REVIEW

# Epigenetic Regulation of Metabolism and Inflammation by Calorie Restriction

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**ABSTRACT**

Chronic caloric restriction (CR) without malnutrition is known to affect different cellular processes such as stem cell function, cell senescence, inflammation, and metabolism. Despite the differences in the implementation of CR, the reduction of calories produces a widespread beneficial effect in noncommunicable chronic diseases, which can be explained by improvements in immuno-metabolic adaptation. Cellular adaptation that occurs in response to dietary patterns can be explained by alterations in epigenetic mechanisms such as DNA methylation, histone modifications, and microRNA. In this review, we define these modifications and systematically summarize the current evidence related to CR and the epigenome. We then explain the significance of genome-wide epigenetic modifications in the context of disease development. Although substantial evidence exists for the widespread effect of CR on longevity, there is no consensus regarding the epigenetic regulations of the underlying cellular mechanisms that lead to improved health. We provide compelling evidence that CR produces long-lasting epigenetic effects that mediate expression of genes related to immuno-metabolic processes. Epigenetic reprogramming of the underlying chronic low-grade inflammation by CR can lead to immuno-metabolic adaptations that enhance quality of life, extend lifespan, and delay chronic disease onset. *Adv Nutr* 2019;10:520–536.

Keywords: energy intake, DNA methylation, histone acetylation, sirtuin, microRNA, dietary restriction

## Introduction

Caloric restriction (CR) without malnutrition constitutes a safe and effective way to promote weight loss, decrease metabolic complications, increase lifespan, and improve quality of life. CR refers to a reduction in the intake of net calories, while meeting the necessary micronutrient requirements. Caloric intake can be restricted in a variety of ways: percentage CR, macronutrient limitation, or exercise-induced restriction. Percentage of restriction of total calories spans from mild (15% energy restriction) to severe (60% restriction) (1–4) and can be supplemented with physical activity (1, 4, 5). Alternatives for CR include changes in the proportion of macronutrients, such as hyperproteic compared with hypoproteic diets (6), or ketogenic diets with a high lipid, low carbohydrate content (7–9). In animal studies, CR can also be achieved by increasing the litter size or access to food, but, although this approach provides a natural method of energy limitation, often the amount of food cannot be measured (10, 11).

CR has been used to treat and prevent chronic disease development. In obese patients, CR provides additional health benefits to weight loss, such as decreased visceral adipose tissue (12) and inflammation (13), while improving kidney function (14, 15) and cellular quality control (16). Moreover, improved insulin sensitivity has also been documented for type 2 diabetic animals (17) and patients (18–22). In addition, a 12-wk CR treatment (30% restriction) reduced the circulating concentration of fetuin-A (a biomarker for multiple metabolic diseases), improved blood pressure in patients, and hepatic steatosis in rats (23). Lastly in preclinical and preliminary clinical studies, CR or fasting can effectively prevent malignancies through a variety of cellular responses and can improve the efficacy of therapeutic agents (24). It has been shown that various forms of CR reduce the progression of cancers with high morbidity rates, including colorectal, pancreas, breast, liver, prostate, esophagus, and kidney malignancies. In monkeys from the Wisconsin National

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Abbreviations used: 5mC, 5-methyl cytosine; CD, cluster determinant; CR, caloric restriction; DNMT, DNA methyltransferase; eIF, eukaryotic translation-initiation factor; FL, full length; HDAC, histone deacetylase; H3K, histone H3 lysine; H4K, histone H4 lysine; miR, microRNA; mTOR, mammalian target of rapamycin; NIA, National Institute of Aging; PBMC, peripheral blood mononuclear cell; PDK4, pyruvate dehydrogenase kinase 4; RISC, RNA-induced silencing complex; SIRT, sirtuin; Sir2, silent mating type information regulation 2; SL, short length; WNPRC, Wisconsin National Primate Research Center.


 

Primate Research Center (WNPRC), 30% lifelong restriction was sufficient to cause a 50% reduction in neoplastic events, gastrointestinal adenocarcinoma being the most common (25). Similarly, CR at a young age (30% restriction) reduced the incidence of cancer in monkeys from the National Institute of Aging (NIA), despite great genetic differences between them and the WNPRC monkeys. Overall, chronic CR has been demonstrated to be an effective dietary intervention for the treatment of many noncommunicable chronic diseases.

Given the impact that CR has on the prevention, development, and treatment of chronic diseases, we sought to review 3 epigenetic mechanisms that might be related to the long-term programming of cellular functions. Epigenetic mechanisms are at the forefront of cellular changes that can be modulated by CR and lead to long-lasting cellular adaptations and improved health outcomes. Modulation of important growth mechanisms that lead to increased longevity [phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR)] among others is known to be regulated by epigenetic factors, but the wide range and complexity of these pathways are not well understood (26). We will provide an overview of different epigenetic mechanisms that can be targeted. We will then explore the effect of CR on such mechanisms. Lastly, we will delineate the epigenetic regulation of immunometabolic processes that leads to improvements for many noncommunicable chronic diseases.

## CR and DNA Methylation

CR has been found to affect the methylation pattern of certain genes involved in biological processes such as metabolism, oxidative stress, senescence, and aging (27–29) (**Figure 1**). CR protocols that compensate for malnutrition can combat chronic disease development by shaping the epigenome during early developmental stages. The timing of the restriction onset as well as the diet formulation (30) are the most important factors that can predict the positive outcomes related to CR (Figure 1A). Moreover, important caveats exist in CR research, where sex-specific and strain-specific effects are observed, and higher restrictions result in improvements of neither lifespan nor health span (31). Thus, a clear consensus is necessary to address the methodological differences between studies regarding percentage restriction and health outcomes.

### Dynamic regulation of DNA methylation

DNA methylation, or the modification of a cytosine nucleotide to 5-methyl cytosine (5mC), is an epigenetic process intricately associated with the regulation of gene expression. DNA methylation has been associated with the control of gene expression at all stages of development (32, 33). DNA methyltransferases (DNMTs) are the enzymes involved in de novo and maintenance methylation of cytosine residues (33, 34). DNMT1 is responsible for the establishment of methylation patterns that define different tissues, whereas DNMT3A and 3B oversee the dynamic turnover of novel methylation marks, or de novo methylation. Other DNMTs include DNMT2, which is involved in the methylation of transfer RNA (tRNA), and DNMT3L, which activates DNMT3A and 3B. Transcriptionally related DNMTs are required for the silencing and transcription of target genes.

The mechanisms and enzymes involved in the process of DNA demethylation are poorly understood. In eukaryotes, the recently described Ten Eleven Translocation family of proteins has been found to play an important role in DNA demethylation (35). The proposed mechanism for DNA demethylation involves Ten Eleven Translocation–mediated oxidation, starting with the conversion of 5mC to 5-hydroxymethyl cytosine, followed by oxidation to 5-formyl cytosine, and finally to 5-carboxyl cytosine (36). Both 5-carboxyl cytosine and 5-formyl cytosine DNA residues are recognized by other proteins that restore the nucleotide to the demethylated cytosine (37).

### CR induces changes in DNA methylation

CR without malnutrition provides protection against chronic illness, produces weight loss, and helps prevent the development of metabolic abnormalities. Comparison of 2 nonhuman primate longitudinal CR studies from the NIA and the WNPRC revealed differences in CR onset and diet formulation (30). In the studies, very early CR onset appears to be linked to lower life expectancy: for instance, in the NIA study, before the control group, CR monkeys reached 80% mortality. Meanwhile, early CR onset did not affect the average body weight as expected: compared with the control group, female CR monkeys in the NIA studies showed no significantly different body weight for any age categories. On the other hand, male CR monkeys in the NIA study weighed significantly less than controls, among the juvenile and adult categories. In addition, early CR onset was also linked to reduced quality of life, whereas mid- to late-onset CR produced significant health benefits (Figure 1B). Hence, we sought to review the effects of mid- and late-onset CR and diet specifications associated with longevity and better quality of life, with an epigenetic focus. In a clinical population, 8 wk of CR-induced weight loss significantly reduced DNA methylation of the inflammatory cytokine tumor necrosis factor (*TNF*), and provided a striking difference in biomarkers for the prediction of weight reduction (38). Moreover, similar methylation patterns for TNF and leptin were observed for obese women who were prescribed an 8-wk-long low-calorie diet (39), thus lessening the inflammatory burden of obese patients. Other genes that are known to be modified by CR-induced weight loss are ATPase phospholipid transporting 10A (*ATP10A*) and cluster determinant (CD) 44 molecule (*CD44*) in overweight and obese men (40). Similarly, in overweight and obese postmenopausal women, CR differentially affected the methylation pattern of genomic loci involved in weight control and insulin secretion, analyzed in adipose tissue biopsies (41). In the group of weight loss maintainers, DNA methylation patterns were also more similar to those of normal-weight individuals, rather than those of the obese counterparts who did not lose weight (42). Interestingly, weight loss methods (CR or bariatric surgery) affect the methylation status differently (43).

