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COMPREHENSIVE INVITED REVIEW

The Redox Basis of Epigenetic Modifications: From Mechanisms to Functional Consequences

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Abstract

Epigenetic modifications represent mechanisms by which cells may effectively translate multiple signaling inputs into phenotypic outputs. Recent research is revealing that redox metabolism is an increasingly important determinant of epigenetic control that may have significant ramifications in both human health and disease. Numerous characterized epigenetic marks, including histone methylation, acetylation, and ADP-ribosylation, as well as DNA methylation, have direct linkages to central metabolism through critical redox intermediates such as NAD^+, S-adenosyl methionine, and 2-oxoglutarate. Fluctuations in these intermediates caused by both normal and pathologic stimuli may thus have direct effects on epigenetic signaling that lead to measurable changes in gene expression. In this comprehensive review, we present surveys of both metabolism-sensitive epigenetic enzymes and the metabolic processes that may play a role in their regulation. To close, we provide a series of clinically relevant illustrations of the communication between metabolism and epigenetics in the pathogenesis of cardiovascular disease, Alzheimer disease, cancer, and environmental toxicity. We anticipate that the regulatory mechanisms described herein will play an increasingly large role in our understanding of human health and disease as epigenetics research progresses. *Antioxid. Redox Signal.* 15, 551–589.

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I. Introduction

Conrad Waddington initially described epigenetics as "the science concerned with the causal analysis of development" in 1938 (319). Waddington was attempting to link the study of genetics with developmental biology, and his theoretical "epigenetic landscape" postulated the derivation of phenotype from genotype. This initial formulation of epigenetics, though exceptionally broad, was a remarkably prescient construct given the lack of knowledge about molecular biology in Waddington's time [DNA was not putatively identified as the genetic material until 1943 (9)]. Perhaps due to the staggering breadth of Waddington's definition, the term "epigenetics" did not generate interest with researchers except as a last resort descriptor for heritable phenomena lacking an easily describable genetic component. These early studies proposed dissimilar definitions of epigenetics that were convenient for the individual researchers, and no unifying description of epigenetic mechanisms was generated until the late 1980s. By this time, DNA methylation and gene imprinting had been identified as heritable regulatory processes in mammalian cells. In a landmark analysis, Robin Holliday identified these mechanisms as epigenetic elements in a restatement of Waddington's theory, now described as "the strategy of genes in unfolding the genetic program for development" (122). Holliday's reintroduction of epigenetics marked the beginning of a major shift in conceptual thought that continues today, and a perusal of the literature suggests that epigenetics as a field is clearly here to stay. For example, searching the PubMed literature database with the terms "epigenetics" and "metabolism" yielded 28,866 hits as this article went to press, with 3696 identified as reviews. This strongly underscores the massive current interest in epigenetic research.

Modern epigenetics, as defined by Adrian Bird, is "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (18). This definition is remarkable for several reasons, but perhaps most notable is the implicit assumption that epigenetic changes comprise an adaptive response to various events rather than *a priori* events that control gene transcription. This is a powerful theoretical concept, because it makes no assumption regarding the source of the stimuli that force epigenetic adaptation. In other words, if the genotype communicates the phenotype through epigenetics, Bird's definition allows the possibility that nongenetic events may also communicate phenotype through epigenetic means. Epigenetics thus potentially represents a mechanism for integrating genetic and environmental stimuli and translating them into phenotypic outcomes (Fig. 1). To conceptualize this, consider the baking of a cake. This requires careful combination of ingredients, such as flour, sugar, and butter, followed by the chemical process of baking for various temperatures and times. In this way, the ingredients and the environment represent vital inputs into a system, and the cake represents a phenotypic outcome. Now consider what happens if you alter the ratios of ingredients, but not their identities, and/or their baking environment—you may end up with cookies, which represents an equally delicious but entirely different phenotypic outcome than cake simply from varying the relative amounts of the same inputs. The difference is the weight that each input is given in contributing to the overall outcome. Similarly, epigenetic regulation of cellular processes provides a weight to any number of cellular stimuli, combining them into one

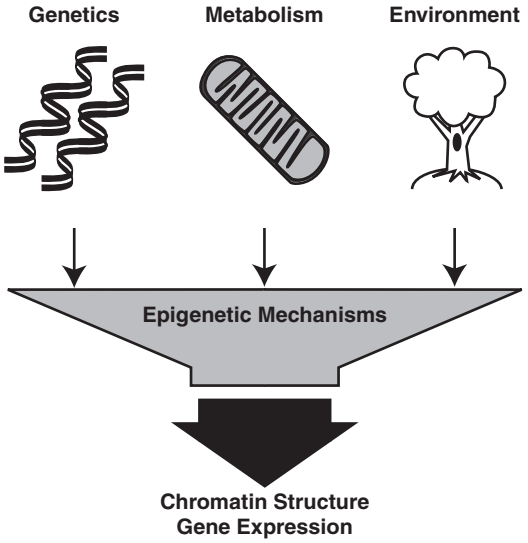

FIG. 1. Epigenetics as an integrating mechanism. Epigenetic processes can be conceptualized as a common effector for multiple different inputs to affect chromatin structure and gene expression. Epigenetics can thus be considered as an integrating mechanism that takes into account signals from diverse processes to produce a phenotypic outcome.

coherent phenotypic product. This model raises several questions, including the nature of the stimuli that can affect epigenetic processes as well as the mechanisms by which these phenomena can produce epigenetic consequences. Recent evidence suggests that one possible mechanism results from the direct influence of redox metabolic processes on epigenetic enzymes.

Broadly, metabolism is the process by which organisms harvest energy from fuel sources to execute vital functions. Redox processes are central to the functionality of all known metabolic systems. Albert Szent-Györgyi, the physiologist credited with identifying ascorbic acid, eloquently described this dependence, stating that the energy derived from electronic transfers is the "energy which drives life" (295). All known organisms share common elements of this redox metabolism, and these metabolic processes are necessary for life. Indeed, contemporary debates about the nature of biogenesis address the concept that primordial reactions comprising metabolic cycles established the requisite materials for all life (81). Given life's absolute reliance on a conserved set of metabolic reactions, the potential for higher organisms to have critical regulatory systems evolve sensitivities to metabolic outputs is obvious. In this review, we will focus attention specifically on the capacity of redox metabolism to modulate phenotype through epigenetic means. We will also address the potential roles that perturbation of these metabolic processes may have in the pathogenesis of human disease.

II. Epigenetic Control of Gene Expression

As a practical matter, epigenetics encompasses both the post-translational modification of histones and chemical modifications of the DNA base cytosine to produce differential transcriptional outcomes without affecting the genomic sequence (119). There is an ever-growing number of identified histone post-translational modifications, including acetylation, methylation, ADP-ribosylation, SUMOylation, phosphorylation, ubiquitylation, and deimination (153). The classic DNA chemical modification is methylation of CpG dinucleotides, producing 5-methylcytosine (5-meC), though the importance of other modifications including 5-hydroxymethylcytosine (5-meOHC) is steadily emerging (16, 156, 296). By integrating the combined signals of these various epigenetic marks, cells produce transcriptional outcomes. The study of the gestalt of epigenetic events has proven to be a challenge; if one were to consider all of the combinatorial possibilities for histone modifications alone, the known modifications on histone H3 alone could produce over 1 million distinct post-translational signatures (Fig. 2). In fact, C. David Allis formulated the "histone code" hypothesis to account for the vast capacity of histones for transient information storage on top of DNA sequence (137). Given the additional information provided by DNA methylation, epigenetic regulation of gene transcription is an exceptionally broad phenomenon. Here, we will focus specifically on histone methylation, histone acetylation, histone poly-ADP-ribosylation, and DNA methylation as targets for direct control through metabolic intermediates.

A. Histone methylation

Histone methylation occurs at lysine (K) and arginine (R) residues of the unstructured N-terminal tails that project from the core histone fold. These residues can be mono-, di-, or trimethylated, with different functional outcomes depending on the number of methyl groups. For example, one of the initially characterized methylation marks, tri-methylation at lysine 4 of histone H3 (H3K4), is strongly associated with transcription when localized to promoter regions (178, 270, 271, 275, 288). Notably, the H3K4me1 mark is associated not with active transcription at promoter regions, but instead is found in enhancers (114). Conversely, the H3K9me3 mark is associated with transcriptional repression and heterochromatin formation, as it provides a scaffold for the binding of heterochromatin protein 1 (161, 213, 217, 244). Much of the current knowledge regarding the importance of these histone marks is summarized in Figure 2. Clearly, histone methylation is a strongly context-dependent modification that promotes a variety of different transcriptional effects depending on the location and context in which it exists. To that end, there are numerous enzymes that function to specifically methylate or demethylate particular histone residues in both temporal and spatially dependent fashions.

1. Histone methyltransferases. Histone methyltransferases (HMTs) collectively are the enzymes that can methylate either lysine or arginine residues on histones. There are two main families of histone lysine methyltransferases: the SET domain-containing methyltransferases, so named for the conserved sequence identified in the three *Drosophila* proteins Su(var)3–9, Enhancer of zeste, and Trithorax (61); and the Dot1/lysine methyltransferase 4 lysine methyltransferases (282). In addition, there are two types of generalized protein arginine methyltransferases that can modify histone tails (282). Each of the different HMTs catalyzes the methylation of specific residues, owing to differences in the size and conformations of

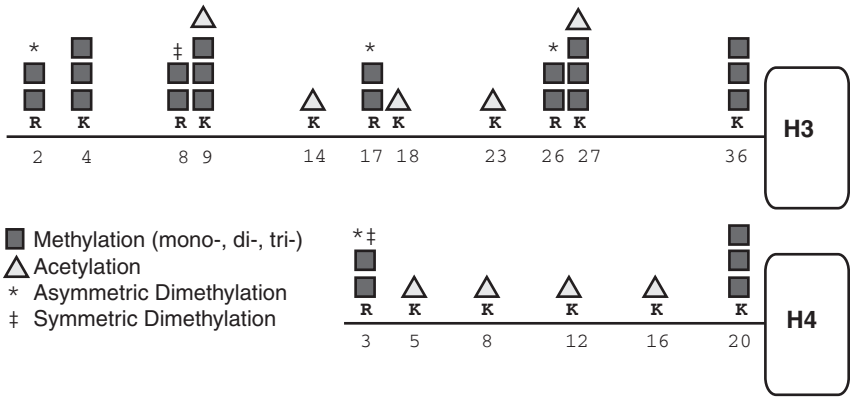

FIG. 2. Example modifications of histones H3 and H4. Post-translational modification of histones can produce myriad different combinations and effects on gene expression. Here we provide illustration of H3 and H4 N-terminal tails and the locations that can be methylated or acetylated as examples. Critically, these marks do not necessarily exist independently of one another, leading to remarkable combinatorial diversity and signaling subtleties. Arginine demethylation is specially denoted as it may be symmetric or asymmetric around the guanidino moiety, with different transcriptional outcomes. See Wysocka *et al.* (330) for further review.

their substrate binding pockets. Despite this difference in substrate specificity, all HMTs utilize a similar reaction mechanism in which *S*-adenosyl methionine (SAM) donates a methyl group to form *N*-methyl protein adducts and the by-product *S*-adenosyl homocysteine (SAH) (Fig. 3) (282). While many of the HMTs were identified in the late 1990s (138), histone methylation itself was considered to be an irreversible mark that required the recycling of the entire histone protein. Recently, however, the discovery of multiple protein families capable of oxidatively demethylating histone tails has suggested that the dynamic regulation of histone methylation is substantially more complex.

2. History of histone demethylation. While biochemical assays had long indicated the potential existence of histone demethylases (233), there were no definitive enzymes established until 2004. At this time, a series of parallel studies demonstrated multiple mechanisms for active histone demethylation. Cuthbert *et al* (52) and Wang *et al.* (322) independently identified that the peptidyl arginine deiminase 4 (PAD4/PADI4) was capable of deiminating monomethyl arginine residues on histone tails, leaving citrulline and producing methylamine. However, this did not represent a true demethylase activity, because the arginine residues were left as citrulline—the significance of which is still being investigated (8, 40). In parallel, Shi *et al.* (279) identified and characterized the first *bona fide* histone demethylase, lysine-specific demethylase 1 (LSD1). These findings represented a watershed moment for histone methylation research, as within months mechanisms for both lysine and arginine demethylation had been definitively identified. Importantly, these initial studies also hinted at the impact that these enzymes would have on transcriptional regulation: both PAD4/PADI4 studies demonstrated that it played a role in the repression of target gene loci through the removal of the permissive methyl-arginine histone marks (52, 322), whereas Shi *et al.* demonstrated that LSD1 targeted H3K4 mono- and di-, but not tri-, methylation (279).

