD45β, a close homologue of GADD45α, mediates systemic gene expression responses in mice during **calorie restriction (CR)** [42], which is associated with longevity. Deletion of *Gadd45a* prolongs lifespan in telomerase-deficient mice [43]. Mechanistically, GADD45α promotes demethylation at the subtelomeric regions of short **telomeres** and its deficiency promotes chromatin compaction and attenuates initiation of a DNA damage response at short telomeres of telomerase-deficient cells.

The finding that impaired C/EBPβ function emerged as a cause of progeria in *Gadd45a/Ing1* DKOs is tantalizing in light of the important nexus between energy metabolism and ageing since C/EBPs are key regulators of glucose and fat metabolism (Box 2), as discussed below.

mTORC1 Controlled C/EBPβ-Isoform Expression in Health and Lifespan Regulation

C/EBPβ was postulated to play a role in health span and lifespan determination ever since studies revealed its role in the regulation of metabolism and energy homeostasis. Transgenic mice in which the *Cebpa* gene is replaced by a second *Cebpb* gene have an increased median lifespan of 20% and show

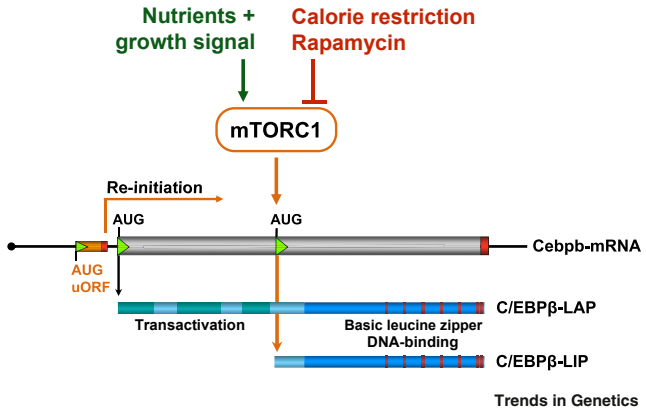

Figure 2. mTORC1-Controlled Translation of *Cebpb*-mRNA.
Cebpb-mRNA is translated into the protein isoforms C/EBPβ–LAP and C/EBPβ–LIP. The transcriptional activator C/EBPβ–LAP is translated by regular translation initiation. Translation into the transcriptional inhibitory protein C/EBPβ–LIP requires an initial translation of the uORF in *cis* and subsequent translation re-initiation at the downstream AUG codon. This process is stimulated by mTORC1 signaling, which in turn is stimulated by nutrient availability and growth factors and suppressed under calorie restriction or pharmacological inhibition by rapamycin. Abbreviations: C/EBPβ, CCAAT/enhancer binding protein β; LAP, liver-enriched activating protein; LIP, liver-enriched inhibitory protein; mTORC1, mammalian/mechanistic target of rapamycin complex 1;. uORF, upstream open reading frame.

reduced fat storage and increased mitochondrial biogenesis in white adipose tissue (WAT) [44,45], suggesting that C/EBPβ acts as a pro-longevity factor. *Cebpb* is a recurring top hit in screens identifying candidate transcriptional regulators of ageing-associated genes [29,46,47]. Besides its transcriptional activities on superenhancers, the regulation of C/EBPβ protein isoform expression by the mTORC1 signaling pathway provides another link to lifespan regulation [48]. The mTORC1 pathway integrates intracellular nutrient and energy availability with growth factor and/or hormonal signals. Its activation stimulates the conversion of nutrients and energy into macromolecules for cell growth while at the same time inhibiting the recycling of cellular constituents back into nutrients through **autophagy**. Inhibition of the mTORC1 pathway in response to CR is seen as a key event that mediates the health- and lifespan-extending effect of CR. Accordingly, reduced signaling through the mTORC1 pathway itself caused by pharmacological inhibition (e.g., by rapamycin) or by genetic mutations of pathway components delays the onset of age-related diseases and extends lifespan in different model organisms [49,50]. Notwithstanding its key role in ageing, little is known of the factors downstream of mTORC1 that mediate the physiological response in terms of gene regulation.