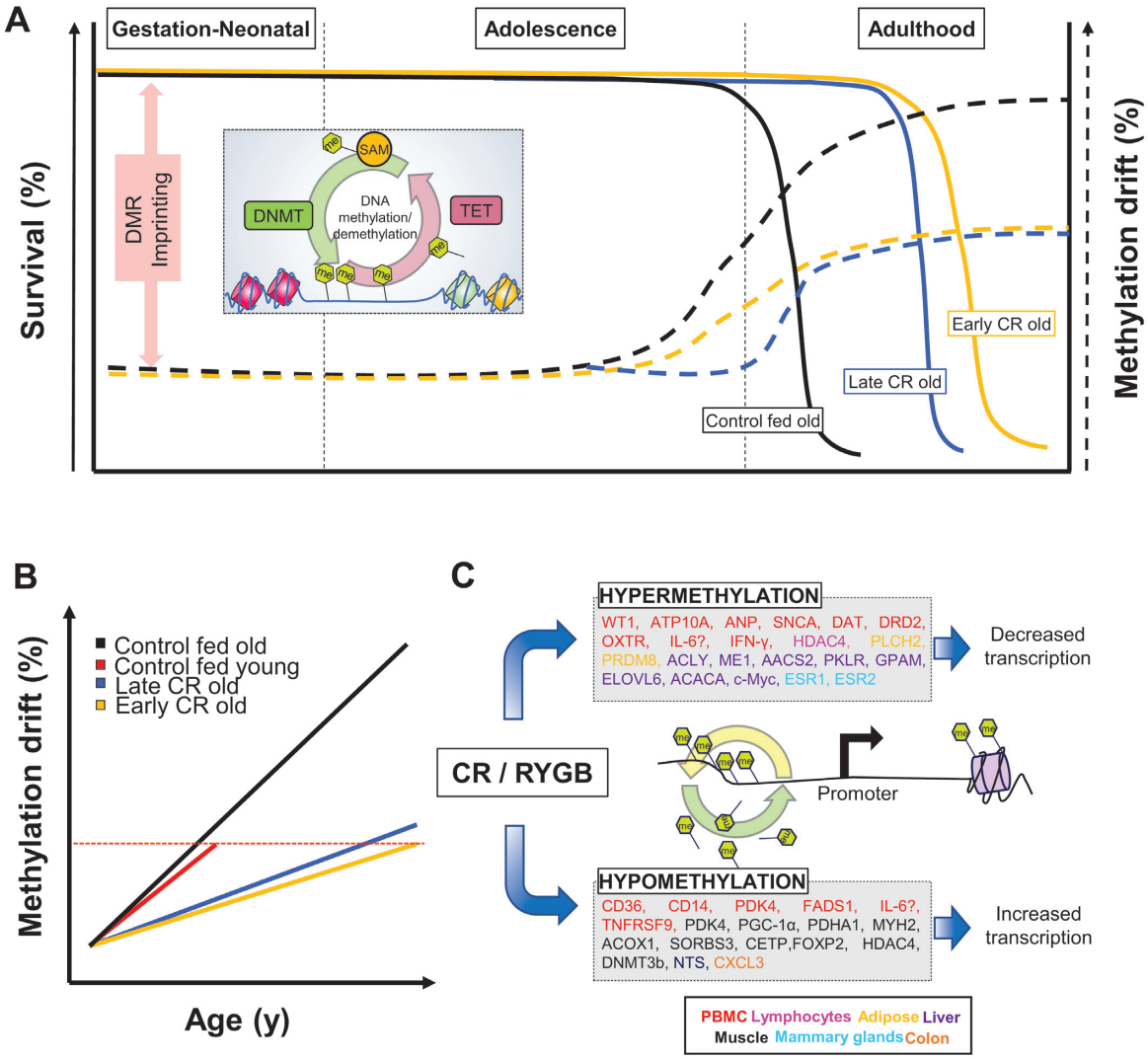


**FIGURE 1** Age-related changes in DNA methylation drift and the effect of CR. (A) DNA methylation is a dynamic process that is regulated during development and throughout life. Early-life DNA methylation patterns are established through genetic and epigenetic imprinting of DMRs. Both early (yellow solid line) and late onset of CR (blue solid line) are able to extend lifespan with differences in health span compared with control without CR (black solid line). Both early (yellow dotted line) and late onsets (blue dotted line) are able to ameliorate age-related methylation drifts. (B) DNA methylation dysregulation with age. (C) Weight loss strategies such as CR and RYGB are able to produce distinct patterns of either hyper- or hypomethylation of many genes (listed in the text boxes) in many metabolic tissues (see color code by tissue), compared with their obese counterparts. AACS2, acetoacetyl-CoA synthetase; ACACA, acetyl-CoA carboxylase $\alpha$; ACLY, ATP citrate lyase; ACOX1, acyl-CoA oxidase 1; ANP, natriuretic peptide A; ATP10A, ATPase phospholipid transporting 10A; CD, cluster determinant; CETP, cholesteryl ester transfer protein; c-MYC, MYC proto-oncogene; CR, caloric restriction; CXCL3, C-X-C motif chemokine ligand 3; DAT, dopamine transporter; DMR, differentially methylated region; DNMT, DNA methyltransferase; DNMT3b, DNMT 3 $\beta$; DRD2, dopamine receptor D2; ELOVL6, fatty acid elongase 6; ESR, estrogen receptor; FADS1, fatty acid desaturase 1; FOXP2, forkhead box P2; GPAM, glycerol-3-phosphate acyltransferase, mitochondrial; HDAC4, histone deacetylase 4; IFN-$\gamma$, interferon-$\gamma$; Me, methyl (CH3); ME1, malic enzyme 1; MYH2, myosin heavy chain 2; NTS, neurotensin; OXTR, oxytocin receptor; PBMC, peripheral blood mononuclear cell; PDHA1, pyruvate dehydrogenase E1 $\alpha$ 1 subunit; PDK4, pyruvate dehydrogenase kinase 4; PGC-1$\alpha$, peroxisome proliferator–activated receptor $\gamma$ coactivator 1 $\alpha$; PKLR, pyruvate kinase L/R; PLCH2, phospholipase C $\eta$ 2; PRDM8, PR/SET domain 8; RYGB, Roux-en-Y gastric bypass; SAM, S-adenosyl methionine; SNCA, synuclein $\alpha$; SORBS3, sorbin and SH3 domain containing 3; TET, Ten Eleven Translocation protein; TNFRSF9, TNF receptor superfamily member 9; WT1, Wilms tumor 1.

### CR-induced DNA methylation changes and immuno-metabolism

In human studies, weight loss by CR has been shown to alter DNA methylation in blood, adipose tissue, and skeletal muscle (Figure 1C). In 1 study, obese and overweight men were subjected to 30% energy restriction for 8 wk (40). Greater weight loss was associated with hypermethylation of genes for Wilms tumor 1 (*WT1*) and *ATP10A* in

peripheral blood mononuclear cells (PBMCs). In a similar study involving overweight women, an 8-wk intervention involving 30% CR resulted in decreased methylation of cluster determinant 36 (*CD36*), cluster determinant 14 (*CD14*), pyruvate dehydrogenase kinase 4 (*PDK4*), and fatty acid desaturase 1 (*FADS1*) in PBMCs (44). Examination of subcutaneous adipose tissue from these women revealed no change in DNA methylation within the leptin (*LEP*) promoter (39). Another study focused on overweight and obese postmenopausal women who underwent a 6-mo CR weight loss program. Subcutaneous adipose tissue biopsies were performed after an additional 4-wk weight stability period. Those participants that had >3% reduction in body fat percentage were found to have hypermethylated loci associated with phospholipase C $\eta$ 2 (*PLCH2*) and PR/SET domain 8 (*PRDM8*) (41). Thus, it appears that weight loss strategies, regardless of the form or severity, are able, to some extent, to alter DNA methylation patterns of metabolism- or immune-related genes, but significant variability in the response is expected.