At this point, the search was on for other enzymes capable of demethylating histone tails. The discovery of the alkane hydroxylase (AlkB) enzyme activity, which oxidatively demethylates alkyl DNA lesions in *E. coli*, provided clues as to potential mechanisms for putative histone demthylases (67, 70, 305). AlkB and related proteins belong to the superfamily of 2-oxoglutarate (2-OG)- and Fe(II)-dependent dioxygenases, which include the prolyl hydroxylase domain (PHD) proteins that modulate the hypoxia-inducible factor (HIF) signaling pathway (231). AlkB mediates the demethylation of both 1-methyladenine and 3-meC by catalyzing the formation of hydroxymethyl adducts, which in certain chemical contexts are inherently unstable. These hydroxymethyl groups then spontaneously produce formaldehyde, leaving unmodified adenine or cytosine (67, 70, 305). After the discovery

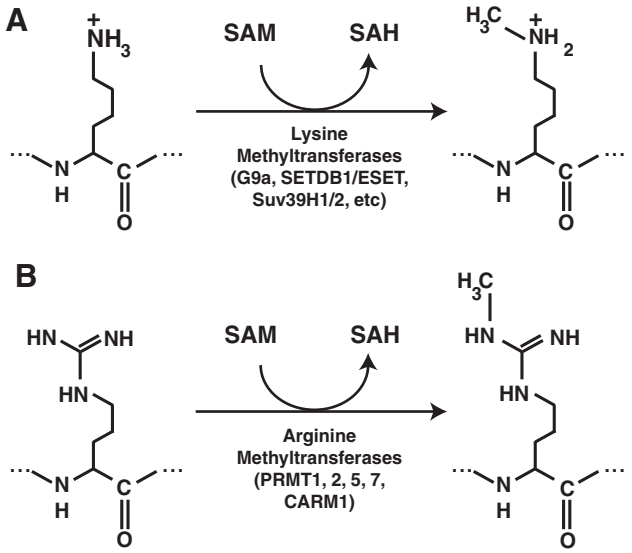

FIG. 3. Histone lysine and arginine methyltransferase mechanisms. (A) Illustration of lysine methylation reaction utilizing SAM as a methyl donor. Individual enzymes determine the specificity for individual histone lysines. Lysines may be mono-, di-, or tri-methylated by serial iteration of methyltransferase reactions (not pictured). **(B)** Arginine methyltransferase reaction. These enzymes also use SAM as a methyl donor, and can catalyze the formation of symmetric or asymmetric dimethylarginine (not pictured) through serial addition of methyl groups. For detailed review of these topics, see Spannhoff *et al.* (287). SAM, *S*-adenosyl methionine.

of this mechanism, many groups openly speculated that histone demethylases might utilize an analogous mechanism (306). The next clue was provided from studies on the gene *Epe1*, which is a key regulator of heterochromatin formation in the fission yeast *Schizosaccharomyces pombe* (11). Epe1 was found to mediate heterochromatin assembly, and significantly, overexpression of Epe1 disrupted methylation of H3K4 (11). Additionally, Epe1 belonged to the jumonji-domain containing (jmjC) family of proteins. The original jumonji protein had been initially characterized as a transcription factor required for neural tube development by mouse gene trapping in 1995 (297), and a characteristic jmjC sequence was subsequently identified in a large number of other genes. Among these was the factor inhibiting HIF, a well-characterized 2-OG- and Fe(II)-dependent dioxygenase also associated with HIF signaling (115). Taken together, these multiple lines of research strongly suggested that jmjC proteins represented excellent candidates for histone demethylases. Tsukada *et al.* confirmed this in 2006, demonstrating that the jmjC protein JHDM1 had 2-OG and Fe(II)-dependent H3K36me2 demethylase activity (307). Since then, a large number of jmjC histone lysine demethylases (KDMs) have been discovered, and as interest in this protein family continues to grow, new naming standards have been established [presented in ref. (4)]. Moreover, as we will illustrate here, the mechanism utilized by jmjC proteins provides a critical target for metabolic regulation.

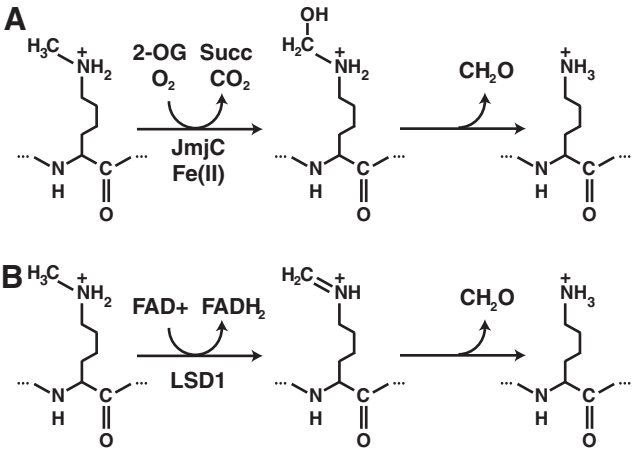

FIG. 4. Histone lysine demethylase mechanisms. (A) JmjC demethylase mechanism. JmjC proteins catalyze the hydroxylation of methyllysine residues in a 2-OG and Fe(II)-dependent manner, releasing succinate and CO_2. In a second, nonenzymatic step, formaldehyde is spontaneously released after the decomposition of the *N*-hydroxymethyl moiety. **(B)** LSD1 demethylase mechanism. LSD1 catalyzes the oxidation of the C-N methylamine bond in an FAD-dependent mechanism, producing an unstable intermediate that decomposes to release formaldehyde. As a consequence of this mechanism, LSD1 cannot eliminate trimethyllysine, whereas jmjC demethylases can. 2-OG, 2-oxoglutarate; jmjC, jumonji-domain containing; LSD1, lysine specific demethylase 1; Succ, succinate.

3. Mechanisms of histone demethylases. The enzymes that result in histone demethylation can broadly be classified into the jmjC demethylases, the LSD1-type KDMs, and the PAD-type arginine demethylases (Fig. 4). Each of these requires distinct cofactors and produces different products. JmjC demethylases, as stated before, are 2-OG- and Fe(II)-dependent dioxygenases (Fig. 4A). Briefly, the Fe(II) at the jmjC active site is coordinated by two conserved histidines and a glutamate residue (49, 218). These aid in catalyzing a one-electron transfer to molecular oxygen, producing Fe(III) and superoxide anion ($O_2^{\bullet -}$). Superoxide then attacks the 2-carbon of 2-OG, resulting in the production of an Fe(IV)-containing peroxyhemiketal bicyclic intermediate (282). This intermediate rapidly decarboxylates, producing succinate, carbon dioxide, and a critical Fe(IV)-oxo intermediate. This moiety oxidizes the methyl carbon of a methylated lysine, producing a chemically unstable hemiaminal (*N*-hydroxymethyl) derivative while recycling back to Fe(II). The remaining hemiaminal then spontaneously decomposes to produce formaldehyde and a lysine with one fewer methyl group (Fig. 4A). Thus, the overall stoichiometry of the reaction requires the consumption of both 2-OG and oxygen and the concomitant production of succinate and CO_2 per methyl group removed as formaldehyde. In terms of substrate specificity, JmjC enzymes have been associated mostly with KDM activity, though at least one family member (JMJD6) has demonstrable arginine demethylase activity (39). Additionally, these enzymes collectively are capable of demethylating trimethyl-lysine substrates, which LSD1 is unable to do.

Indeed, LSD1 utilizes an entirely different mechanism to catalyze the oxidative demethylation of histones (Fig. 4B). LSD1 belongs to the family of flavin adenine dinucleotide (FAD)-dependent amine oxidases (78). In the rate-limiting step, LSD1 catalyzes the 2-electron oxidation of the C-N methylamine bond concomitant with the 2-electron reduction of the FAD cofactor. The resultant imine group is chemically unstable, and nonenzymatically reacts with water to result in the production of formaldehyde and the removal of one methyl group (79). The reduced FAD is subsequently reoxidized by O_2, producing H_2O_2 as a byproduct, though the capacity of the enzyme to reduce other substrates has been questioned due to relatively low rates of reaction with O_2 (79). Notably, since LSD1 requires a free electron pair on an amine nitrogen for catalysis, it is unreactive toward trimethyl-lysine residues.

While the mechanisms for both jmjC and LSD1 demethylases have been largely accepted, questions still remain about the PAD4/PADI4 deiminase and its capacity to remove methyl groups. Multiple laboratories have produced compelling *in vitro* evidence suggesting instead that methylated arginines make poor substrates for PAD4. Moreover, the associated mechanism produces citrulline, which likely represents a distinct post-translational modification with its own functional ramifications. For these confounding reasons, we will not cover the PAD4 protein further; for more detailed review, please see references (139, 302).

4. Kinetic considerations of histone demethylases. As might be expected from the markedly different modes of catalysis, jmjC and LSD1 demethylases have significantly different apparent catalytic rates. Under *in vitro* conditions, the jmjC demethylase JMJD2A/KDM3a has an apparent catalytic rate k_{cat} of $\sim 0.01\,\text{min}^{-1}$ for methylated histone substrates (49). On the other hand, LSD1 has a measured k_{cat} of

~3.1, approximately three orders of magnitude higher than KDM3a (78). Both of these apparent catalytic rates are remarkably small, especially in comparison to other enzymes that share similar reaction mechanisms. For example, other characterized 2-OG-/Fe(II)-dependent dioxygenases have k_{cat} values 2–4 orders of magnitude higher than KDM3a, whereas other amine oxidases have k_{cat} values 2–3 orders of magnitude higher than LSD1 (78, 282). This strongly suggests that the *in vitro* assays may be inadequate for appropriately describing histone demethylase activity. Protein–protein interactions and local environmental considerations must be taken into account for these enzymes to reasonably function at a biologically significant level. Smith and Denu also noted in their review of mechanisms of histone modifications that these measurements suggest that as-yet-undiscovered demethylase enzymes might be playing a role in the regulation of histone methylation status (282).

B. Histone acetylation

It has been known for decades that histones could potentially be acetylated at lysine residues, though the enzymes responsible for this mark were unidentified until the mid-1990s (193). Notably, histone deacetylase (HDAC) enzymes were almost immediately identified after histone acetyltransferases (HATs), in stark contrast to similar genes involved in histone methylation (159). Also unlike methylation, histone acetylation is an obligate transcriptional activator—mechanistically, it has been proposed that the acetyl groups disrupt the tight association of DNA with normally basic histone residues (153). Acetylation generally is found at the N-terminal tails of histones, though recent studies have demonstrated that H3K56, a conserved residue within the core histone domain, is also target for acetylation and is critical for multiple regulatory functions (230, 337). Importantly, there are a number of distinct families of both HATs and HDACs, each of which present distinct possibilities for mechanistic regulation.

1. Histone acetyltransferases.

HATs typically belong to one of three main families, named for their founding members. These are the GNAT (Gcn5-related *N*-acetyltransferase), MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60), and p300/CBP families (258). While each of these general families has nuanced differences in active site residues and geometry that may be associated with substrate specificity, the overall kinetic mechanisms are broadly shared among the three classes (258, 282). Briefly, these enzymes catalyze the abstraction of a proton from the ε-amino group of lysine, which then is primed for nucleophilic attack on the keto-carbon of acetylated coenzyme A. This intermediate structure then decomposes, releasing free coenzyme A and acetyl-lysine (Fig. 5). Generally speaking, HATs are not absolutely specific for particular lysine residues, instead promoting broad acetylation of histone tails when recruited as part of coactivating complexes (159, 258, 320).

2. Histone deacetylases.

Enzymes with HDAC activity are currently divided based on both mechanistic similarity and phylogenetic comparison. Under old classification systems, there were two distinct classes of metal ion-dependent HDACs and a single class of nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes (called sirtuins; see below). In 2004, Gregoretti *et al* performed a comprehensive phylogenetic analysis of HDAC-like sequences across all sequenced genomes, establishing that nonsirtuin HDAC enzymes are best classified into three distinct groups: Class I, II, and IV (99). For consistency's sake, sirtuin-like enzymes remained defined as Class III.

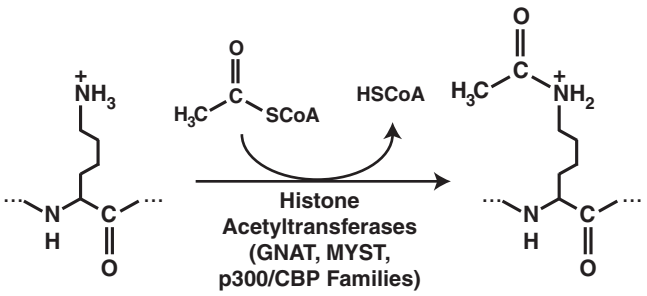

FIG. 5. HAT mechanism. Broadly, HAT enzymes each use a similar mechanism, with acetyl-CoA serving as the acetyl donor. Notably, HAT enzymes are not generally specific for lysine residues on histones, but rather are responsible for pan-acetylation reactions. HAT, histone acetyltransferase.

3. Nonsirtuin HDACs.

Class I, II, and IV HDACs share a similar catalytic mechanism that involves the coordination of a divalent metal ion. Initial studies suggested that the catalytic metal was Zn(II), but recent findings have indicated that Fe(II) may play a more significant role (86). Regardless of the nature of the divalent ion, mechanistic studies indicate that the enzyme-coordinated metal and a conserved tyrosine (for Class I, IIb, and IV) or histidine (Class IIa) polarizes the carbonyl oxygen, priming the carbonyl carbon of the acetyl group. Elsewhere in the active site, conserved histidines activate a water molecule for nucleophilic attack on the carbonyl carbon; the resulting tetrahedral intermediate is stabilized by the metal ion before decomposing into acetate and unmodified lysine (Fig. 6A) (282).

Class I HDACs include human HDAC1–3 and HDAC8 (99). HDAC1–3 are known members of important nuclear repressor complexes such CoREST, NURD, SIN3, N-COR, and SMRT, which play multiple roles in gene transcription (3, 123, 326). HDAC8, though phylogenetically grouped with class I HDACs, shares many features of both class I and II HDAC enzymes (69). While it has not to date been identified as part of any known nuclear complexes, HDAC8 notably was the first HDAC crystal structure to be examined and has provided the basis for much of our *in vitro* knowledge of class I, II, and IV HDAC activity (69, 99, 286).

Class II HDAC family members may be further grouped into classes IIA and IIB. Class IIA HDACs include human HDAC 4, 5, 7, and 9, which share a functionally significant N-terminal regulatory domain that enables both nuclear-cytoplasmic shuttling activity and binding to a variety of transcription factors in the nucleus (194, 328). Class IIB includes HDAC6 and HDAC10. HDAC6 is a major cytoplasmic protein deacetylase whose substrates include α-tubulin and heat shock protein 90 (HSP90); it also plays a role in diverse cellular functions such as aggresome formation and epidermal growth factor receptor signaling, independent of deacetylase activity (87, 124, 141, 154). Comparatively little is known about HDAC10 and its role in transcriptional regulation.