mTORC1 controls the translation of the *Cebpb*-mRNA into its two functionally different protein isoforms, the transcriptional activator liver-enriched activating protein (LAP) and the transcriptional inhibitor liver-enriched inhibitory protein (LIP), and thereby adjusts metabolic gene regulation downstream of C/EBPβ to energy/nutrient availability [48]. mTORC1 stimulates the expression of the inhibitory C/EBPβ isoform LIP through a translational mechanism that requires an **upstream open reading frame (uORF)** within the *Cebpb*-mRNA that serves as a *cis*-regulatory element. Thus, reduced mTORC1 activity results in lower C/EBPβ–LIP expression and thereby in reduced inhibitory C/EBPβ function. Since the activating C/EBPβ isoform C/EBPβ–LAP is not affected by mTORC1, the net effect of mTORC1 inhibition is an increase in C/EBPβ transcriptional activity (Figure 2).

Strong support for a role of differential C/EBPβ isoform expression in health and lifespan regulation comes from knock-in mice that contain a mutation of the *cis*-regulatory *Cebpb*-uORF required for mTORC1-mediated LIP expression. In these $Cebpb^{\Delta uORF}$ mice C/EBPβ–LIP expression is abrogated and as a consequence the transcriptional C/EBPβ function is increased (C/EBPβ super-mice), which mimics reduced mTORC1 signaling at the level of C/EBPβ. These C/EBPβ super-mice display an