In extreme cases of obesity, bariatric surgery may be used as a weight loss strategy to reduce the capacity of the stomach and reduce food intake, thus providing some form of artificial CR. One study found that gastric bypass patients had higher methylation of *PDK4* in whole blood at 12 mo after surgery (45). In skeletal muscle biopsies 6 mo after surgery, gastric bypass patients had normalized DNA methylation in 11 metabolic gene promoters, including *PDK4*, peroxisome proliferator–activated receptor $\gamma$ coactivator 1 $\alpha$ (*PPARGC1A*), pyruvate dehydrogenase E1 $\alpha$ 1 subunit (*PDHA1*), myosin heavy chain 2 (*MYH2*), acyl-CoA oxidase 1 (*ACOX1*), and others (46). Another study found reduced methylation in skeletal muscle at 30 CpGs associated with sorbin and SH3 domain containing 3 (*SORBS3*) after Roux-en-Y gastric bypass surgery (47). In addition to muscle, DNA methylation profiles of omentum and subcutaneous adipose tissue were altered by gastric bypass surgery (48). Results showed 3601 differentially methylated CpGs in subcutaneous adipose tissue and 15 in omentum. CpGs were associated with genes involved in obesity and epigenetic regulation, such as cholesteryl ester transfer protein (CETP), forkhead box P2 (FOXP2), histone deacetylase (HDAC) 4, and DNMT3B. Overall, CR via bariatric surgery mediates DNA methylation of metabolic genes from metabolic tissues such as muscle and adipose tissue in humans.

Whereas human studies have primarily focused on CR as a means of weight loss, animal models have examined CR in the context of longevity and cancer. Previous studies have discussed the role of CR on lifespan through modification of DNA methylation changes (49, 50). Studies in *Drosophila* found dietary restriction to extend the lifespan without changing methylation patterns, whereas several others have shown that CR mitigates age-associated DNA methylation in a range of tissues (28, 51–53). A study in mice used 60% energy restriction starting at 12 wk of age and examined genome-wide methylation in the liver (54). Not only did restricted animals have longer lifespans, but dietary restriction was also shown to ameliorate age-related hepatic DNA methylation changes. In a set of 1,167,959 bins covering 29 million CpGs, 3176 bins showed a significant methylation difference. Additional analysis revealed hypermethylation of gene bodies. CR-induced methylation was enriched for fatty acid, TG, and ketone body metabolism-related genes, including ATP-citrate lyase (Acly), malic enzyme 1 (Me1), acetoacetyl-CoA synthetase (Aacs2), pyruvate kinase (Pklr), glycerol-3-phosphate acyltransferase (Gpam), fatty acid elongase 6 (Elovl6), and acetyl-CoA carboxylase 1 (Acaca). In hippocampal tissue of mice exposed to 40% CR, >30% of age-related differentially methylated CpGs were prevented by CR (55). Genes affected by CR were enriched for pathways related to energy regulation, inflammation, and phagocytosis. DNA methylation in other brain regions also depends on age and diet. In mouse cerebellum, aging induced a significant increase in 5mC immunoreactivity (56). A 15% calorie reduction had no effect on Purkinje cell methylation in 12-mo-old mice. However, the same CR regimen decreased global DNA methylation in Purkinje cells of 24-mo-old mice. In normal WI-38 lung fibroblasts, glucose restriction extended the lifespan and downregulated expression of the senescence gene p16 (57). This effect might be due to elevated DNMT activity and hypermethylation of the promoter of p16 in the restricted cells. In addition to metabolic and aging pathways, CR also affects methylation of cancer-related genes. One study showed that the livers of aging mice steadily decreased methylation in the promoter and increased methylation in the gene body of *proto-oncogene* (58). After both 11 mo and 21 mo of 42% CR, age-related methylation changes surrounding MYC proto-oncogene (*Myc*) were significantly diminished. Thus, CR improves health span by altering DNA methylation in a variety of tissues.

The duration of CR appears to be critical in producing methylation changes. Female mice were 30% calorie restricted starting at 6–8 wk of age, and genome-wide DNA methylation in mammary tissue was measured at 5 mo and 22 mo (59). After 5 mo of dietary treatment, there were 511 significantly more methylated along with 248 significantly less methylated CpGs in CR compared with control mice. After prolonged treatment, the number of differentially methylated loci substantially increased to 7552 with the majority (6901) being hypermethylated in the CR group. Closer investigation of Estrogen receptor 1 and 2 (*Esr1* and *Esr2*) uncovered minimal methylation differences at 5 mo. However, in aged animals, CR resulted in greater methylation at 3 loci within the first intron. Aged CR mice also showed hypermethylation upstream and downstream of the *Esr2* gene body. Another study in mice found that 40% CR for 4 mo was sufficient to observe an increase in neurotensin (*Nts*) expression in the colon as well as lower promoter DNA methylation in restricted mice. Interestingly, this DNA methylation change persisted even when mice were switched back to an ad libitum diet for 5 mo (27).

Although CR is known to regulate systemic inflammation, it remains unclear how DNA methylation contributes to altered cytokine concentrations. In humans, weight loss studies have revealed various changes across adipose tissue and blood. One report found that after an 8-wk 30% energy-restricted intervention there was no change in promoter DNA methylation of *TNF* subcutaneous adipose tissue of obese women (39). However, results in whole blood were contradictory, as gastric bypass patients had elevated methylation of *IL-1B*, *IL-6*, and *TNF* 12 mo after surgery (45). Findings regarding *IL-6* were reproduced in white blood cells, because women subjected to 6 mo of 30% energy restriction had increased IL-6 methylation (43). However, *IL-6* methylation in bariatric surgery patients decreased. Interestingly, these changes were not correlated with circulating concentrations of the cytokines. In another study, obese and overweight men were subjected to 30% energy restriction for 8 wk. Analysis revealed hypomethylation of TNF receptor superfamily member 9 (*TNFRSF9*) and hypermethylation of interferon-$\gamma$ (*IFNG*) in PBMCs (40). Overall, investigation of CR-mediated DNA methylation of inflammatory genes has yielded inconsistent results in humans.

In animal models, CR and DNA methylation in inflammatory pathways has been examined in a limited number of studies. One study showed that age-related DNA methylation drift is accelerated under conditions of chronic inflammation (60). In ulcerative colitis patients there was hypermethylation in age-related CpG islands in colon epithelial cells (this observation was found in 12 patients out of 18 cases, with 5 controls). These results suggest an association between CR, DNA methylation, and inflammation. Indeed, in mouse hippocampus, CR resulted in hypermethylation of CpGs that fell within genes that were enriched for inflammatory pathways, including FC $\varepsilon$ receptor signaling, signaling by the B cell receptor (BCR), antigen activation of B cell receptor leading to generation of second messengers, FC $\gamma$ receptor–dependent phagocytosis, and IL-2 signaling (55). Similarly, in monkey liver, CR reduced age-related DNA methylation drift associated with several genes including the neutrophil chemoattractant C-X-C motif chemokine ligand 3 (*CXCL3*) (28). Collectively, evidence suggests an association between CR and inflammation as well as CR and DNA methylation; however, more work is necessary to uncover the role of DNA methylation in mediating inflammatory outcomes in CR.