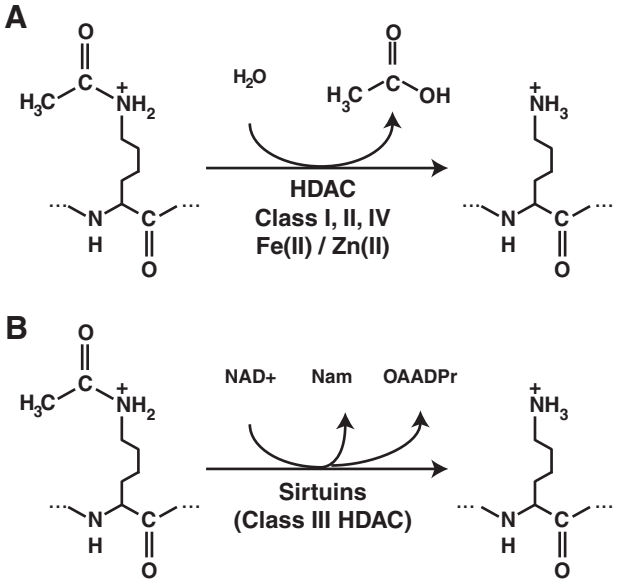

FIG. 6. Histone deacetylase mechanisms. (A) HDAC class I, II, IV mechanism. Classical HDAC enzymes utilize a redox-active metal [Zn(II) or Fe(II)] to coordinate the hydrolysis of acetate from lysine residues. **(B)** Sirtuin enzyme mechanism. Sirtuins remove acetyl groups at the expense of NAD, releasing nicotinamide and the unique metabolite O-acetyl-ADP-ribose. Both HDAC and sirtuin enzymes may catalyze the deacetylation of substrates other than histones. HDAC, histone deacetylase; OAADPr, 2′-*O*-acetyl-ADP-ribose; Nam, nicotinamide.

Only one Class IV HDAC, HDAC11, has been identified to date. HDAC11 shares similarities between both class I and II HDACs, but specific molecular characterizations have not been extensively studied. However, recent literature suggests a role for HDAC11 in both immune system modulation and glial cell biology (183, 317).

4. Sirtuin deacetylases. The class III HDACs, or sirtuins, are mechanistically and structurally unrelated to any of the other HDAC classes. Instead, these enzymes share homology with the yeast enzyme silent information regulator 2 (Sir2), initially characterized in 1984 as a protein important for position-effect control of yeast genes (280). In the 1990s, it became apparent through sequence comparison that humans have a stable of related enzymes, subsequently known as the sirtuins. Sirtuins were initially characterized as ADP-ribosyltransferases by a handful of independent laboratories (82, 300). A series of studies published thereafter demonstrated evidence of NAD^+-dependent protein deacetylase activity (128, 164, 165, 285). Tanner *et al* settled the apparent contradiction in 2000, when they definitively demonstrated the sirtuin reaction mechanism and suggested that ADP-ribosyltransferase activity likely represents a low-flux side reaction of NAD^+-dependent deacetylase function (299).

Humans have seven sirtuin family members, named SIRT1–7. Each shares a conserved catalytic sirtuin core domain, but differs in C- and N-terminal sequence. These divergent domains promote multiple specific enzymatic activities, subcellular localizations, and binding partners among different sirtuin enzymes, which in turn give each unique functionality [reviewed in ref. (103)]. Despite these divergent functions, the sirtuins all share a unique mechanism for deacetylation. Briefly, sirtuins catalyze the NAD^+-dependent deacetylation of acetyl-lysine residues, releasing free nicotinamide, deacetylated lysine, and the unique metabolite of unknown significance, 2′-*O*-acetyl-ADP-ribose (299). First, acetylated lysine must bind within the active pocket before NAD^+. The presence of acetyl-lysine then forces the nicotinamide ring of NAD^+ into a conserved hydrophobic pocket, positioning it for catalysis. Once both substrates are in place, the carbonyl oxygen of the acetyl group attacks the 1′-carbon of the nicotinamide ribose, forming an *O*-alkylamidate intermediate and ejecting the nicotinamide ring. A series of successive elimination steps then occurs to release deacetylated lysine and 2′-*O*-acetyl-ADP-ribose (Fig. 6B). For more detailed review of the sirtuin mechanism, please see reference (283).

C. Histone ADP-ribosylation

In the past 50 years, it has become increasingly apparent that ADP-ribosylation is an important post-translational modification in the nucleus. It was first discovered in the early 1960s, when Paul Mandel's group identified an NAD^+-dependent nuclear enzyme activity producing an RNA-like polymer of adenine residues (38). Multiple groups independently identified this structure as poly(ADP-ribose), which notably consists of two ribose moieties per adenine (260). These studies led to the identification of the poly(ADP-ribose) polymerase (PARP) family of enzymes, which currently are attracting a great deal of interest among epigenetics researchers. PARP family members broadly are capable of both initiating the post-translational transfer of ADP-ribose to multiple proteins as well as the generation of complex polymers of poly(ADP-ribose) that have myriad effects on the regulation of protein function (110). Notably, histones were among the first proteins to be associated with PARP activity, through both the identification of poly(ADP-ribosyl)ated histones (312) and the strong association of histones themselves with polymerized ADP ribose itself (229). This research continues today, and although ADP-ribosylation and poly (ADP-ribosyl)ation were not initially considered as part of the canonical histone code, evidence strongly suggests that they play a role in the dynamic regulation of the epigenetic status of histones (137, 251).

1. PARP activity and functionality. PARP family members catalyze the addition of ADP-ribose moieties to proteins, generally at E or K residues, and further catalyze the complex polymerization producing poly(ADP-ribosyl)ated proteins (105, 109, 110). This process consumes NAD^+, releasing nicotinamide as a reaction byproduct (Fig. 7). Under conditions suitable for maximal PARP activity, poly(ADP-ribosyl)ation is capable of rapidly depleting intracellular NAD^+, which can radically alter cellular metabolism (for further discussion, see below) (15). The founding member PARP1 was initially identified in association with DNA damage and repair pathways. The canonical model for PARP1 activity holds that it is activated upon binding to DNA strand breaks. At this point, it auto poly(ADP-ribosyl)ates at multiple sites, producing a complex network of ADP-ribose residues extending from the core protein structure (109, 110). This network is capable of interfering with protein–protein interactions and

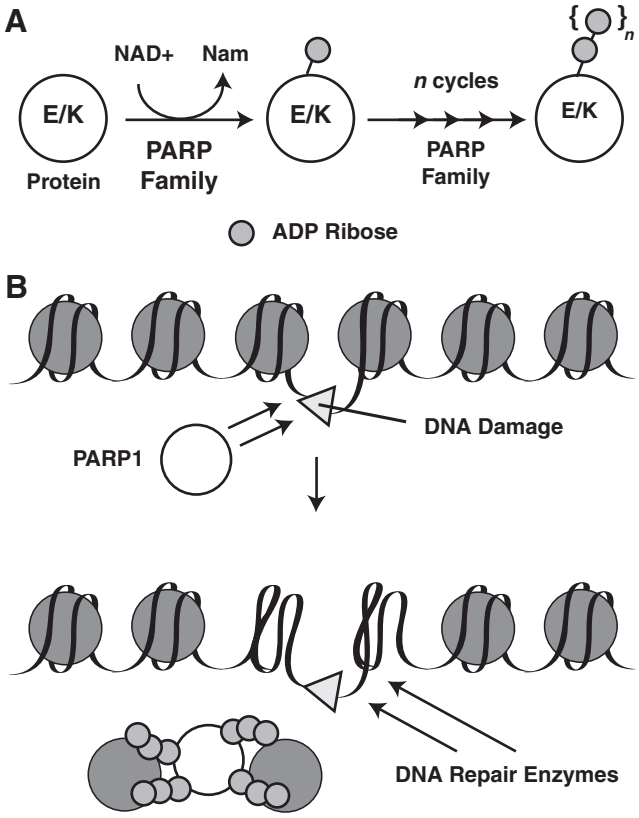

FIG. 7. PARP activity and histone shuttling. (A) Canonical model for PARP family activity. PARP enzymes consume NAD^+ to generate the poly ADP-ribose mark, releasing nicotinamide as a byproduct. This can target both glutamate and lysine residues on proteins. **(B)** Histone shuttling diagram. PARP1 is a nuclear PARP that recognizes DNA damage, promoting autocatalysis that results in the poly (ADP-ribosyl)ation of PARP1 itself. Poly(ADP-ribose) is a strong competitor for DNA binding to histones, which shuttles histones away from DNA lesions and allows repair enzymes access to the damaged region. PARP, poly(ADP-ribose) polymerase.

protein–DNA interactions in the immediate vicinity, including the association of histones with DNA. Histones were found to bind with an affinity to nuclear poly(ADP-ribose) networks that could not be predicted based solely upon electrostatic interaction, providing a remarkably efficient competitive stimulus to remove histones from DNA (5). This model, termed "histone shuttling," suggests that this subsequently frees DNA local to the site of damage for efficient implementation of DNA repair processes (Fig. 7). This is further supported by data that PARP deletion is associated with base excision repair (BER) deficiencies (54).

PARP family members have also been associated with post-translational modifications of histones. Numerous studies have identified native mono- and poly(ADP-ribosyl)ated histone core proteins from mammalian nuclear extracts (95, 202, 312). Moreover, PARP activity is differentially associated with different histone proteins depending on local nuclear conditions. For example, under conditions with high local NAD^+ concentrations, the linker histone H1 is the primary target for ADP-ribosylation (259). This relieves H1 constriction of local chromatin, and subsequently promotes a more relaxed chromatin environment as evidenced by the classic "beads-on-a-string" appearance on electron microscopy (57). However, under nonoptimal reaction conditions (*e.g.*, with less NAD^+ availability), histones H2A and H2B are predominant targets for ADP-ribosylation, resulting in more subtle chromatin effects (148). Critically, there is some debate as to whether these reactions produce poly(ADP-ribosyl)ated histones *in vivo* but mono-ADP-ribosylation is, nevertheless, accepted as a distinct and relevant post-translational modification mediated by PARP family members (109).

While the ADP-ribosylation of histones has been effectively demonstrated *in vivo*, the functional significance of this mark remains to be generally determined. To date, it has been associated with contrasting chromatin functions, including the maintenance of facultative heterochromatin at the X chromosome (221) in addition to the relaxation of chromatin in association with polytene chromosome "puffs" in *Drosophila* (308–310). Additionally, the actual target residues for modification on histone tails have been in question. Until recently, only modification of glutamate residues had been mapped on histone tails, but work from the Hottiger laboratory has demonstrated that lysines present in each of the core histone N-terminal tails also are targets for ADP-ribosylation (202). The significance of this in terms of epigenetic events is critical, as lysines are the targets for many other established epigenetic modifications, and thus ADP-ribosylation may change the dynamics of the histone code on target histones. Additionally, the authors note that the chemical nature of the ADP-ribose changes the net ionic state of the lysine tail from +1 to −2, which may precipitate relatively massive changes in the interactions of histones with DNA and/or other chromatin-associated proteins. This is in stark contrast with other modifications such as acetylation, which merely eliminate a positive charge but do not produce a net negative ionization. What is clear is that ADP-ribosylation of histones is an often-overlooked modification, and that evidence is mounting that ADP-ribosylation should be considered an epigenetic element in the histone code.

2. Indirect effects of ADP-ribosylation. In addition to the direct modification of histones with ADP-ribosyl moieties, there is considerable documented evidence that ADP-ribosylation events within the nucleus can indirectly control the implementation of other epigenetic events. The Caiafa laboratory has focused extensively on the linkage between DNA methylation and PARP activity. Using the PARP inhibitor 3-aminobenzamide, they demonstrated over a series of publications in the late 1990s that abrogation of PARP activity was associated with broadly aberrant DNA hypermethylation in the mouse fibroblast cell lines NIH/3T3 and L929 (55, 339, 340, 342). Notably, this hypermethylation extended to exogenously induced plasmid DNA, suggesting that increased *de novo* DNA methylation was taking place (340). Ultimately, they proposed a model by which auto poly(ADP-ribosyl)ated PARP1 promotes the upregulation of DNA methyltransferase 1 (DNMT1) in a cell-cycle-specific fashion, leading to enhanced PCNA-DNMT1 complex formation at the G1/S border and subsequently higher DNA methylation (341). Notably, this model does not exclude the possibility that the poly(ADP-ribosyl) network extending from PARP1 is directly promoting an enhanced protein–protein interaction between

DNMT1 and PCNA, which would also facilitate increased DNA methylation.

Poly(ADP-ribosyl)ation through PARP enzymes has also been proposed to communicate with histone acetylation through sirtuins, which also consume NAD^+. Competition for NAD^+ in the nuclear environment may prove to be a critical locus for metabolic regulation of these enzymes, as data have already indicated that PARPs are capable of rapidly consuming NAD^+ pools (15). In addition to this broad metabolic mechanism, multiple studies have also illustrated that mono-ADP-ribosylation of histone H4 occurs preferentially when H4 is hyperacetylated (21, 95). Taken together with NAD^+ regulation, these data strongly suggest a dynamic interplay between ADP-ribosylation and histone acetylation that merits further study.