Key Figure

Nexus between C/EBPβ, DNA Methylation, and Longevity Pathways

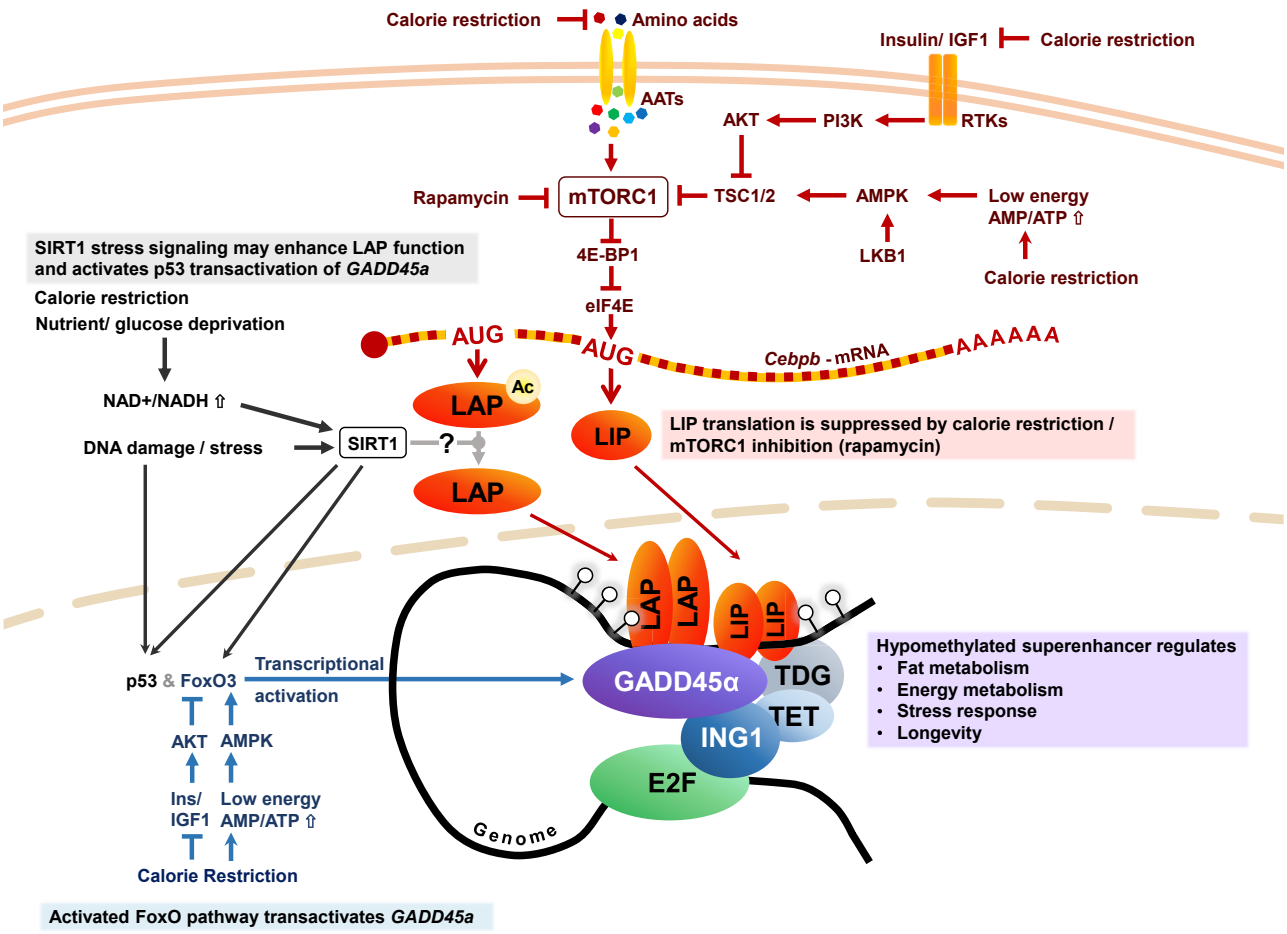

Figure 3. The activating C/EBPβ–LAP and inhibitory C/EBPβ–LIP transcription factors bind to hypomethylated superenhancers to modulate gene expression. The LIP:LAP ratio is regulated by mTORC1 signaling, which integrates nutrient, energy, and growth factor signaling. mTORC1 signaling and C/EBPβ–LIP expression are suppressed under conditions of nutrient and/or energy deprivation, such as calorie restriction. Occupancy of C/EBPβ sites is determined by DNA demethylation of superenhancers residing in chromatin loops with E2F-associated promotors. TET/TDG-mediated demethylation of C/EBPβ binding sites is targeted by GADD45α associated with C/EBPβ binding sites and ING1 bound at E2F-associated promotor sequences. Connections to other longevity proteins including SIRT1, p53, and FoxO3 is depicted. The involvement of SIRT1-mediated deacetylation is hypothetical, and based on SIRT1–C/EBPα regulation. Abbreviations: AAT, amino acid transporter; Ac, protein lysine-acetylation; AKT, also known as protein kinase B (PKB); AMPK, AMP-activated protein kinase; 4E-BP, initiation factor eIF4E binding protein; C/EBPβ, CCAAT/enhancer binding protein β; eIF4E, translation initiation factor 4E; FoxO3, Forkhead box O3; GADD45α, growth arrest and DNA damage protein 45α; IGF-1, insulin-like growth factor 1; ING1, inhibitor of growth family member 1; LAP, liver-enriched activating protein, C/EBPβ protein isoform; LIP, liver-enriched inhibitory protein, C/EBPβ protein isoform; LKB1, liver kinase B1, also known as serine/threonine kinase 11 (STK11); mTORC1, mammalian/mechanistic target of rapamycin complex 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; RTK, receptor tyrosine kinase; SIRT1, sirtuin 1, silent mating type information regulation 2 homolog 1; TDG, thymine DNA glycosylase; TET, ten-eleven translocation. Open lollipops, unmethylated CpGs at C/EBPβ sites.