## Histone Modifications and CR

### The basis of histone remodeling

The chromatin landscape determines the availability of the genome, whether it is found in an open and accessible conformation (euchromatin) or in a tightly packed, closed arrangement (heterochromatin). Chromatin changes are mainly driven by covalent modifications to nucleosomal histones, producing a change in the availability of genes for gene expression; specific amino acid residues within histones can be modified covalently, thus altering the interaction with the DNA that winds around the histone octamer (61). Modifications to the histones, such as acetylation, methylation, phosphorylation, or addition of small ubiquitin-like modifers (SUMOylation), can interact with each other and other factors (61) to control the rate of transcription, which in turn dictates tissue-specific gene expression. One such modification, histone acetylation, is a posttranscriptional addition of an acetyl group facilitated by histone acetyltransferases, and removed by HDACs. Histone acetyltransferases transfer acetyl groups from acetyl CoA onto the histone tails (lysine residues), neutralizing their positive charge and causing DNA to decondense to allow transcription, mainly driven by an increased frequency of transcription factor binding within the target genes. For instance, the addition of acetyl groups to histone H4 lysine (H4K) 16 increases transcription in vivo and in vitro (62, 63). Conversely, HDACs remove acetyl groups from the histones and restore the positive charge on the histone, restoring the heterochromatic state (64, 65). Other histone modifications have been described and their activating or repressive mechanisms have been reviewed previously (61, 66). Furthermore, the dynamic regulation of histone modifications and their modifiers has been established previously (67–69) and, thus, will not be the focus of this review. Important aging pathways regulated by sirtuins (SIRTs) include the PI3K/Akt/mTOR axis (70, 71), which highlights the great therapeutic potential of CR and SIRTs. Studies that investigate the relation between nutrient sensing and chromatin modifications are reviewed here to illustrate the potential effects of CR on the health span (72).

### CR as regulator of protein modifications

During CR, energy depletion in the cell is evidenced by the increased catabolic state, alteration of nutrient-sensing pathways (73), and increased $NAD^+$ production (74, 75). Alteration of the nutrient pool and its sensors will in turn influence nuclear gene transcription, which can be mediated by protein, and specifically histone modifiers susceptible to CR (67, 76). CR is known to affect SIRTs, a family of nutrient-sensing HDACs (77) (**Figure 2**). SIRTs are $NAD^+$-dependent protein deacetylases (78) and exert their function on lysine residues found mostly on the histone tails. The first identified SIRT in *Saccharomyces cerevisiae*, Silent mating type information regulation 2 (Sir2), is indicated in lifespan extension through silencing ribosomal DNA, decreasing the frequency of ribosomal DNA circles that cause aging in yeast (79). Deletion of Sir2 results in a shorter lifespan (80). In addition, telomeres, the genomic regions that protect the ends of each chromosome from deterioration, are shortened when Sir2 expression is low (81). When Sir2 is overexpressed, the longevity phenotype is restored. Increased cellular stress coming from increased ribosomal circles, shorter telomeres, or epigenomic insults could lead to cell senescence and long-term exposure could affect aging, and Sir2 appears to be a strong regulator of this process.

The SIRT family is comprised of 7 mammalian isoforms, which localize in the nucleus (SIRT1, SIRT2, SIRT6) where they influence histones and other trans-activating factors, in the mitochondria (SIRT3–5) where they can participate

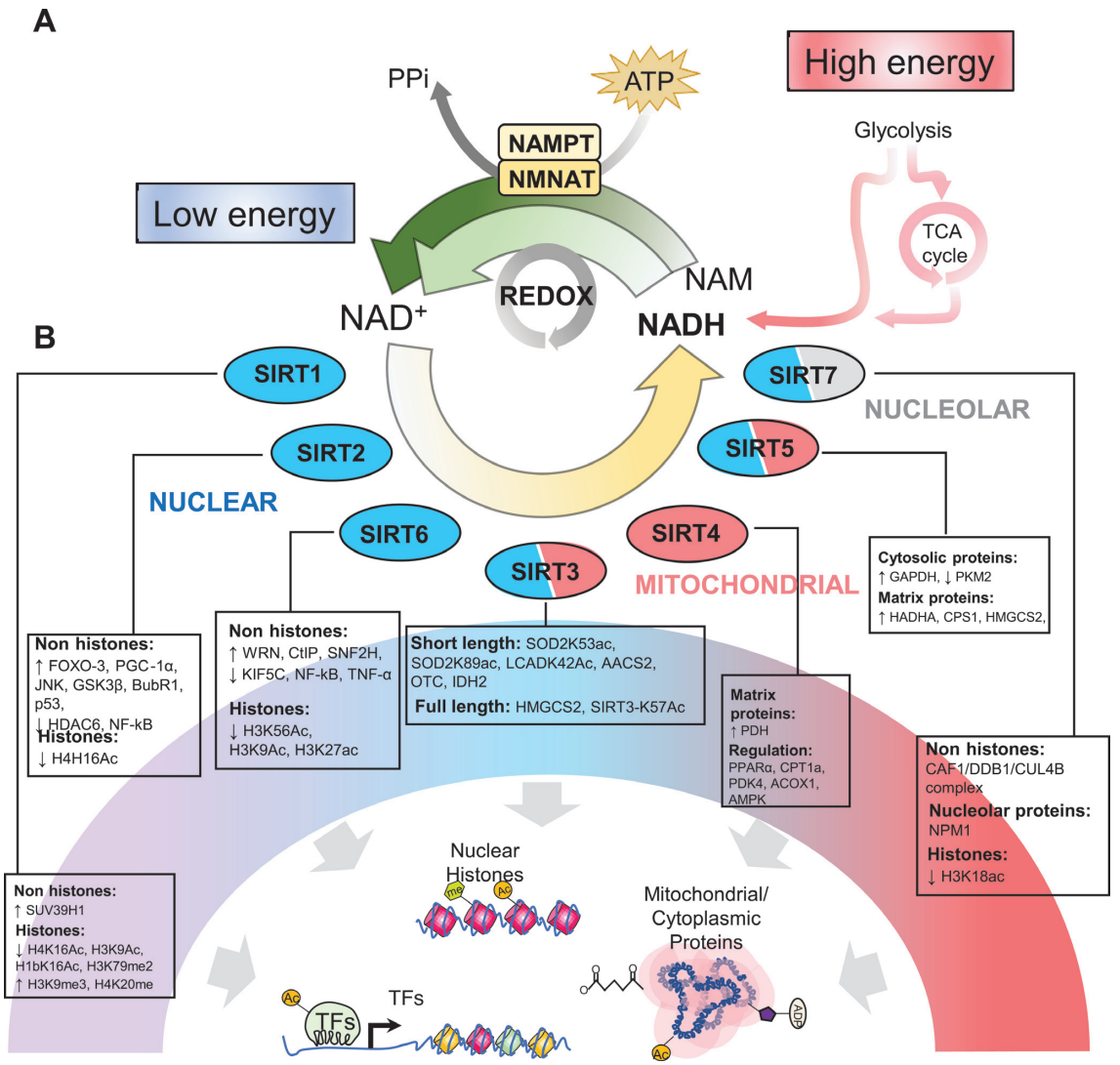


**FIGURE 2** Epigenetic and genetic regulation of sirtuins by caloric restriction. (A) High energy levels after a feeding period contribute directly to the elevated concentrations of NAM and NADH originated from catabolic pathways. Diverse cells and cellular processes deplete the concentration of NADH, and together with the biosynthetic transformation by NAMPT and NMNAT, high intracellular concentrations of $NAD^+$ are produced. (B) Intracellular $NAD^+$ is sensed by $NAD^+$-dependent enzymes, such as sirtuins that add or remove posttranslational protein modifications from nuclear (blue), cytosolic, nucleolar (grey), and mitochondrial (red) proteins. Seven sirtuins, or SIRTs, have been defined in mammals, and participate in deacetylation, mono ADP-ribosylation, and defatty-acylation [demyristoylation, desuccinylation, demalonylation, deglutarylation, demethylglutarylation, and de-3-hydroxy-3-methylglutaryl(HMG)-ation] of nuclear transcription factors, nucleosomal histones, and various nuclear, nucleolar, and mitochondrial proteins. AACS2, acetoacetyl-CoA synthetase; ACOX1, acyl-CoA oxidase 1; AMPK, AMP-activated protein kinase; BubR1, BUB1 mitotic checkpoint serine/threonine kinase B; CAF1/DDB1/CUL4B, ubiquitin complex CAF1/DDB1/CUL4B; CPS1, carbamoyl-phosphate synthase 1; CPT1a, carnitine palmitoyltransferase 1A; CtIP, C-terminal-binding protein interacting protein; FOXO-3, forkhead box O3; GSK3$\beta$, glycogen synthase kinase 3 $\beta$; HADHA, hydroxyacyl-CoA dehydrogenase subunit $\alpha$; HDAC6, histone deacetylase 6; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; H1bK16Ac, histone 1b lysine 16 acetylation; H3K9Ac, histone 3 lysine 9 acetylation; H3K9me3, histone 3 lysine 9 trimethylation; H3K18ac, histone 3 lysine 18 acetylation; H3K56Ac, histone H3 lysine 56 acetylation; H3K79me2, histone 3 lysine 79 dimethylation; H4H16Ac, histone 4 histidine 16 acetylation; H4K16Ac, histone 4 lysine 16 acetylation; H4K20me, histone 4 lysine 20 monomethylation; IDH2, isocitrate dehydrogenase 2, mitochondrial; JNK, JUN N-terminal kinase; KIF5C, kinesin family member 5C; LCADK42Ac, acyl-CoA dehydrogenase long chain lysine 42 acetylation; NAM, nicotinamide; NAMPT, nicotinamide phosphoribosyltransferase; NF-kB, nuclear factor $\kappa$B; NMNAT, nicotinamide mononucleotide adenylyltransferase 1; NPM1, nucleophosmin 1; OTC, ornithine carbamoyltransferase; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; PGC-1$\alpha$, peroxisome proliferator–activated receptor $\gamma$ coactivator 1 $\alpha$; PKM2, pyruvate kinase M1/2; PPAR$\alpha$, peroxisome proliferator–activated receptor $\alpha$; p53, tumor protein P53; SIRT, sirtuin; SIRT3-K57Ac, sirtuin 3 lysine 57 acetylation; SNF2H, sucrose nonfermenting protein 2 homolog; SOD2K53ac, superoxide dismutase lysine 53 acetylation; SOD2K89ac, superoxide dismutase lysine 89 acetylation; SUV39H1, suppressor of variegation 3-9 homolog 1; TCA cycle, tricarboxylic acid cycle; TF, transcription factor; TNF, tumor necrosis factor; WRN, Werner Syndrome RecQ-like helicase; H3K27ac, histone H3 lysine 27 acetylation; Ac, acetylation; Me, metylation; PPi, pyrophosphate.