D. DNA methylation

DNA methylation is a nonhistone epigenetic phenomenon that plays a role in transcriptional regulation. In all eukaryotes, methylation takes place on cytosine residues, producing 5-meC, but the local context of the methylation event differs among organisms (294). In mammals, the primary targets for methylation are cytosine bases immediately preceding guanosine bases (CpG dinucleotide), which confers strand reciprocity. Most CpG dinucleotides are methylated in mammals, and they are distributed throughout multiple genetic elements, including genes, endogenous repeats, and transposable elements (127). Notably, CpG dinucleotides occur at a frequency ~21% of that predicted by random chance, as 5-meC bases spontaneously deaminate to produce thymine (163). However, there are local exceptions to this rule. Short sequences (~1kb) in which CpGs appear in high density in a mostly unmethylated form are termed CpG islands (CGIs). CGIs, though only representative of a fraction of all CpG dinucleotides, occur in the promoter sequences of a vast number of human genes (127). These islands are thought to persist based upon methylation status; since they remain largely unmethylated (through as yet unclear mechanisms), minimal deamination takes place and the islands maintain their integrity. Recent work aimed at computationally identifying the nature of CGIs in the genome identified that ~72% of genes have these canonical CGIs closely associated with their promoters, whereas the remaining 28% have background levels of CpG dinucleotide occurrence (273). Notably, those genes with low CpG frequency were heavily associated with differentiation and cell-type-specific function, whereas there was a significant overrepresentation of broadly expressed housekeeping genes within the canonical CGI population (273). These data clearly suggest an important regulatory role for both individual CpG dinucleotides and CGIs, and genome-level approaches are yielding new information routinely (294). An excellent example of this is the recently published work by the Costello group. In Maunakea *et al.* (196), the authors generated a DNA methylation map encompassing the majority of the CpG sites within the human genome utilizing a combination of methyl-CpG immunoprecipitation and methylation-sensitive restriction digests. They demonstrated that the majority of methylated CGIs existed in intra- and intergenic regions, as opposed to within canonical promoters. Critically, these intra- and intergenic CGIs corresponded with RNA markers of transcription start sites, suggesting that these constructs could play a role in the regulation of alternative transcripts. Indeed, this was found to be the case with the experimental locus *SHANK3*, which has alternative transcripts expressed in a tissue- and cell-specific fashion based on the status of its intragenic CGIs. These data suggest an expanded role for CGI regulation that is not merely limited to whole gene repression, but a more finely tuned process for selection of appropriate transcript utilization. However, these data do not change the overall endpoint for CpG methylation.

From a broad regulatory standpoint, cytosine methylation is heavily associated with transcriptional repression. This happens through two basic mechanisms, including direct interference with transcription factor binding and the recruitment of specialized methyl-DNA binding proteins. For the former, many transcription factors have methylation status-specific interactions with DNA, including E2F, Krox-20, and CREB (31, 126, 252). Methylation of CpG nucleotides abrogates normal binding of these proteins to DNA, leading to transcriptional repression. For the latter mechanism, there is extensive literature about 5-meC binding proteins, including methyl CpG binding protein 2 and associated methyl-binding domain (MBD) family members MBD1–4, as well as the recently discovered Kaiso zinc finger domain proteins (20). Collectively, these proteins bind to 5-meC bases and provide a protein scaffold that recruits other factors that mediate transcriptional repression, including multiple families of histone modifying enzymes.

1. DNA methyltransferases.

In mammals, two main types of DNMTs mediate the establishment and maintenance of cytosine methylation. DNMT1 is associated with the post-DNA replication maintenance of established methyl groups, as it preferentially recognizes hemimethylated CpG substrates (133). The DNMT3 family has several members and is responsible for *de novo* methylation (281). DNMT2, though the most highly phylogenetically conserved of any of the DNMT enzymes, is relatively poorly characterized, and is thought to play a larger role in RNA processing than in DNA methylation (274). All DNMT enzymes share a basic common mechanism analogous to the HMT enzymes, involving methyl transfer from the donor SAM to the 5-carbon of the cytosine ring, yielding SAH (Fig. 8). This occurs through enzyme-linked thiolate attack of the 6-carbon, which primes the cytosine ring for enamine attack on the methyl group of SAM. The resulting enzyme-linked product then undergoes β-elimination to release 5-meC and regenerate active DNMT (281).

2. DNA demethylases.

While it has long been recognized that plants have an extensive network of enzymes that actively demethylate DNA, the search for a mammalian active demethylase system has proven exceptionally difficult. Ooi and Bestor provide an excellent historical perspective on this topic in their 2008 review (226). The current dogma holds that DNA glycosylases and the BER pathway play a major role in the active demethylation of mammalian DNA, but this poses kinetic difficulties given the rapidity with which demethylation has been observed in model systems (352). Recent evidence from Hajkova *et al*. (104) demonstrates that while BER has significant importance on active demethylation in germ cells, the putative mechanism preceding BER-related demethylation needs to be re-evaluated. The authors

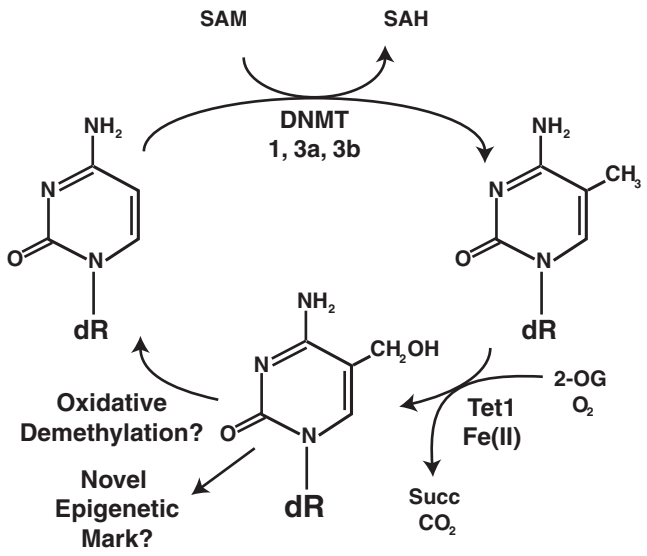

FIG. 8. DNA methyltransferase and putative demethylase. Cytosine methylation is catalyzed by the action of the DNMT enzymes in a context-dependent fashion (see text for further information). The recently discovered Tet1 protein is a 2-OG and Fe(II)-dependent dioxygenase that catalyzes the formation of hydroxymethylcytosine, which may represent a critical step in active oxidative DNA demethylation or may itself comprise a novel epigenetic mark. DNMT, DNA methyltransferase; dR, deoxyribose.

illustrated that in mouse primordial germ cells (PGCs), functional BER is necessary for the genome-wide demethylation event that occurs normally in embryonic cells (104). Critically, this was not accompanied by significant expression of enzymes linked to cytosine deamination, which had previously been associated with CpG demethylation (104). However, the data demonstrated that TET1 expression was significantly elevated in PGCs, providing a potential alternative route to active demethylation (104).

TET1 is an acute myeloid leukemia-associated gene that was recently identified as a 5-meC hydroxylase. Studies by Tahiliani *et al.* (296) demonstrated that TET1 is a 2-OG and Fe(II)-dependent dioxygenase that actively oxidizes 5-meC to 5-meOHC, in a reaction analogous to that utilized by the jmjC family histone demethylases (Fig. 8). While the subsequent elimination of formaldehyde has not been demonstrated effectively, this presents a potential mechanism for active DNA demethylation. Importantly, the chemical milieu of the hydroxymethyl group is different in this case, given that it is attached to a ring carbon rather than nitrogen as with lysine, leading to different susceptibility to spontaneous demethylation. However, the Tet1 catalytic step may represent merely the first section of a multistep oxidative process, leading to the ultimate removal of methyl groups from DNA as we have previously discussed (118). Recent findings illustrating that 5-meOHC is present at high levels in specific neuronal populations lend credence to this hypothesis (156). Epigenetic regulation is becoming increasingly important in helping to explain both complex behavioral patterns and memory, and active demethylation of DNA in neurons may play a vital role in these processes (206).

E. Noncoding RNA and epigenetic regulation

Since the initial discovery of RNA interference in plants in 1986 (68), an intense research effort has been focused on establishing the mechanisms and biological significance of RNA-mediated modulation of gene expression. The initially characterized systems were illustrative of post-transcriptional silencing, mediated by the RNA-induced silencing complex. These findings led to the widespread utilization of siRNA and shRNA technology as powerful tools to both transiently and permanently silence gene expression in experimental models. In the past decade, it has become apparent that noncoding RNA (ncRNA) may also mediate longer term gene silencing through modulation of epigenetic phenomena at targeted promoter regions. This may occur through a variety of mechanisms, involving multiple classes of ncRNA and distinct modes of inactivation. Here, we will briefly introduce some of the known linkages between ncRNA modulation of gene expression and coordinate epigenetic regulation.

1. Long ncRNAs. Long ncRNAs are involved in long-term silencing in many developmentally critical events through interactions with the polycomb group (PcG) proteins, which are associated with the formation of repressive heterochromatin states through recruitment of histone modifying complexes (211). There are several prime examples of biologically significant long ncRNAs, including those that interact with the *HOX* gene cluster in higher eukaryotes and those that mediate X-chromosome inactivation. *HOX* genes are critical for the development of complex multicellular eukaryotes, and there are a large number of ncRNA transcripts that are antisense to *HOX* genetic sequences. For example, the *HOX* antisense intergenic RNA (HOTAIR), a 2.2-kb fully processed transcript, is capable of mediating *HOXD* in *trans* through interaction with PcG proteins and subsequent methylation at H3K27 (255). X-inactivation is another critical developmental process that mediates dosage regulation of X-chromosome genes in (X, X) females. In humans, this is mediated by a 17-kb RNA transcript called the X-inactivation-specific transcript (Xist) at the center of an exceptionally complex regulatory network. Although a thorough discussion of the coordinate regulation of X-inactivation and the *XIST* genetic locus is beyond the scope of this review, it does involve epigenetic regulation of heterochromatin and several PcG proteins. For a more detailed examination of ncRNA and X-inactivation, please see reference (171).

2. Short ncRNAs. Short ncRNAs may also mediate transcriptional silencing, and they may do so through a variety of different mechanisms. The classical pathway for ncRNA-dependent silencing, involving the binding of short ncRNA (or microRNA when expressed endogeneously) to the 3′-UTR of target transcripts, may be utilized to direct the expression levels of epigenetic enzymes themselves. This is becoming an increasingly important research topic as more powerful bioinformatic methodologies are developed, enabling high-throughput screening for microRNA targets. For example, both SIRT1, which is a critical nuclear protein deacetylase, and EZH2, which is a histone methyltransferase, have recently been associated with microRNA regulation. MiR-34a, an identified tumor suppressor microRNA, is associated with the repression of SIRT1 activity (331). This re-

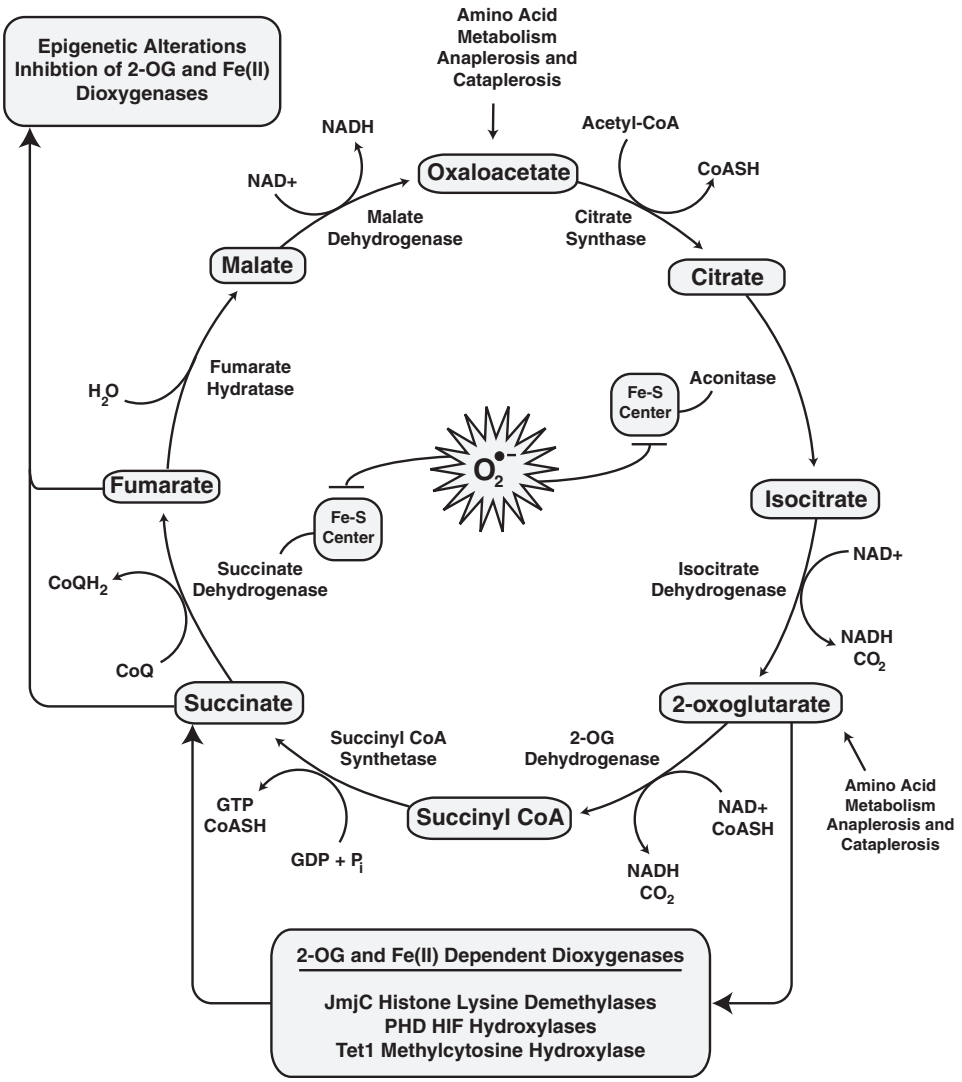

FIG. 9. The citric acid cycle and key metabolic intersections. The citric acid cycle is a critical intersection between catabolic and anabolic processes that has many implications for epigenetic events. To supplement the basic 8-reaction cycle, we have included the utilization of 2-OG as a substrate by the 2-OG and Fe(II)-dependent dioxygenases, as well as the ability of both succinate and fumarate to inhibit these enzymes. Additionally, superoxide produced during conditions of oxidative stress may interfere with both SDH and aconitase, leading to different substrate balances that could affect downstream processes. SDH, succinate dehydrogenase.

pression was associated with enhanced apoptosis in colon cancer in association with enhanced p53 acetylation, providing a putative mechanism for the tumor-suppressive qualities of miR-34a (331). EHZ2, on the other hand, is negatively regulated by miR-101, another tumor suppressor microRNA (80). In tumors lacking miR-101, EHZ2 is overexpressed, leading to aberrant patterns of histone methylation that likely contributes to observed epigenetic plasticity in tumor cells (316). These findings are critical because they highlight mechanisms by which malignant cells may coopt the epigenetic machinery to promote a more favorably environment for enhanced tumorigenesis. We anticipate that as research linking microRNAs and epigenetic targets accumulates, this will prove to be a generalized paradigm applicable to multiple players in epigenetic regulation. Moreover, as we will discuss later on, the metabolic derangements in tumor cells may additionally promote epigenetic disturbances, prompting a synergistic disruption of normal epigenetic regulation.