improved metabolic phenotype with features observed under CR [51], including leanness, increased insulin sensitivity and glucose tolerance, enhanced fatty acid oxidation, reduced steatosis, and higher adiponectin levels [48]. At gene regulation level, C/EBPβ super-mice show an increased expression of genes connected to both *de novo* **lipogenesis** and **lipolysis** in WAT, while in liver lipogenesis, genes tend to be repressed and genes involved in β-oxidation are upregulated. The same gene expression pattern is observed in mice under CR suggesting that, in C/EBPβ super-mice, a similar metabolic roundabout is induced as in mice under CR. In CR-fed mice, ingested carbohydrates are first converted into fat that is subsequently broken down again through lipolysis into fatty acids, which undergo β-oxidation in peripheral tissues for energy production. In other words, mice under CR burn more fat than they eat [51]. Although this metabolic detour with increased fat production at first sight seems not to agree with the reduced food availability under CR conditions, it is proposed to be at least partially responsible for the beneficial effects of CR [51] and therefore could also contribute to the increased metabolic health in C/EBPβ super-mice.

Female C/EBPβ super-mice show a markedly increased median lifespan of 20%, that is partially due to a decrease and delay in cancer incidence. Aged C/EBPβ super-mice appear rejuvenated, with better motor coordination, a more juvenile **memory:naïve T cell ratio**, and a reduction in ageing-associated interindividual variation in gene expression, particularly of genes related to fatty acid metabolism and **oxidative phosphorylation**. Since these genes are either known or anticipated C/EBPβ target genes, their increased variation in expression levels upon ageing in wild-type mice might be due to ageing-associated fluctuation in C/EBPβ–LIP/LAP isoform ratios, which is prevented in the C/EBPβ super-mice. C/EBPβ–LIP expression is indeed elevated in aged liver and fat tissue [52–54], suggesting that ageing-associated deregulation of C/EBPβ–LIP may contribute to the risk of developing age-related conditions. This idea is supported by a mouse model that only expresses the C/EBPβ–LIP isoform but lacks C/EBPβ–LAP isoform expression as these mice display an earlier onset of ageing-associated tumorigenesis and a reduction in lifespan [55]. Altogether, the studies demonstrate that an increased transcriptional function of C/EBPβ promotes health and lifespan in mice.

Potential Role of C/EBPα in Health and Lifespan Regulation

Similar to C/EBPβ, C/EBPα is an important metabolic regulator with partially overlapping target genes and functions with respect to C/EBPβ. Similar to *Cebpb*, the *Cebpa*-mRNA contains a *cis*-regulatory uORF and is translated into different protein isoforms, termed C/EBPα-p42 and C/EBPα-p30. As for C/EBPβ–LIP, the expression of C/EBPα–p30 is under control of mTORC1 signaling [56], indicating a role for C/EBPα in mTORC1 downstream effects.

Besides mTORC1, another nutrient-sensitive regulator connected with the control of health and lifespan, the **NAD^+**-dependent deacetylase SIRT1, affects C/EBPα activity [57]. Fluctuations in NAD^+ levels due to nutrition, exercise, circadian rhythm, or ageing regulate SIRT1 activity. Initially, the homologous yeast Sir2 was described as extending lifespan, but further studies in worms, flies, and mice have challenged this interpretation and led to the current prevailing view that SIRT1 activity plays a role in health maintenance and stress response [58–60]. Increased expression of the SIRT1 homologues in yeast, flies and *Caenorhabditis elegans* results in lifespan extension, and mammalian SIRT1 activity plays a role in health maintenance and stress response, acting to extend lifespan by rescuing from the effects of life-shortening stress [58–60]. SIRT1 deacetylates C/EBPα, which is required for the elevation of mitochondrial mass and respiratory function in response to glucose deprivation, suggesting that C/EBPα is a critical downstream mediator of SIRT1 function. This is supported by the finding that expression of a deacetylation-mimicking C/EBPα mutant is sufficient to increase the expression of mitochondrial genes and enhance mitochondrial respiration in the absence of SIRT1 or nutrient deprivation [57]. Mouse models addressing these mTORC1- or SIRT1-dependent modes of C/EBPα regulation should give more evidence for a possible role of C/EBPα in health and lifespan regulation in the future.