in metabolic-related processes and modulate oxidative stress (72), or in the nucleolus (SIRT7) where they aid in cell division (72). SIRTs function as intracellular nutrient sensors allowing them to detect the energy status of the cell, which then is coupled with deacetylation of target proteins (such as histones within target genes or target proteins), as well as ADP-ribosylation in the cytosol (82) or deacylation of mitochondrial matrix proteins (83).

Nutrient and energy sensing within the cells can be achieved through the monitoring of cellular energetics during feeding and fasting periods. Among the determinants of cellular energetics, the ratio of $NAD^+$:NADH is an adequate indicator of the energy status. The $NAD^+$:NADH ratio is high when energy levels are low, whereas the $NAD^+$:NADH ratio decreases when nutrient and energy availability is high. High concentrations of NADH provide reducing agents for oxidized substrates (redox reactions), and together with biosynthetic pathways that use a nicotinamide phosphoribosyl transferase and nicotinamide nucleotide adenylyl transferase to convert nicotinamide, both can yield high amounts of oxidized $NAD^+$ (Figure 2A). During fasting or CR, the concentrations of $NAD^+$ increase and $NAD^+$ catalyzes reactions mediated by all SIRTs (Figure 2B), given that during each round of deacetylation, SIRTs consume 1 $NAD^+$ molecule (84). After activation of the SIRTs, a variety of posttranslational modifications are removed (acetyl, acyl, myristoyl, succinyl, etc.) or added (ADP-ribosylation) to proteins and histones within the nucleus, mitochondria, and nucleolus. We have subdivided the functions of SIRTs according to their subcellular localization, and we will give a brief overview of their effects on metabolism and immunity through epigenetic modifications.

***SIRT1.***

SIRT1 is the mammalian homolog of yeast Sir2 (78) and it is thought to have a major role in extending lifespan with CR in mammals. It localizes within the nucleus and cytosol and is involved in deacetylation reactions of different substrates such as cytosolic proteins, and nuclear histones and transcription factors. Although SIRT1 has many targets within the cytosol, the long-lasting effects of CR that are known to be mediated by SIRT1 are thought to be linked to its HDAC role in the nucleus. In general, SIRT1 maintains a heterochromatic environment by deacetylation of histone H1, H3, and H4 at specific lysine residues. The combined reactions result in the recruitment of histone H1 and the loss of the active transcription mark histone H3 lysine (H3K) 79 methylation (85). In addition, SIRT1 interacts with histone methyltransferase (HMT) suppressor of variegation 3-9 homolog 1 (SUV39H1) to allow the methylation of H3K9 (H3K9me3) and H4K20 (H4K20me), both hallmark histone modifications of heterochromatin (86, 87). Interestingly, the state of constitutive and facultative chromatin is responsive to both micronutrient and macronutrient availability, as we have previously reviewed (88). SIRT1 provides stability to facultative heterochromatin, as it deacetylates H3K9Ac and H4K16Ac marks, and is able to interact with linker histone modification H1bK26Ac and recruit it to promote higher-order organization (85), which in turn is modified by Enhancer of zeste 2 (EZH2) to generate H1bK26me (67). Thus, it seems that SIRT1 can respond to nutrient availability through the increased $NAD^+$ concentration in CR and induce several changes within the acetylation landscape of cytosolic and nuclear proteins and histones to mount an adaptive response to the low energy status.

***SIRT2.***

SIRT2 is regarded as the most conserved SIRT across species and, owing to this feature, it is thought to regulate important cellular processes that are shared by multiple organisms. SIRT2 localizes within the nucleus and cytosol where it can act on cell cycle control and cell division as well as metabolism of fatty acids, which is thought to be mediated through its control on acetylation and myristoylation. This SIRT deacetylates and activates the transcription factors forkhead box o3 (FOXO-3), PPARG coactivator 1 $\alpha$ (PGC-1$\alpha$), p53, and NF-$\kappa$B, key regulatory kinases Jun kinase (JNK) and glycogen synthase kinase 3b (GSK3$\beta$), and HDAC6. The main function of SIRT2 is to localize to microtubules where it deacetylates the $\alpha$-tubulin, thus promoting their polymerization/depolymerization cycle. This process is sensitive to the concentrations of $NAD^+$ (89), which is of particular importance given that during CR the concentrations of $NAD^+$ rise and can directly regulate the SIRT2-mediated deacetylation of $\alpha$-tubulin. Moreover, the deletion of SIRT2 in mouse oocytes results in higher rates of spindle defects, chromosome disorganization, and impaired kinetochore interaction with the centromere (90). This function appears to be intricately related to the direct acetylation of a component of the spindle assembly checkpoint complex BubR1 (BubR1-K243) (91) and deacetylation of histone H4 at K16 (90). CR may prevent genomic instability by regulating mitotic and spindle control, inspecting cell division checkpoints, and promoting the fidelity of the chromosomal distribution.

***SIRT3.***

The response of mitochondrial protein deacetylase SIRT3 to the cell's energy status is primordial for the coupling of metabolic reactions and preservation of mitochondrial integrity (92, 93). SIRT3 exists in 2 forms, the full-length form (FL, 40 kD) and the mitochondrial-exclusive short-length form (SL, 28 kD). FL SIRT3 is known to act as an HDAC. Although the regulation of stress-response genes is important in the context of disease, limited information is available on the genome-wide binding targets of the FL SIRT3 form and its regulation during CR. The SL, mitochondrial form of SIRT3 has been well characterized in relation to the mitochondrial acetylome (mitochondria-wide acetylation/deacetylation) and its regulation. In the mitochondrial matrix, the SL SIRT3 is able to interact with a myriad of factors that are involved in energy metabolism, such as the electron transport chain, tricarboxylic acid cycle, $\beta$-oxidation, and ketogenesis, as well as stress resilience and reactive oxygen species quenching (94–99). It appears

that the effects of SL SIRT3 revolve around mitochondrial-performance enhancement and increasing resistance to stress. Interestingly, higher $NAD^+$ availability in CR is able to activate SL SIRT3 that in turn directs the deacetylation of 2 lysine residues within superoxide dismutase 2 (SOD2) (K53 and K89), thus improving the response to oxidative stress (97). Similarly, fasting or CR could increase fatty acid $\beta$-oxidation by deacetylating long-chain acyl CoA dehydrogenase (LCAD) at lysine 42 (K42) (98). Moreover, in addition to LCAD, SL SIRT3 is known to directly deacetylate acetyl-CoA synthetase 2 (ACSS2), ornithine carbamoyltransferase (OTC), and isocitrate dehydrogenase 2 (IDH2) (99, 100), to promote acetate and urea metabolism and antioxidant defenses.