It is becoming increasingly clear that short ncRNAs may mediate transcriptional events themselves, in stark contrast with the classical post-transcriptional regulation of gene expression *via* ncRNAs. Multiple groups have demonstrated that antisense RNA directed against promoter regions, rather than transcripts, can silence gene expression [reviewed in ref. (191)]. Critically, this repressive environment can substantially outlast exposure to siRNA. Hawkins *et al.* demonstrated that a 1-week induction of shRNA mediates transcriptional silencing of the human ubiquitin C gene for up to 1 month (112). This silencing was dependent on the activity of several repressive epigenetic enzymes, including HDAC1, DNMT1, and DNMT3a, strongly supporting an epigenetic role in the establishment of a repressed state (112).

Collectively, these studies have established a role for epigenetic machinery in the regulation and maintenance of RNA-mediated gene silencing. Although the specific nature of the interactions between RNA and proteins leading to the recruitment and activity of DNMTs, HDACs, HMTs, and other critical epigenetic regulatory enzymes has yet to be determined, ncRNA-mediated epigenetic events will likely garner increasing interest as further research is undertaken. We have only briefly touched upon these issues here, as an in-depth description of the field is beyond the scope of this review. For further reading on the topic of epigenetic mechanisms

involved in ncRNA-mediated gene silencing, please see references (47, 100, 155, 191, 291).

III. Epigenetic Regulation and Redox Metabolism

The epigenetic mechanisms described above represent multiple distinct approaches for cells to effectively regulate gene transcription. Although the establishment and maintenance of histone methylation, histone acetylation, and DNA methylation are all governed by different enzymatic mechanisms, there is considerable interplay between these epigenetic processes. For example, H3K9me3 is a histone mark that recruits heterochromatin protein 1, which in turn recruits DNMT3a and DNMT1 to promote enhanced methylation of local CpG dinucleotides (84). The converse is also true: the binding of MBD1 to 5-meC results in the recruitment of the Suv39h1 HMT, enabling DNA methylation to promote subsequent histone methylation (83). These particular reciprocal events illustrate how histone methylation can regulate *de novo* DNA methylation and *vice versa* to promote a synergistic epigenetic signal. Importantly, they also illustrate the inherent complexity of the epigenetic machinery. The regulation (or, conversely, deregulation) of any one element in the epigenetic process has the potential to broadly affect other epigenetic marks, effectively amplifying perturbations to one element in the system. Coordinate regulation of dynamic epigenetic events is thus of critical importance. Inputs from cellular metabolism represent one such mechanism for this coordinate regulation.

Metabolism is an absolute requirement for life, and metabolic processes likely predate epigenetic phenomena by billions of years. Given that the evolution of epigenetic regulation occurred in the context of an ancient series of conserved metabolic reactions, we speculate that epigenetic processes might initially have existed as a means to transduce metabolic events into phenotypic results as organisms became more complex. Regardless of the evolutionary origins of epigenetic modification, the functional significance of metabolic contributions to epigenetic signaling is becoming ever clearer. In the following sections, we will profile some of the metabolic inputs that are known to have roles in the maintenance of the epigenome, and discuss the mechanisms associated with this maintenance. In particular, we will focus on elements of redox metabolism and biology.

IV. Redox Metabolism

Cellular metabolic processes employ a multitude of redox processes in both catabolic and anabolic systems. The electron shuttling in these vital reactions is critical, because the energy derived from the exchange of electrons to a donor molecule is large in comparison to the energy gained from nonredox processes. This is obvious when comparing oxidative *versus* solely glycolytic metabolism of a glucose molecule. Simple glycolysis yields a net of two adenosine triphosphate (ATP) molecules, whereas the full oxidation of glucose to carbon dioxide (CO_2) through mitochondrial respiration yields a net of ~36 ATP (254). Redox processes are also necessary for the production of complex biomolecules, including fatty acids and other lipids, complex carbohydrates, and proteins. With the vast number of metabolic events taking place simultaneously within a cell, the trafficking of electrons at any one time is significant. The large amounts of energy at play also means that utilizing the energy of electrons to drive metabolism is not without substantial risk for cellular damage. Consequently, numerous mechanisms ensure that electrons are harnessed in a controlled fashion. Here, we will address a subset of critical metabolic systems that play specific roles in the maintenance of epigenetic processes.

A. The citric acid cycle and intermediates of central metabolism

The citric acid cycle (CAC) is a series of eight reactions in the mitochondrial matrix that result in both the full oxidation of an activated acetyl group (acetylated coenzyme A) to CO_2 and the regeneration of the 4-carbon carrier molecule oxaloacetate (Fig. 9). The CAC represents a critical nexus of metabolism, both because it provides entry points for the electrons of complex biomolecules into the respiratory cascade and because it provides critical intermediates for the anabolic processes that lead to new biomolecules. The ebb and flow of the steady-state levels of molecules within the cycle is described by the terms anaplerosis and cataplerosis. Anaplerosis is the process by which the intermediate carrier molecules in the CAC are replenished; cataplerosis refers to the depletion of these intermediate molecules. In a closed system, the CAC would replenish the carrier molecules with absolute efficiency, but some estimates hold that ~1%–2% of intermediate substrates are lost (26). This is particularly important when considering actively respiring cells, as several studies have shown that supplementation with anaplerotic substrates (those that are capable of replenishing intermediate pools) is required for maintenance of full respiratory capacity (261, 262). These results indicate that the maintenance of the pools of CAC intermediates is a dynamic process that is tightly regulated to ensure appropriate metabolic functionality. Moreover, the net input and output of substrates into the CAC strongly regulates the tissue-specific production of more complex biomolecules. For example, in hepatocytes, an influx of anaplerotic substrates can be utilized to drive gluconeogenesis, whereas in the brain, anaplerosis is associated with the production of neurotransmitters. Overall, the broad connections between anaplerotic and cataplerotic mechanisms are critical for further discussion of epigenetic modification, because both of these processes provide regulatory input for epigenetic enzyme functionality.

There are several main physiologic portals for both anaplerosis and cataplerosis in the CAC, including succinyl-CoA, 2-OG, and oxaloacetate. Succinyl-CoA, which in the CAC is derived from the oxidative decarboxylation of 2-OG by 2-OG dehydrogenase, also can be derived from multiple routes of amino acid catabolism (28, 304). The next enzyme of the CAC, succinyl CoA synthetase, drives the production of succinate and CoASH, releasing enough energy to catalyze the substrate-level phosphorylation of a GTP from GDP and inorganic phosphate (Fig. 9). Succinate is a critical metabolic intermediate that plays an established role in the regulation of 2-OG and Fe(II)-dependent dioxygenases. Examination of the reaction mechanisms for these enzymes (Figs. 4 and 8) reveals how this may occur. A buildup of succinate can produce a product-level inhibition of these enzymes, preventing the catalytic process from taking place. This has been demonstrated in several model systems, but is most well characterized for the HIF regulatory axis. The 2-OG and

Fe(II)-dependent PHD enzymes are competitively inhibited by succinate, producing a pseudo-hypoxic stabilization of HIF in the absence of true hypoxia (147, 276, 277). This phenomenon was established through study of tumors harboring succinate dehydrogenase (SDH) mutations.

SDH is a 4-subunit complex (SDHA, SDHB, SDHC, and SDHD) that catalyzes the oxidation of succinate to fumarate in a flavin-dependent manner. It plays a role both as a vital CAC enzyme and as complex II in the electron transport chain, as it passes the electrons from succinate directly to coenzyme Q in the mitochondrial inner membrane. Inherited mutations in any of the four subunits of SDH result in a spectrum of human disease: SDHA mutations result in Leigh Syndrome, a rare and serious necrotizing encephalopathy, whereas mutations in SDHB, SDHC, or SDHD all result in familial paraganglioma syndromes characterized by pheochromocytoma and related tumors of the neuroendocrine chromaffin cells (75, 240) [mutations in SDH5, a recently identified accessory gene responsible for the flavination of the SDHA subunit, have additionally been associated with paraganglioma syndromes (108)].

Critically, tumors with SDH mutations displayed enhanced HIF stability in addition to a pattern of gene expression suggestive of HIF activation (93, 94). In 2005, Selak *et al.* linked this phenomenon to inhibition of the PHD enzymes secondary to succinate buildup (276). Succinate levels were significantly increased after the RNAi-mediated silencing of the SDHD subunit. This was linked to reduced PHD activity and enhanced HIF-1α stability in these cell lines, mirroring the behavior of cells treated solely with the cell-permeant succinate analog dimethylsuccinate (276). This critical finding provided a novel mechanism linking metabolic perturbation to oncogenesis, and established the framework for how other intermediates might also interfere with downstream epigenetic processes. Appropriately, fumarate was also identified as a critical metabolic intermediate that interferes with 2-OG and Fe(II)-dependent dioxygenase function (130). Isaacs *et al.* (130) established that fumarate also efficiently stabilizes HIF-1α through interference with PHD enzymes, and that mutations in fumarate hydratase (FH) in renal cell carcinoma correlate with enhanced HIF stability (130). Clearly, the dysregulation of CAC intermediates secondary to mutations in CAC enzymes can produce deleterious effects on signaling processes closely related to epigenetic regulation. The evidence that these intermediates can directly disrupt epigenetic phenomena is building rapidly.

In 2007, Smith *et al.* directly linked these intermediates to epigenetic enzymes, demonstrating conclusively that succinate addition in yeast can promote histone hypermethylation that is partially alleviated by 2-OG, suggesting that jmjC histone demethylases are modulated by succinate much like PHD enzymes (284). They further reproduced this hypermethylated outcome using yeast deficient in *jhd1*, a jmjC histone demethylase, or *sdh2*, a yeast component of SDH, providing exceptional support for the succinate-based inhibition of jmjC proteins (284). A similar phenomenon was seen in mammalian cells treated with the SDH inhibitor thenoyltrifluoroacetate (37). Cervera *et al.* (37) treated a panel of human cell lines with thenoyltrifluoroacetate, and utilized a complementary genetic knockdown of SDHD or SDHB to demonstrate enhanced histone methylation in both cases. These key studies collectively demonstrate that perturbations in succinate metabolism can regulate epigenetic processes in a broad spectrum of cells. The fact that this perturbation is conserved from yeast through mammalian cells also strongly suggests that metabolic regulation of epigenetic processes is not merely a byproduct of perturbations, but may in fact be a conserved mechanism for metabolism to communicate effectively with transcriptional events in the nucleus (Fig. 10).

Another of the critical intermediates in the CAC is 2-OG itself. Numerous anabolic pathways make use of this substrate to generate more complex biomolecules, and a similarly large number of catabolic pathways result in the production of 2-OG for use as mitochondrial fuel. Levels of this intermediate also may play significant roles in epigenetic processes, again through modulation of both jmjC and Tet1 2-OG and Fe(II)-dependent dioxygenases. An abundance of 2-OG secondary to anaplerotic pathways may drive the reaction in the forward direction, forcing a higher rate of epigenetic hydroxylation reactions. While this has not been conclusively shown as a driving force for epigenetic change *in vivo*, at least one study has illustrated that the addition of cell-permeable 2-OG derivatives in cell culture alters the activity of 2-OG and Fe(II)-dependent dioxygenases, suggesting this as a possible regulatory mode (190). This becomes a significant and relevant mechanism considering that 2-OG dehydrogenase, which metabolizes 2-OG in the CAC to produce succinyl CoA, is a tightly regulated enzyme currently thought to be associated with multiple disease states (304). Conversely, a reduction in the levels of 2-OG secondary to cataplerotic pathways would remove a critical substrate for jmjC or Tet1 functionality, limiting the rate at which histone demethylation or 5-meC hydroxylation could occur. Indeed, this was suggested as a potential mechanism for epigenetic derangements in cancer cells harboring specific isocitrate dehydrogenase (IDH) mutations that limit the production of 2-OG upstream in the CAC (350). In this study, the authors noted that there were significant reductions in 2-OG levels at steady state when the glioma-derived IDH mutation R132H was introduced into U-87MG glioma cell culture models. This was associated with increased HIF stability, mediated through a 2-OG and Fe(II)-dependent system analogous to that utilized by the aforementioned epigenetic enzymes (350). Later research indicated that the reduction in 2-OG amount was accompanied by an overproduction of the oncometabolite D-2-hydroxyglutarate (D-2-HG), which offers another explanation for why HIF stability and potentially epigenetic regulation is altered in cells bearing an IDH mutation (53).