Other C/EBPs in Ageing

It is likely that other C/EBP proteins also play a role in ageing, given their overlapping function and binding site specificity. C/EBPδ mediates **DNA-damage response**, and similar to GADD45α and

ING1, C/EBPδ deficiency sensitizes mice to ionizing radiation [61]. Loss of C/EBPδ exacerbates cognitive decline in aged mice exposed to radiation due to impaired oxidative stress response [62]. Relatedly, the worm orthologue of CEBPβ regulates a neuronal gene clusters in ageing nematodes [63]. Furthermore, coexpression screening of a seed list of genes overexpressed with age yielded *Cebpa*, *Cebpb*, and *Cebpd* as the top hits, and C/EBP motifs were found to be enriched in the promoters of ageing-associated genes [46]. It is important to consider that different C/EBP family members form heterodimers and share DNA recognition sites, but differ in expression patterns and upstream regulatory pathways. Due to this complexity, little is known about an integrated C/EBP function coming from the combined action of the different C/EBP family members and its involvement in the ageing process. This will be an important topic of further studies.

Concluding Remarks

Whether changes in epigenetic DNA methylation can drive organismal ageing is an unresolved question. Progeric *Gadd45a/Ing1* DKO mice show impaired binding of C/EBPβ due to a defect in TET-mediated DNA demethylation of C/EBPβ superenhancers. This suggests a GADD45α–ING1–C/EBPβ axis regulating energy metabolism and in turn longevity. Hence, changes in DNA methylation are not only excellent markers for biological age but actually can cause ageing when interfering with normal C/EBPβ activity. Independent evidence for C/EBPβ regulating ageing comes from mouse mutants deficient in the inhibitory C/EBPβ–LIP isoform (C/EBPβ super-mice) that show CR-type metabolic adaptations paired with a prolonged health and lifespan, which highlights that increased transcriptional function of C/EBPβ delays the ageing process. C/EBPα and -β are crucial transcriptional regulators of energy metabolism, a pivot in the control of organismal ageing. Effectors changing the ratio of the inhibiting/activating LIP/LAP C/EBPβ and possibly p30/p42 C/EBPα isoforms therefore also may delay organismal ageing. C/EBPβ as well as GADD45A and ING1 are all part of gene regulatory networks with well-established roles in ageing, including mTORC1, SIRT1, p53, and FOXO3 (Figure 3, Key Figure).

Notwithstanding open questions (see Outstanding Questions), there may be enough ground to build on strategies manipulating C/EBPβ activity to attenuate ageing-related diseases. For example, adifovir dipivoxil reduces C/EBPβ–LIP levels and induces fatty acid β oxidation in cell culture as a potential CR mimetic [64]. Progeria resulting from site-specific demethylation defects raises the possibility that drugs might promote healthy ageing. Furthermore, the likely involvement of other C/EBP proteins besides C/EBPβ might provide additional therapeutic targets to promote healthy ageing.

Outstanding Questions

What is the biological significance of ageing DMRs? Are they associated with C/EBP motifs?

What is the role of TET demethylases in aging? Can aging be targeted by drugs affecting (specific) DNA methylation?

Is the expression/activity of *Gadd45a* or *Ing1* reduced upon ageing and does overexpression of *Gadd45a* or *Ing1* increase organismic life span?

What is the role of C/EBPα in ageing? Do $Cebpa^{\Delta uORF}$ mice show lifespan extension like $Cebpb^{\Delta uORF}$ mice?

Is the C/EBPβ activity regulated by SIRT1-mediated deacetylation as was shown for C/EBPα and does SIRT1-mediated deacetylation of C/EBPα and/or -β play a role in health and lifespan regulation?

GADD45α, ING1, and C/EBPβ promote cellular senescence, whose suppression can enhance lifespan [86]. Does their senescence function affect their role in ageing or does the GADD45α–ING1–C/EBP axis affect ageing solely via energy metabolism?

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