SIRT3 is highly responsive to CR through various regulation pathways. SIRT3 is able to direct the deacetylation of 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2), thus increasing its activity and enabling the generation of ketone bodies during fasting and CR (101). Moreover, SIRT3 modifies mitochondrial enzymes that participate in the hallmark processes of CR (102), which makes it an attractive target for therapeutic strategies. SIRT3 is also able to reshape the entire acetylome of the liver in response to CR with 3 different types of acetyl residues: those that are responsive to CR and are acted on by SIRT3, those that are responsive to CR and are unresponsive to SIRT3 (class II), and those that are unresponsive to both CR and SIRT3 (class III) (101). Furthermore, evidence also suggests that SIRT3 activity may be inactivated by other SIRTs. In obese and aged mice, SIRT1 concentrations are reduced and SIRT3 becomes hyperacetylated owing to the inability of SIRT1 to remove SIRT3-K57Ac (103).

***SIRT4.***

Unlike the other mitochondrial SIRTs, SIRT4 is strictly localized within the matrix and its primary function does not involve the deacetylation of target proteins, but rather entails ADP-ribosylation and deacylation, mainly removing glutaryl, methylglutaryl, and de-3-hydroxy-3-methylglutaryl (HMGyl) residues from mitochondrial proteins. Unlike other SIRTs, SIRT4 is negatively regulated by CR and its crystal structure indicates a higher inhibitory potential of NADH due to its higher preference over $NAD^+$ (104).

The effects of SIRT4 oppose that of CR. Studies indicate that it participates in crucial steps of glycolysis by controlling the pyruvate dehydrogenase complex by removing lipoyl and biotinyl residues from the complex (105). Moreover, SIRT4 is known to regulate fatty acid $\beta$-oxidation in the liver by controlling PPAR-$\alpha$, the master regulator of fatty acid oxidation, which controls carnitine palmitoyl transferase 1a (CPT1a), PDK4, and ACOX1 (106), as well as AMP-activated protein kinase (AMPK) (107). During fasting conditions or prolonged nutrient limitation, SIRT4 is suppressed and SIRT1 is upregulated to initiate the oxidative program of the mitochondria.

***SIRT5.***

The final "mitochondrial" SIRT actor is SIRT5, a particularly interesting SIRT whose function spans not only mitochondrial protein modifications, but nuclear and cytosolic ones as well. SIRT5 does not possess deacetylase activity, which sets it apart from other SIRTs. Rather it removes glutaryl, malonyl, and succinyl residues from its target proteins (83). SIRT5 (and SIRT4) is involved in deglutarylation of proteins belonging to oxidation/reduction, generation of precursor metabolites and energy, fatty acid and coenzyme metabolism, as well as aerobic respiration (108). SIRT5 targets carbamoyl phosphate synthase 1 (CPS1), and possibly other glutarylation targets such as hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit $\alpha$ (HADHA). Regarding energy metabolism, SIRT5 is known to affect the lysine (K) malonylation of several glycolytic enzymes such as GAPDH and pyruvate kinase (PK), thus activating glycolytic flux, as well as urea cycle and other mitochondrial enzymes (109). Likewise, SIRT5 is also able to regulate several mitochondrial proteins related to $\beta$-oxidation and ketogenesis; for instance, SIRT5 removes succinyl residues from 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), thus regulating the critical step in ketogenesis (110). SIRT5 not only regulates the mitochondrial acylome, but also protects against mitochondrial fragmentation and mitophagy (mitochondrial degradation). Thus, it is vital for starvation-induced (CR) mitochondrial elongation (111). Finally, although the role of SIRT5 in metabolism has been defined, several avenues of research indicate the great potential of this SIRT in health and age-related diseases (112–115).

***SIRT6.***

SIRT6 primarily remains in the nuclear compartment where it is involved in deacetylation, ADP-ribosylation, and defatty-acylation (myristoyl residues) of numerous proteins related to cell cycle and metabolism. Interestingly, unlike other SIRTs, SIRT6 is capable of binding $NAD^+$ in the absence of acetylated substrate, implying that this SIRT might act as an $NAD^+$ metabolite sensor (116). $NAD^+$ sensing constitutes a key function of SIRT6 in health and disease (117). SIRT6 deacetylates and activates the C-terminal-binding protein interacting protein (CtIP) and recruits sucrose nonfermenting protein 2 homolog (SNF2H) to promote double-strand break and resection, which in turn facilitates homologous recombination of chromosomes in collaboration with breast cancer type 1 susceptibility protein (BRCA1) (118, 119).

In addition to chromosomal stability, SIRT6 also cooperates with different telomeric maintenance systems to protect these important regions. In the nucleus, SIRT6 is able to deacetylate H3K9Ac and H3K56Ac within telomeric regions and facilitates the recruitment of Werner Syndrome RecQ-like helicase to aid in telomere capping during cell division (67, 120). Moreover, upon oxidative damage, SIRT6 promotes directional telomeric movement, which grants protection and is related to telomeric length conservation (121). In a similar way, SIRT6 can act on equally important genomic

regions such as enhancers; SIRT6 deacetylates H3K27ac and thus activates cis-regulatory loci (122, 123). Another function that has been described for SIRT6 is related to energy and nutrient metabolism in liver, muscle, and brain (124). As an $NAD^+$-dependent deacetylase, SIRT6 can affect insulin secretion in response to glucose by deacetylating H3K56Ac within the thioredoxin interacting protein (*Txnip*) promoter in pancreatic $\beta$-cells to enhance insulin secretion (125). SIRT6 downregulates microRNA (miR)-122 in the liver by deacetylating H3K56Ac within the promoter, prevents the inhibitory effect from miR-122, and thus increases fatty acid $\beta$-oxidation (126). Lastly, SIRT6 inhibits adipogenesis by blocking mitotic clonal expansion through the inactivation of kinesin family member 5C (KIF5C), thus preventing hyperplasia in adipose tissue (127).

When $NAD^+$ is present, SIRT6 deacetylates myristoyl residues, and this process mediates the secretion of various proteins (128). SIRT6 regulates secretion of TNF-$\alpha$, a potent inflammatory and signaling cytokine, through removal of fatty acyl modifications on K19 and K20, thus stimulating its cellular export in macrophages (129), other immune cells (130), and pancreatic cancer cells (131). SIRT6 deacetylates H3K9Ac tails within p65 (NF-$\kappa$B)-responsive regions, thus impeding binding of this transcription factor and inhibiting aging-associated inflammation (132). Finally, SIRT6-mediated inactivation of cytokines might be related to the potent inhibition of inflammation by CR in aged animals (133) and humans (16), which is sensitive to cellular $NAD^+$ concentrations.

### *SIRT7.*

The last member of the SIRT family is one of the least understood and most understudied $NAD^+$-dependent deacetylases. Nucleolar SIRT7 is depleted in senescent cells, which indicates that SIRT7 is related to replicative senescence (134). Disorganized spindles and disruption of chromosomal syzygy are observed in SIRT7-depleted cells and obese mice (135), but overexpression of SIRT7 is associated with cancer growth (136, 137). The latter is thought to be regulated through miR-125b-5p and miR-340 (136, 138), or CCAAT enhancer binding protein (C/EBP$\alpha$)-mediated HDAC3 recruitment (139), which negatively regulates SIRT7 expression. Deacetylation of histone H3K18 by SIRT7 decreases mRNA transcription mediated by the Pol II machinery, which demonstrates its effect on chromatin and ability to promote cell transformation and tumorigenesis (137). Further, SIRT7 interacts with and represses the RNA pol I and other nucleolar chromatin remodeling complexes, which emphasizes the role of SIRT7 in transcription (140).