1. 2-Hydroxyglutarate: oncometabolite or normal regulator? 2-HG is a particularly interesting metabolic intermediate that is centrally involved in the pathogenesis of several inherited inborn errors of metabolism. It has two stereoisomers, the D (or R) form and the L (or S) form, that differ in the orientation of the hydroxyl group on the 2-carbon of glutamic acid. Notably, there are multiple routes to these metabolites that occur under natural processes in addition to a number of detoxification pathways (Fig. 11). Disease results when there are imbalances in the production or removal of the 2-HG stereoisomers, leading to a change in steady-state levels of these metabolites. For example, the disease L-2-hydroxyglutaric aciduria, which causes severe nervous system pathology leading to mental retardation, results from a deficiency in the FAD-dependent enzyme L-2-HG dehydrogenase (263). Similarly, a deficiency in the enzyme D-2-HG dehydrogenase

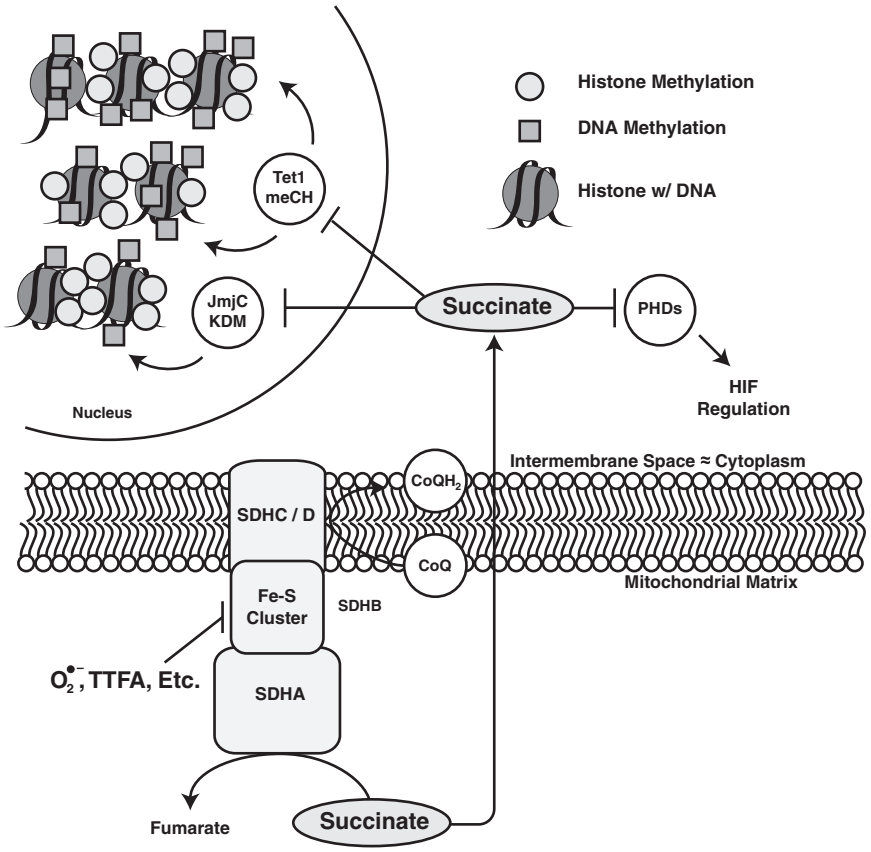

FIG. 10. SDH and regulation of dioxygenases. SDH is an inner-membrane-bound protein in the mitochondrion and its activity may be altered by a number of stressors such as thenoyltrifluoroacetate and superoxide. In this case, succinate buildup would occur, leading to increased succinate levels in the cytoplasm which could negatively impact the activity of multiple 2-OG and Fe(II)-dependent dioxygenases, including the jmjC histone demethylases, the Tet1 methylcytosine hydroxylase (MeCH), and the PHD HIF hydroxylases. Regulation of any of these processes could have potentially large effects on downstream gene expression. HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase domain; TTFA, thenoyltrifluoroacetate.

causes D-2-hydroxyglutaric aciduria, though the outcomes are substantially more pleiotrophic, ranging from migraine headaches to mental retardation (289). Significantly, many case studies have noted that there is an increased occurrence of central nervous system malignancy in patients with L-2-HG aciduria, suggesting a role for this metabolite in the pathogenesis of brain tumors (2, 106, 212, 232). This, coupled with the fact that identified IDH mutations promote an overproduction of D-2-HG and differential pathogenesis in glioma, strongly suggests that 2-HG needs to be comprehensively evaluated as a potential clinical target. Further support for this comes from recent studies identifying similar gain-of-function IDH mutations in acute myelogenous leukemia with concomitant 2-HG production, suggesting that this metabolic derangement may be common to the pathogenesis of multiple tumors (97, 101, 151, 192, 237).

The mechanism by which 2-HG may alter the activity of 2-OG and Fe(II) dependent dioxygenases is apparent when comparing the structural similarities of 2-HG and 2-OG. A vital driving force in the 2-OG-dependent hydroxylation reactions catalyzed by this superfamily is the elimination of CO_2 from 2-OG, yielding succinate. This step is critical for the generation of the Fe(IV)-oxo intermediate that eventually hydroxylates the target substrates of these enzymes. We hypothesize that the reduced 2-OG derivative 2-HG may hinder this process by disallowing appropriate catalysis, preventing the generation of the critical Fe(IV)-oxo intermediate. This mechanism would account for the increased HIF stability observed in glioma tissue samples in patients with 2-HG-producing IDH mutations, as PHD enzymes in the *EGLN* family would be inhibited by high levels of 2-HG (53, 350). Moreover, since the jmjC histone demethylases utilize a comparable mechanism, it is likely that these would additionally have altered activity. While epigenetic derangements in gliomas and leukemias harboring IDH mutations have not, to our knowledge, been explored extensively, these models may prove to be valuable starting points for studies of epigenetic derangement secondary to metabolic perturbation.

A potential secondary mechanism for 2-HG interference with signaling processes involves the generation of ROS; several studies from Latini *et al.* have suggested that both stereoisomers of 2-HG produce enhanced oxidative stress in rat brain as measured by enhanced lipid oxidation and protein carbonyl formation (167, 168). Oxidative stress itself can deregulate many epigenetic enzymes through both direct and indirect means; these include deregulation of the labile iron pool (LIP), derangement of glutathione (GSH) metabolism, and alteration of the NAD^+/NADH ratios, each of which will be discussed in greater detail below. This same group later demonstrated that 2-HG impairs mitochondrial energy metabolism, potentially through an uncoupling mechanism (166). Mitochondrial uncoupling would promote rapid electron flux through the electron transport complexes of the inner mitochondrial membrane, potentially speeding up the rate at which the CAC turns through a cycle due to rapid recycling of the electron shuttles NADH and NADPH. In the presence of a defective IDH, increased CAC turnover would lead to increased production of 2-HG, resulting in a potentially destructive positive feedback loop.

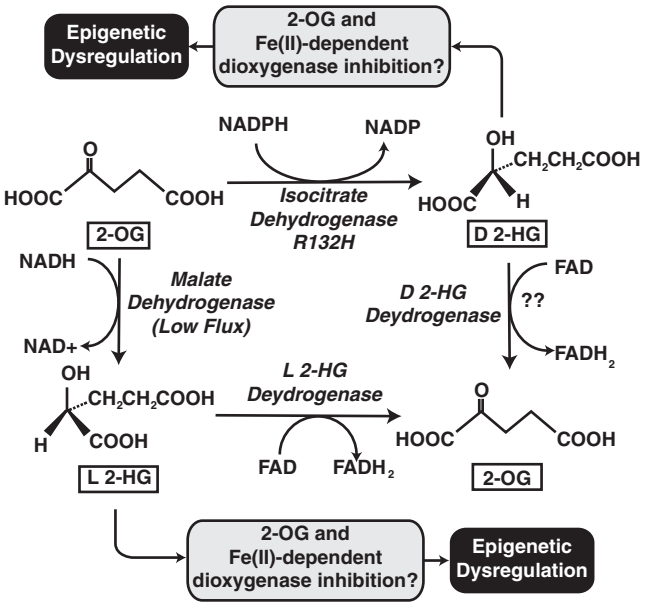

FIG. 11. 2-Hydroxyglutarate metabolism and potential epigenetic effects. L-2-HG is produced normally in low amounts by malate dehydrogenase in the mitochondrial matrix, and is normally metabolized back to 2-OG by the L-2-HG dehydrogenase. D-2-HG is an oncometabolite produced by the indicated isocitrate dehydrogenase mutation identified from malignant gliomas, and is also produced from various glyoxalate detoxification pathways (not shown). A D-2-HG dehydrogenase metabolizes D-2-HG back to 2-OG. When 2-HG production exceeds the capacity of the dehydrogenases to convert back to 2-OG, we hypothesize that 2-HG may interfere with the 2-OG and Fe(II)-dependent dioxygenases, thereby perturbing epigenetic modifications governed by jmjC and Tet1 proteins. This may also represent a normal signaling axis (for details, see text). L-2-HG, L-2-hydroxyglutarate.

Although the recent interest in 2-HG as a potential oncometabolite has focused needed attention on this important intermediate, there still is a dearth of research into the normal function of 2-HG in metabolic processes. The prevailing hypothesis among the groups that study this metabolite in hydroxyglutaric aciduria is that 2-HG represents an error of normal metabolism that is corrected by the appropriate 2-HG dehydrogenase enzymes (264, 289, 290, 314). The evidence for this comes from studies into the chemical origin of 2-HG in normal tissues; substrate labeling studies have indicated that the L-2-HG stereoisomer is derived from 2-OG that is aberrantly reduced by the downstream CAC enzyme malate dehydrogenase (25). While this suggests that 2-HG may be derived from an erroneous enzymatic process, the potential regulatory downstream effects on both epigenetic and metabolic processes that have been noted by other groups strongly suggest that there may be other roles for 2-HG. Given these new data, we hypothesize that the dynamic regulation of 2-HG may represent a critical locus for metabolic intermediates to communicate with higher order downstream regulatory processes through modulation of critical epigenetic enzymes (Fig. 11). In this model, 2-HG is not an erroneously generated metabolite, but instead a signaling molecule akin to fructose-2,6-bisphosphate (F-2,6-BP), a critical regulator of the glycolytic pathway. In this analogous system, small amounts of fructose 6-phosphate (F-6-P) are diverted from the central glycolytic pathway by the dual-function enzyme phosphofructokinase 2/F-2,6-BPase, which regulates levels of F-2,6-BP depending on other metabolic signaling inputs (225). F-2,6-BP is a critical metabolite that serves as an allosteric activator of phosphofructokinase 1, the key regulatory enzyme in glycolysis that commits glucose to energy production. 2-HG may function similarly by producing downstream effects secondary to flux in CAC substrate pools. For example, if 2-OG is abundant, 2-HG might similarly be abundant, leading to downstream changes through 2-HG effects on other enzyme systems. This would comprise a novel regulatory mechanism for central metabolism to tune gene transcription under normal circumstances. This construct would also help explain why the perturbation of this signaling pathway could lead to diverse pathologic consequences in different cell types; if 2-HG is involved in normal epigenetic signaling, imbalances in normal homeostatic mechanisms could have significant and immediate consequences on context-dependent cellular epigenetic mechanisms.

B. GSH and the recycling of SAM

GSH is a critical tripeptide that helps to maintain the intracellular environment in a reduced state. It accomplishes this through its ability to cycle between a reduced form (GSH) and an oxidized form, consisting of two GSH molecules linked by an intermolecular disulfide bridge (GSSG). The generation of GSSG from two GSH frees two vital electrons that participate in a variety of critical reduction reactions that occur in virtually all intracellular compartments. Additionally, a single GSH molecule may be conjugated to other substrates through the action of a family of GSH S-transferases, resulting in detoxification of harmful agents (60). To maintain GSH pools at the appropriate levels [which in most mammalian cells is on the order of 5 m*M* (201)], there are several main mechanisms. GSH reductase is an NADPH-dependent enzyme that recycles 2GSH from GSSG, closing the loop on the redox cycle (200). This is the major route for the replenishment of GSH in most cell types, and utilizes the majority of the NADPH generated by the oxidative branch of the pentose phosphate shunt of central metabolism (321). A secondary mechanism is through anaplerotic addition of substrates to the γ-glutamyl cycle, in which the glutamate residue of GSH is γ-linked to a free amino acid to facilitate transport across a cell membrane (Fig. 12) (256). Through a concerted series of reactions, GSH is analogous to a carrier molecule that gets replenished at the end of the cycle. Just as addition of substrates at key points in the CAC provides critical carbon skeletons for the generation of more complex biomolecules, an abundance of substrates required for the regeneration of GSH at the end of the cycle results in a net production of GSH. Importantly, the pull of intracellular demand for GSH can also play a large role in this case, diverting resources from other intersecting pathways to maintain a reducing environment in the cell. The consumption of these resources can have a critical impact on epigenetic maintenance.

The rate-limiting substrate for GSH biosynthesis is cysteine, which provides the critical sulfhydryl moiety (Fig. 12) (188). Local cysteine bioavailability strongly regulates the

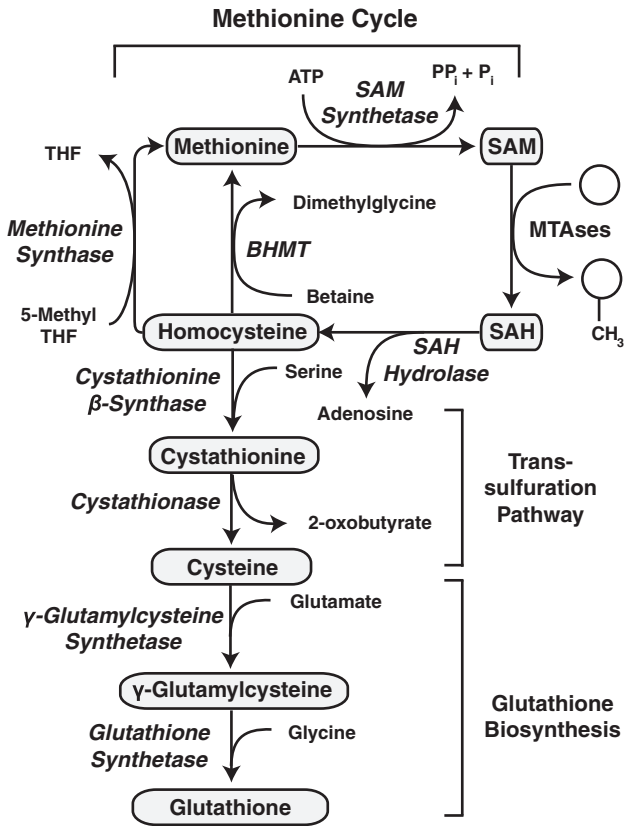

FIG. 12. Methionine cycle and glutathione biosynthesis. Methionine serves as the starting material for a variety of different biologically important compounds, including SAM, SAH, and cysteine. Under normal conditions, regular dietary intake of methionine offsets losses to the transsulfuration pathway. Under conditions of oxidative stress, when the demand for glutathione may exceed the capacity of the cell to produce it, there is a net reduction of the methionine pool, leading to an insufficiency of SAM and SAH that likely has significant effects on downstream epigenetic events. BHMT, betaine-homocysteine methyltransferase; MTase, generalized methyltransferase. SAH, *S*-adenosyl homocysteine.

production of GSH, and can be affected by multiple different elements, including dietary consumption and membrane transport system integrity (187). Critically, the linkage between pools of cysteine and GSH works in both directions. GSH is considered to be a reservoir for cysteine storage, as cysteine itself is unstable and readily auto-oxidizes to form the disulfide-linked amino acid cystine. Intracellular incorporation of cysteine into GSH maintains its redox status, and the cysteine in GSH can ultimately be freed for usage in proteins or other biological processes through the γ-glutamyl cycle (188, 200). Although the trafficking of cysteine is heavily regulated, it is not strictly an essential amino acid; *de novo* synthesis of cysteine can be accomplished by hepatocytes by consuming methionine in a process called transsulfuration (Fig. 12) (188). Methionine, which is an essential amino acid, is converted to cysteine through a multistep series of reactions that involves the intermediates SAM, SAH, homocysteine, and cystathionine. In the liver, a majority of dietary methionine is converted into SAM, which is a critical locus for one-carbon metabolism as the methyl donor for most known methyltransferase enzymes (188). This is especially important when considering epigenetic regulation, because SAM is required for methylation of DNA and histones by DNMT and HMTs, respectively. These reactions produce SAH, which is hydrolyzed to produce homocysteine and adenosine. Homocysteine represents a crossroads between methionine and cysteine metabolism. It may proceed through transsulfuration, or it may be recycled to methionine through betaine homocysteine methyltransferase (BHMT), which is expressed primarily in liver and kidney (293), or methionine synthase (MS), which is broadly expressed (188). This network of reactions provides a mechanism for communication between the critical methylation intermediate SAM and the production of GSH through cysteine metabolism, allowing the redox buffering status of the cell to have an influence on epigenetic regulation through modulation of SAM pools. Studies to date indicate that this is a significant mechanism for alteration of epigenetic marks.

When cells are exposed to prolonged conditions of oxidative stress, GSH pools can be significantly depleted. This occurs through both active cellular export of GSSG, which can mediate oxidation reactions, and through conjugation of GSH directly to intracellular molecules to protect from further oxidation (187). When NADPH becomes depleted in these situations, cells can no longer recycle GSSG to GSH, and so *de novo* GSH production becomes a primary mode of redox maintenance (188). As cysteine is vital for GSH synthesis, it is rapidly depleted under these conditions. A clinically relevant illustration of this phenomenon is acute acetaminophen overdose, which involves rapid depletion of GSH to detoxify the harmful acetaminophen metabolite *N*-acetyl-*p*-benzoquinone imine. Rapid treatment with the cysteine prodrug *N*-acetyl cysteine produces increased flux to GSH, providing a protective effect that stabilizes liver GSH (132). Without the addition of exogenous cysteine sources, the drainage of cysteine for GSH production produces a metabolic sink for methionine through the liver transsulfuration pathway. This results in well-established deficiencies in nonepigenetic methyltransferase reactions that are dependent on SAM as a cofactor (143), but also can produce significant alterations in epigenetic profiles. The Scimeca group explored this at multiple levels utilizing an animal model for GSH depletion. In a series of publications from the mid-1990s, they employed bromobenzene as a GSH-depleting toxin in Syrian hamsters, and examined several metabolic and epigenetic endpoints. They demonstrated that liver methionine pools were reduced by 12% in bromobenzene treated animals over control (173), and that this reduction was associated with both reduced thymidylate (the product of uracil transmethylation by folate-dependent thymidylate synthase) (174) and reduced cytosine methylation (175). Notably, these findings could be reversed with administration of labeled methionine, definitively establishing the linkage between methionine metabolism and GSH depletion in this system. To our knowledge, similar experiments have not been undertaken to examine linkages between histone methylation and GSH depletion, but we hypothesize that SAM depletion secondary to GSH reduction would similarly produce a hypomethlyated state through reduced activity of HMT enzymes. Should this prove to be a general mechanism, it would represent an additional and under-characterized pathway for redox biology to affect gene expression through epigenetic means. Given the alterations in DNA methylation patterns in cancer cells (discussed in detail

later) including genome wide demethylation, this model provides an attractive hypothesis to explain this phenomenon at least in part.

C. The NAD^+/NADH ratio

NAD^+ is an important cofactor for a number of pathways in cellular metabolism, and forms one half of the critical NAD^+/NADH redox couple. NAD^+ can be synthesized through two basic means: the *de novo* synthetic pathway, in which NAD^+ is ultimately derived from tryptophan in a complex series of reactions, and the salvage pathway, in which nicotinamide from catabolic processes is recycled back to NAD^+ (Fig. 13) (180). While both pathways have important demonstrable roles in the maintenance of NAD^+ levels, it appears clear that the salvage pathway is quantitatively more significant. This result comes from gene deletion studies in yeast that demonstrated no effect when a *de novo* NAD^+ synthesis enzyme was eliminated, whereas elimination of a salvage enzyme resulted in a 2.5-fold reduction in NAD^+ levels (269). After its synthesis NAD^+ is utilized in a large

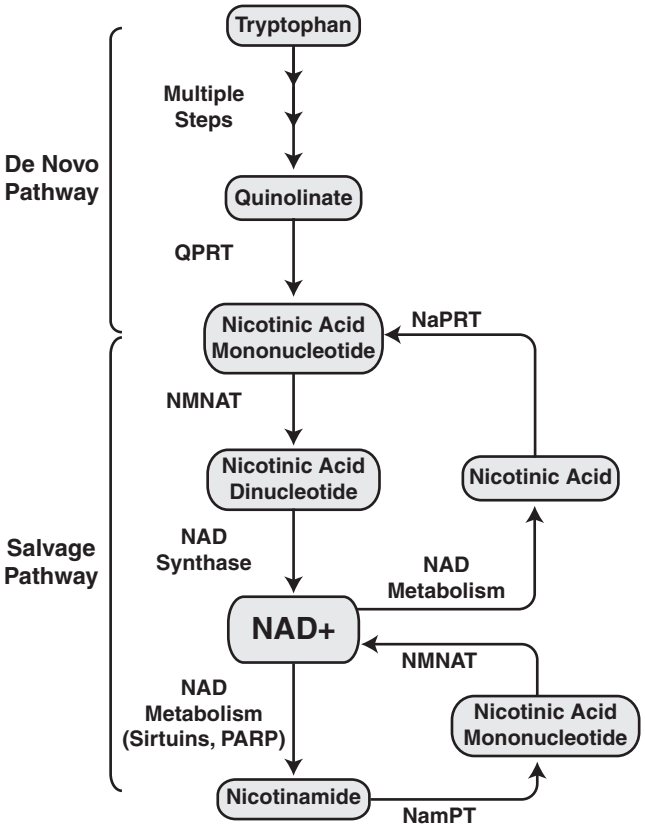

FIG. 13. NAD^+ biosynthesis and salvage pathways. NAD^+ is a critical metabolite for numerous metabolic processes, and both *de novo* biosynthesis and salvage of NAD^+ byproducts are important for maintaining overall pools of NAD. Importantly, both the sirtuins and PARPs consume NAD^+ and produce nicotinamide as a byproduct, which suggests that the salvage pathway is particularly important when considering the function of these enzymes. NaPRT, nicotinic acid phosphoribosyl transferase; NMNAT, nicotinic acid/nicotinamide mononucleotide adenylyltransferase; QPRT, quinolinate phosphoribosyl transferase.

number of enzymatic reactions throughout all cellular compartments. These can be divided into broad categories based upon the function of NAD^+ in these processes; here, we will refer to two main types of NAD^+-dependent reactions: those that involve redox cycling between NAD^+ and NADH, and those that physically consume NAD^+ for other purposes. The former category encompasses a large number of energy-producing reactions in central metabolism, whereas the latter includes the activities of multiple epigenetic enzymes that we have previously discussed, including the PARP family and the sirtuins. The common requirement of these functionally diverse processes for NAD^+ provides a potential regulatory linkage between central metabolism and epigenetic processes and *vice versa*.

1. The NAD^+/NADH ratio and central metabolism. In the main catabolic processes of central metabolism, NAD^+ provides a critical electron sink that drives the oxidation of fuel substrates. In glycolysis, this occurs through the action of glyceraldehyde-3-phosphate dehydrogenase, which is the sole oxidative step of the glycolytic pathway. In this reaction, glyceraldehyde-3-phosphate (Gly-3-P) is oxidized to 1,3-bisphosphoglycerate (1,3-BPG), producing NADH in the process. Similarly, in the CAC in the mitochondrial matrix, NAD^+ is used as an electron sink for the oxidation of multiple substrates, including isocitrate, 2-OG, and malate (Fig. 9). Normal cells have multiple mechanisms for metabolizing NADH back to NAD^+. In the cytosol, the action of lactate dehydrogenase reduces pyruvate to lactate and regenerates NAD^+. In the mitochondrion, the primary mode of recycling NADH to NAD^+ is through the NADH-Coenzyme Q oxidoreducatse (Complex I), which is the first major component of the electron transport chain. The utilization of NADH in this manner leads to the production of the vast majority of ATP in actively respiring cells. Reflecting this importance are the large number of cytosol-mitochondria shuttle mechanisms that transport electrons from cytosolic NADH to mitochondrial NAD^+, allowing cells to maximally harvest the potential energy stored in cytosolic NADH (180).

Given the importance of NAD^+ in the generation of energy through central metabolism, it is unsurprising that the NAD^+/NADH ratio is sensitive to diverse metabolic stimuli (208, 222, 223, 253). Indeed, this ratio is considered to be a surrogate output for respiratory metabolism: if the ratio is high, oxidative metabolism through the mitochondrial electron transport chain is likely high, whereas if the ratio is low, the converse is true. Measurement of this ratio can be accomplished through a number of different means (180). Measurement of the lactate/pyruvate ratio in the cytoplasm serves as an effective surrogate for the measurement of the NAD^+/NADH ratio, due to the action of lactate dehydrogenase (180). Using this method, recent data suggests that the cytoplasmic NAD^+/NADH ratio is $\sim$500–600 under normal conditions (346). Notably, this accounts only for *free* NAD^+ and NADH, and not for bound or otherwise sequestered pools, which collectively make up the vast majority of the intracellular NAD^+/NADH (180, 346). The measured values for the total NAD^+/NADH ratio vary from 3 to 10 in mammals, depending on the tissue type (180). These relatively low ratios indicate that variation in NAD^+ levels may serve as likely regulators of cellular function. In fact, this is precisely the mode of regulation for a variety of different central

metabolic processes. For example, whether glyceraldehyde-3-phosphate dehydrogenase proceeds in the oxidation or reduction direction depends heavily on the NAD^+/NADH ratio. It also regulates the entry of glycolytic products into the CAC, through modulation of pyruvate dehydrogenase activity, and multiple NAD^+ -utilizing enzymes in the CAC itself. However, recent research indicates that the regulatory role of the NAD^+/NADH ratio extends beyond the control of central catabolic metabolism. These theories focus on the contributions of the second broad group of NAD^+ utilizing enzymes—those that actually consume NAD^+ and degrade it.

2. Caloric restriction, the NAD^+/NADH ratio, and sirtuins. In recent years, caloric restriction (CR) has become a major focus of aging research. CR in organisms from yeast through mammals can demonstrably increase lifespan, and in mammals is the only known stimulus to promote this outcome (102). Although many of the specific mechanisms remain elusive, this process is dependent on the activity of sirtuin deacetylases (19, 128, 165, 179, 181). Given the sirtuin mechanism, it is unsurprising that CR-mediated lifespan extension is also dependent on availability of NAD^+ (102, 148, 179, 269, 347). Lin *et al* (181) demonstrated that CR was associated with enhanced oxidative respiration, and that lifespan effects were reproduced by genetic manipulations that promoted mitochondrial oxidative respiration. The authors postulated that this effect was due to enhanced recycling of NADH to NAD^+ by complex I, driving the NAD^+/NADH ratio up and providing essential substrates for sirtuin enzymes. Anderson *et al.* (6) further demonstrated that in the absence of CR, overexpression of the NAD^+ salvage enzyme nicotinate phosphoribosyltransferase 1 promoted longer lifespan in a yeast model. While this was not associated with increased amounts of NAD^+, it was associated with enhanced levels of sirtuin-dependent silencing. The authors reasoned that the increased flux of metabolites through the NAD^+ salvage pathway promoted sirtuin activity by increasing availability of substrate, if not overall amounts of substrate (6). Subsequent research demonstrating that efficient mitochondrial function, which promotes rapid oxidation of NADH to NAD^+, is also associated with enhanced lifespan has served to further cement the regulatory roles of NAD^+ in the CR pathway (241, 242, 248).

It is clear from these results that sirtuin-mediated effects on lifespan extension are heavily dependent on the availability of NAD^+. Since sirtuins are not able to utilize NADH, the NAD^+/NADH ratio plays a significant role in sirtuin functionality in these circumstances. If we step back and consider non-CR situations, we can generalize this type of metabolic control. Since the NAD^+/NADH ratio is a strong regulatory element in the control of central metabolic processes, and is capable of locally fluctuating to control specific enzyme activities under normal circumstances, it follows that sirtuins are likely also subject to the same metabolic regulation. This becomes a much more significant mode of regulation when considering that sirtuins, unlike many of the other characterized HDAC enzymes, have a considerably broader array of targets than just histones (102, 107, 116, 283). Although a discussion of nonhistone targets is outside of the scope of this review, multiple studies have demonstrated direct epigenetic signaling consequences of sirtuin activation. An exceptional example of this is the ribosomal DNA (rDNA) locus, which is under strict epigenetic control (216, 266, 269). In mammalian cells, SIRT1 is a key component of the energy-dependent nucleolar silencing complex, which mediates silencing of individual genes contained in the rDNA cluster (216). SIRT1 grants the complex NAD^+/NADH sensing capacity, allowing for metabolism-sensitive regulation of ribosomal biogenesis. As research in this field proceeds, it is likely that many more examples of sirtuin-based metabolic regulation of epigenetic loci will become apparent.