SIRT7 is known to be affected differently by aging and CR in a tissue-specific manner (141). SIRT7 and SIRT6 appear to have a shared proportion of protein targets that are related to DNA repair, chromatin assembly, and aging (142). Furthermore, aging-dependent nucleophosmin (NPM1) acetylation is dependent on SIRT6–SIRT7, thus shedding light on the antiaging effects of SIRT7. Finally, SIRT7 is known to play a role in fat uptake, opposing the effects of SIRT1, SIRT3, and SIRT6 in fat utilization (143). SIRT7 enhances fat uptake by upregulating hepatic *CD36* and promotes TG synthesis and storage through the elevation of *Mogat*, Monoacylglycerol O-Acyltransferase; *Cidea*, death-inducing DFFA-like effector A; *Cidec*, cell death-inducing DFFA-like effector C (144). Moreover, SIRT7 was identified as a direct inhibitor of the E3-ubiquitin complex CAF1/DDB1/CUL4B, which is known to target the nuclear receptor testicular receptor 4 (TR4) (145). In turn, TR4 activates genes involved in fat uptake and lipid storage in the liver (144). SIRT7 regulation therefore might constitute an attractive target to counteract the effects of a high-fat diet to prevent fatty liver disease.

## Small Noncoding RNA

### Epigenetic basis of small noncoding RNA

When discussing the epigenetic regulation of genes, one must consider the ubiquitous and silencing/activating nature of small noncoding RNAs. miRs are small noncoding RNA molecules that regulate posttranscriptional stability of genes through base pair recognition within the 3'-UTR in the target gene. Technologies such as microarray, q-PCR–based, or sequencing approaches have allowed for the identification of numerous miRs present in vivo (146). The binding of miRs to the target gene recruits the multiprotein complex RNA-induced silencing complex (RISC) that cleaves the target gene through one of its components, Argonaute (147–149). This highly orchestrated process is responsible for the posttranscriptional stability of mRNAs in the cytosol. Therefore, environmental stimuli that affect a particular set of miRs will determine the gene expression pattern. During early eukaryotic mRNA translation, cytoplasmic poly(A)-binding protein (PABPC) and the poly(A) tail of the mature mRNA interact to form a complex that can then associate with the eukaryotic translation-initiation factor (eIF) 4G to protect the mRNA, but miRs hinder the interaction of PABPC and eIF4G at the early stages of translation. Binding of miRs to 3'-UTR recruits the RISC complex, which activates one of its components, trinucleotide repeat containing 6A (GW182) (150). GW182 activation recruits the CAF1-CCR4-NOT deadenylase complex, causing deadenylation of the mRNA, leading to mRNA degradation (151). RISC-mediated degradation is a fine-tuned degradation machinery, but its specificity is ultimately dependent on the presence of the miRs.

Owing to the ubiquitous nature of miRs (151), research in this area can provide valuable knowledge in the exploration of the etiology of human diseases (152). In time, miRs could be used as a predictive biomarker for different tissues given their adaptability to different environmental stimuli (153–155). The mechanisms by which CR promotes health benefits are thought to be mediated through alteration of miR patterns in different tissues.

### CR and miRs

The effects of CR on miR expression patterns appear to be conserved in different species, from *Caenorhabditis elegans*

and *Drosophila*, to rodents (*Rattus norvegicus* and *Mus musculus*), Rhesus macaques (*Macaca mulatta*), and humans (*Homo sapiens*). Different CR concentrations and protocols have been investigated for their miR-modulating effect in many tissue types (**Figure 3**). In this section, we will review all the evidence regarding CR using a systematic review algorithm in PubMed (**Supplemental Table 1**).

In *C. elegans*, 12-h starvation is sufficient to produce significant changes in miRs whose target genes are related to metabolism, development, and oogenesis processes (156) (Figure 3A; Supplemental Table 1). Further, 2-d deprivation of food produced whole-body miR changes that were related to longevity, and such changes were dependent on *Drosha* ortholog (DRSH-1) (157). In addition, 12-h deprivation of food induced physiological changes seen in higher organisms such as decreased lipid accumulation, reduced reproductive function, and increased lifespan (156) (Figure 3A; Supplemental Table 1). Limited evidence exists for miR regulation by CR in fruit flies (*Drosophila melanogaster*), pointing to metabolic improvements and an increased lifespan. Comparing high- with low-nutrient diets (CR mimic), CR flies had altered expression of miR-184, let-7, miR-125, and miR-100 (out of a total of 18 miRs examined), but it was let-7 overexpression in female nervous tissue that was responsible for an ∼22% increase in lifespan (158). Finally, starvation-induced downregulation of miR-305 activates Dp53 (p53) in the fat body and thus produces metabolic adaptations through nutrient-sensing pathways (159). Altogether, CR in lower organisms highlights the great contribution of nutrient-sensing pathways to lifespan and health span.

In rodents, different tissue types with varied restriction protocols have demonstrated the effectiveness of CR at controlling miR production and exocytosis (Figure 3C; Supplemental Table 1). In long-lived B6C3F1 mice, 40% CR for 27 mo produced an miR pattern in serum that is related to longevity (160). Certain miRs appear to be genotype-specific and age-specific in long-lived Ames dwarf mice. In addition, the pathways these miRs affect are related to tumor suppression, inflammation, WNT, wingless/integrated-, insulin-, mTOR-, and MAPK-signaling pathways (161). Similarly, miRs that are commonly observed in serum (160) were upregulated in liver in C57B6J mice after increasing CR from 10% to 30% for 2 y, and miR-125a-5p was identified as a direct contributor to age-related CR effects (162). When compared with metformin, another life-extending intervention, CR was able to produce a distinguishable signature in liver of mice leading to the alteration of miR-20a, miR-34a, miR-130a, miR-106b, miR-125, and let-7 expression (out of a total of 64 miRs examined) (163) (Figure 3C; Supplemental Table 1).

In colon and colon mucosa, CR seems to be related to anti-inflammatory and anticarcinogenic effects. In a murine colon cancer model, 10 miRs were found to be significantly differentially expressed between the 3 treatment groups: CR, diet-induced obese, and control (164). In particular, miR-150 was upregulated, whereas miR-155 was downregulated by CR. Interestingly, another study showed that one of the functions of miR-150 is increasing cell susceptibility to apoptosis and reducing cell proliferation through decreasing cell cycle progression via initiator of the eukaryotic translation protein, eIF5A (165). A previous study indicated a potential mechanism by which eIF5A is used to regulate apoptosis by upregulating p53 protein expression, which in turn increases *Bax*, Bcl-2 associated X expression [a preapoptotic member from the B-cell lymphoma 2 (Bcl-2) family] while decreasing expression of *Bcl2* (166). Consequently, p53-dependent apoptosis is promoted by the activation of *Bax*, while cell survival signals are repressed (167). A balance between these BCL2/BAX and BAX/BAX homodimer formations in mammalian cells is necessary to regulate survival and death signals (168). Thus, the upregulation of miR-150 by CR might be linked to the modulation of apoptosis and alteration of cell proliferation. CR significantly downregulates the expression of miR-155 in colon, which is linked to cell apoptosis (169) and proliferation (170). Interestingly, miR-155 targets tumor protein P53 inducible nuclear protein 1 (TP53INP1), which is a proapoptotic stress-induced gene that activates p53 (Figure 3C; Supplemental Table 1).

In breast tissue, CR is sufficient to produce miR patterns that are associated with longevity and aging (149). This study demonstrated several miRs that were altered after CR treatment, among which the most significantly increased were miR-29c, miR-203, miR-150, and miR-30 (out of an analyzed data set containing >100 miRs generated from 7.0 MiRNA microarray analysis by LC Sciences on 5 mg of total RNA). Co-transfection assays suggested that miR-203 can downregulate the translation of Caveolin-1 (*Cav-1*). Cav-1 is a scaffolding protein that functionally interacts with and regulates signaling molecules such as protein kinase A (PKA), protein kinase C (PKC), H-Ras, epidermal growth factor receptor (EGFR), and G-protein $\alpha$ subunit, and its interaction with such proteins is related to Ku70-mediated apoptosis regulation (171). In a similar way, other miRs such as miR-10a, miR-10b, miR-21, miR-124, miR-125b, miR-126, miR-145, and miR-200a were also identified (out of 101 miRs examined by an Affymetrix GeneChip miRNA 2.0 array) to be associated with mammary tumorigenesis (172). Specifically, miR-200a concentration is elevated throughout cancer progression and it has a pro-proliferative function in mammary cancer cells. Interestingly, miR-200a was found to be downregulated significantly in mammary tumor of CR rats. Considering the function of miR-200a, CR may reduce mammary tumor burden by suppressing miR-200a expression, which consequently inhibits cellular proliferation. Importantly, miR-200a may be useful for the detection of stage-specific breast cancer and may constitute a beneficial CR target for breast cancer patients.

Besides the beneficial effects of CR in peripheral tissues, it also plays a neuroprotective role by modulating expression of age-dependent miRs, thus establishing a balance between proapoptotic and survival signals in the brain. Data has shown age-dependent miRs, miR-181a-1, miR-30e, and miR-34a (56 miRs were examined out of 367 antisense mature

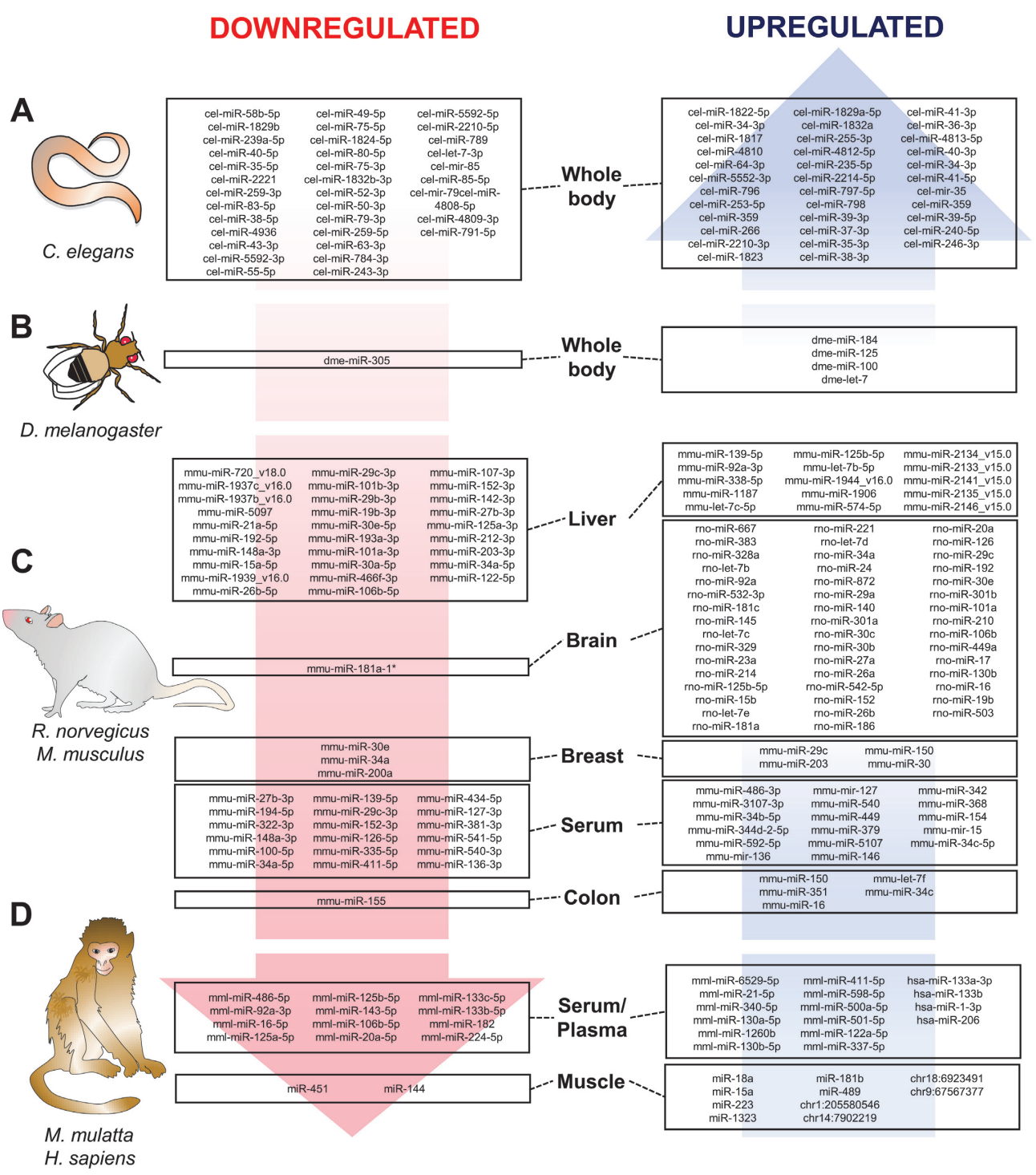


**FIGURE 3** CR conservation of miR changes across species and tissues. CR and starvation are able to upregulate or downregulate miR signatures in (A) *Caenorhabditis elegans*, (B) *Drosophila melanogaster*, (C) rodents (*Rattus norvegicus* and *Mus musculus*), and (D) Rhesus macaques (*Macaca mulatta*) and humans (*Homo sapiens*). miR signatures associated with CR are involved in antiaging pathways such as immuno-metabolic regulation in different peripheral and central tissues. CR, caloric restriction; miR, the mature for of the miRNA; mir, the pre-miRNA and the pri-miRNA; *, asterisk following the name indicate the mature species found at low levels from the opposite arm of a hairpin.

miR sequences, scanned by an Expression 1680 scanner, and analyzed using Array-Pro Analyzer 4.5 software), to be downregulated by CR in brain tissue, which correspond with the upregulation of *Bcl-2* and downregulation of *Bax*, leading to apoptosome inhibition (173) (Figure 3C; Supplemental Table 1). In mouse breast tissue, miR-30 was discovered to be significantly upregulated by CR (149). miR-30 downregulates its 2 target genes ubiquitin-conjugating enzyme 9 (UBC9) and integrin $\beta$3 (ITGB3) by translational repression (174). UBC9 promotes transcription of the *Bcl-2* gene and translation of the Bcl-2 protein. ITGB3 is indicated to have a proapoptotic effect resulting from unligated integrin-mediated cell death, a pathway induced by unligated integrins acting as a negative modulator of cell survival. Therefore, CR-induced expression of miR-30 is implicated in cellular stemness and senescence. CR is related to cell development and physiology in primary cerebromicrovascular endothelial cells, where CR reduced oxidative stress, enhanced nuclear factor erythroid 2 (*Nrf2*) function, and increased miRs related to angiogenic, proliferative, adhesive, antiapoptotic, and anti-inflammatory processes (175). Collectively, evidence from rodent models shows that CR modulates the expression of several immuno-metabolic and oncogenic miRs across tissue types.

Although the evidence in distinct animal models is strong, clinical trials that aim to identify markers relevant to human populations are needed to provide efficacious and sensitive miR biomarkers (Figure 3D; Supplemental Table 1). Relevant studies have been conducted in Rhesus monkeys to assess the CR miR signature, showing a conserved miR pattern related to growth and insulin signaling as well as regulation of ribosomal, mitochondrial, and spliceosomal pathways (176). Similarly, a study analyzing old monkeys revealed an age-dependent decline in muscle-specific miRs (35 significantly regulated miRs out of a total of 451 miRs examined by Ingenuity Pathway Analysis), but CR improved health span and rescued the expression of miR-181b and chr1:205580546, while decreasing miR-451 and miR-144 concentrations (177). Finally, one of the only interventions in humans revealed that whole-body protein synthesis was inversely related with circulating concentrations of muscle-specific miRs (myomiRs) (miR-1-3p, miR-133a-3p, miR-133b, and miR-206) in energy-restricted (35 d) overweight men (178). Altogether, the evidence suggests that CR-based therapies could target muscle miRs and muscle immuno-metabolism to prevent age-related comorbidities.

Through the investigations using several animal models, it is clear that CR treatment affects cellular processes and the cell cycle via regulating miR expression. This knowledge can help build a better understanding of how CR modifies the epigenome and will shed light on future discoveries of treatments and medicines aimed at antiaging and inhibiting tumorigenesis.

## Conclusion

Modulation of age-related decline by CR is robust and is related to genetic and epigenetic adaptations to nutrient availability. Short- and long-term CRs produce significant changes in different tissues and across species, in some animal models even with sex-specific effects, thus indicating that CR acts through conserved mechanisms, such as immuno-metabolic pathways. Notably, early CR onset may cause a different and even an opposite effect on physiological outcomes in animal models such as body weight. Furthermore, CR directly affects the DNA methylation/demethylation cycle, histone and protein modifiers like SIRTs, and miRs, to orchestrate the adaptive and long-lasting response, leading to increased lifespan and health span.


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