3. PARP, NAD^+, and sirtuin activity. Critically, the sirtuins are not the only nuclear proteins that consume NAD^+. As discussed above, the PARP family enzymes utilize NAD^+ to fix complex polymers of poly(ADP-ribose) to protein targets, including PARP1 itself and many of the core histone proteins. Like the sirtuins, PARPs also depend on local concentrations of NAD^+ for appropriate functionality, setting up a direct competition between PARP and sirtuin activities in nuclear settings. This is a particularly important relationship due to the generally opposed natures of sirtuin and PARP modifications to histones; whereas PARP is associated broadly with chromatin relaxation, histone acetylation, and minimal DNA methylation (110, 251), sirtuins are broadly associated with the removal of the permissive histone acetylation mark, promoting gene silencing and chromatin compaction (103) (Fig. 14). Supporting the importance of these competing activities, multiple groups have identified regulatory interactions between PARP and sirtuin enzymes. Pillai *et al*. (247) demonstrated that PARP-1 functionally depletes NAD^+ pools, attenuating nuclear sirtuin activity in a model of cardiac myocyte cell death in heart failure. In the extreme,

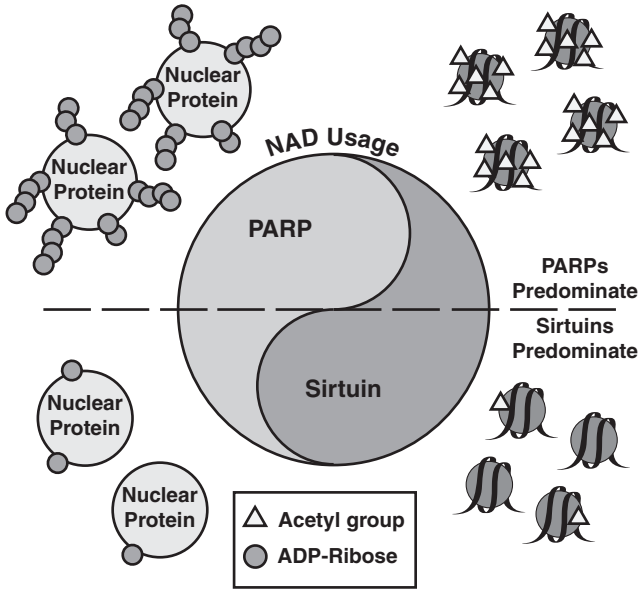

FIG. 14. Relationship between PARP and sirtuin enzymes in a local environment. As both PARPs and sirtuins consume NAD, the relative activity of either enzyme family affects the other within a local environment such as the nucleus. When PARPs are highly active, sirtuin activity is likely blunted, and vice-versa, suggesting a potential regulatory linkage that was initially proposed by Jie Zhang in 2003 (345).

PARP-mediated NAD^+ depletion may be considered a mechanism for "programmed cell necrosis" (109), but this finding illustrated that nonlethal functional depletion of NAD^+ can interact with sirtuin function in pathologic settings. Additional evidence of PARP-sirtuin regulatory axes comes from deletion studies. Kolthur-Seetharam *et al.* (150) found that SIRT1 negatively regulates PARP-1 activity, and that deletion of SIRT1 resulted in enhanced levels of poly (ADP-ribosyl)ation and subsequent apoptosis. Corroborating an interaction, El Ramy *et al.* (68a) showed that concomitant disruption of both PARP1 and SIRT1 in mouse rescued some of the abnormal chromatin phenotype seen with solely SIRT1 knockout (68a). This included a rescue of the nucleolar architecture, which harbors the SIRT1-controlled rDNA loci. These findings, though not demonstrative of a specific regulatory mechanism, definitively demonstrate an interaction between PARP and sirtuin enzymes. Collectively, the data to date support a model for concerted coregulation of both PARP and sirtuin family members through local modification of the NAD^+/NADH ratio (Fig. 14), which was initially proposed by Zhang in 2003 (345). While the model we illustrate here is grossly simplified, the usage of NAD^+ as a substrate by both sirtuin and PARP enzymes in the same cellular localization establishes a competition that likely has far-reaching regulatory ramifications that requires further extensive characterization in both physiologic and pathologic conditions.

4. Plasma membrane redox system and NAD^+. Under conditions of mitochondrial dysregulation or other oxidative stress, cells are capable of compensating for disrupted NAD^+/NADH ratios through the action of the plasma membrane redox system (PMRS). This is a multicomponent system that allows the transfer of electrons from intracellular sources to the extracellular milieu, supporting an appropriate redox balance inside the cell. Significant research has illustrated that these systems play a role in both CR-mediated lifespan extension and sirtuin activity, but the mechanistic description of these elements is beyond the scope of this review. For discussion of the PMRS and the methods by which it can interact with redox epigenetics, please see the review by Hyun *et al*. (125).

D. Maintenance of the intracellular iron redox status and epigenetic enzymes

Iron plays a critical role in many biological processes due to its intrinsic utility in redox reactions. It readily cycles between the Fe(II) and Fe(III) redox states, and in some enzymatic contexts, can exist as the rare Fe(IV) in transition states. These potential states are harnessed by diverse protein systems throughout essentially all life to drive diverse metabolic processes ranging from the detoxification of harmful radical species to the sequestration of oxygen from other enzymatic systems. However, the same properties that render iron particularly useful for biochemical processes also make it an exceptionally dangerous species when its regulation is disturbed. Free iron is of particular importance, because it participates in Fenton/Haber-Weiss chemistry that results in the production of the immensely toxic hydroxyl radical (Equations 1–3). In the Fenton reaction, free Fe(II) reacts with H_2O_2, producing Fe(III), hydroxyl radical ($^{\bullet}OH$), and hydroxide ion (OH^-) [1]. Fe(II) may be regenerated when Fe(III) accepts an electron from $O_2^{\bullet -}$ [2], completing the iron redox cycle. The net reaction is termed the Haber-Weiss reaction [3].

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^- \quad [1]$$

$$Fe(III) + O_2^{\bullet -} \rightarrow Fe(II) + O_2 \quad [2]$$

$$H_2O_2 + O_2^{\bullet -} \rightarrow {}^{\bullet}OH + OH^- + O_2 \quad [3]$$

If intracellular iron were allowed to participate in these reactions, the resultant ROS production would be disastrous for metabolic processes. To mitigate the threat of iron-related ROS production from Haber-Weiss chemistry while simultaneously harnessing its properties for favorable outcomes, several regulatory mechanisms tightly control the availability of iron for biological processes. This network is remarkably complex, and a thorough discussion of the details is beyond the scope of this review—for several succinct examinations of mammalian iron homeostasis, see reviews by Zhang and Enns (344), Salahudeen and Bruick (265), and Theil and Goss (301). Here, we will focus primarily on subjects in iron metabolism that have direct implications for epigenetic enzymes. These include the LIP, the incorporation of iron into iron–sulfur cluster proteins, and the direct utilization of iron as a cofactor by epigenetic enzymes.

1. Labile iron and oxidative stress. The LIP is defined broadly as a low-molecular weight pool of iron, likely both in ionic forms and weakly bound to intracellular ligands. Importantly, any single iron atom only transiently exists in the LIP—this rapid passage helps to regulate the availability of these species for extraneous reactions. LIP only comprises 3%–5% of the total cellular iron, but in recent years it has been studied extensively for its potential regulatory actions in iron homeostasis (157). This has been a controversial topic; as discussed above, free iron may participate in deleterious ROS producing Haber-Weiss chemistry, and so the existence of an LIP strongly suggests that cells will have to be significantly invested in antioxidant defenses. The iron that comprises the LIP comes from both extracellular and intracellular sources. The extracellular iron fraction is imported through two main mechanisms: iron that is chelated to transferrin for plasma transport is internalized through interactions with the transferrin receptor on the cell surface, whereas free iron ions may be imported through the actions of the divalent metal transporter family of membrane proteins (157). Intracellular sources of iron include the iron sequestered in heme prosthetic groups of diverse proteins as well as nonheme iron incorporated into a number of other distinct protein families. If we consider the extracellular and intracellular iron sources as separate entities, we can envision the LIP as a communication between the two (Fig. 15). This is an important concept, because for adequate homeostasis iron must flow reversibly between multiple sources depending on the local need. An excellent example of this is the molecule ferritin, which can either diminish or replenish the LIP depending on the physiologic circumstances.

A single ferritin molecule can sequester on the order of 4500 Fe(III) atoms, which has significant ramifications for the LIP. Picard *et al.* (246) demonstrated that overexpression of the ferritin heavy chain in murine erythroleukemia cells reduced the LIP almost threefold, effectively demonstrating that ferritin levels could realistically modulate the LIP. Cozzi *et al.*

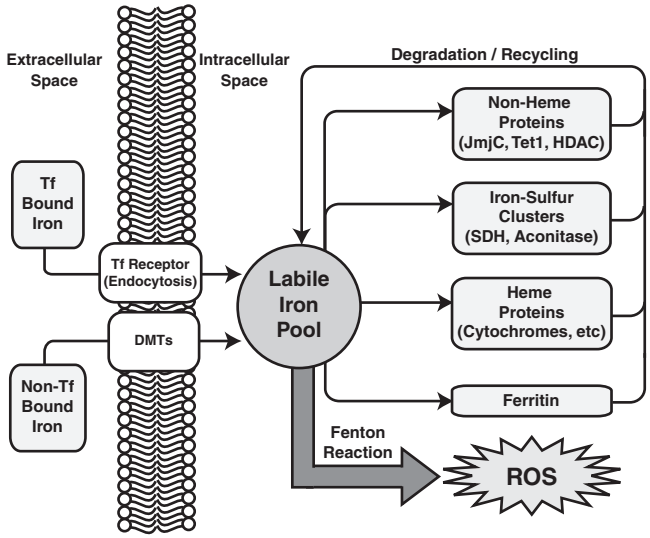

FIG. 15. The labile iron pool and iron homeostasis. Before being utilized in intracellular processes, iron that is imported into the cell passes through the labile iron pool. This transient and normally low-concentration pool is a critical player in redox homeostasis, because labile iron may participate in harmful Haber-Weiss and Fenton reactions, leading to ROS production. Consequently, cells have strict regulatory controls on labile iron to ensure that it does not damage local processes. Tf, transferrin; DMT, divalent metal transporter.

(50) extended this finding to functional oxidative stress endpoints, demonstrating that ferritin heavy chain overexpression in HeLa cells granted enhanced resistance to H_2O_2 toxicity, presumably through removal of redox-active iron that could participate in Haber-Weiss chemistry. Despite the LIP-lowering effects seen in these studies, others have demonstrated that ferritin may also serve as a source for LIP depending on the context. Meyron-Holtz *et al.* (204) demonstrated that extracellular iron-loaded ferritin is taken into cells *via* ferritin receptor, followed by rapid degradation of the endosomal ferritin and an increase in the LIP. Corroborating the ferritin-as-LIP source idea, Konijn *et al.* (152) found that blocking the degradation of ferritin with protease inhibitors attenuated the reestablishment of the LIP in LIP-depleted cells. Collectively, these results indicate the important role of ferritin in the maintenance of the LIP. Through this and other regulatory strategies, cells are able to maintain tight control over the amount of free iron that is available for incorporation into critical cellular processes while limiting the amount of iron that is available for harmful Haber-Weiss processes.

Recent data hint at how inappropriate regulation of the LIP may have a direct role on genetic and epigenetic modifications through the production of hydroxyl radicals. Kawai *et al.* (142) showed that methionine sulfoxide reacts with $^{\bullet}OH$ to produce methyl radicals that ultimately nonenzymatically methylate cytosine and guanine residues in DNA. This is an intriguing result for multiple reasons. Methionine sulfoxide is a result of oxidative damage to methionine residues in proteins, and is normally mitigated through the action of a family of selenium-containing methionine sulfoxide reductases (MSRs) (170). Notably, aberrant expression or mutation of MSR genes has been found in association with cancer (17, 56), and at least one group has hypothesized that an MSR gene on chromosome 8 is a potential metastasis suppressor gene (172). The finding that methionine sulfoxide may promote aberrant DNA hypermethylation through chemical means suggests a potential mechanism for its putative tumor suppressive activity. Iron homeostasis would play a significant role in the development of this hypermethylation state, as $^{\bullet}OH$ radicals were essential for the chemical production of 5-meC in the experimental system. If the LIP were increased by aberrant regulation of iron homeostasis proteins, the potential increase in $^{\bullet}OH$ radical production could mediate the formation of methyl radicals in the presence of methionine sulfoxide, leading to potential DNA hypermethylation in addition to other DNA lesions, including strand breaks. This represents a tantalizing mechanism for aberrant DNA methylation in disease processes that are associated with LIP dysregulation, and more work needs to be done to explore this exciting potential.

Another important way that LIP can directly affect epigenetic modification is through the availability of iron required for the activity of diverse epigenetic enzymes, including jmjC demethylases and the putative Tet1 DNA demethylase. We hypothesize that a reduction in the LIP could potentially have direct suppressive effects on these enzyme activities, promoting aberrant epigenetic modification in an iron-depleted status. Although this represents a potentially tractable hypothesis, it would be exceptionally difficult to directly assay experimentally secondary to the myriad other effects that a reduction in the LIP would certainly exert on the cell. For example, a reduction in the iron available for iron–sulfur cluster assembly in critical metabolic proteins, which we discuss below, also would have potentially measurable effects on the cellular epigenetic status. As assays for measuring the LIP and downstream effects of LIP modulation are developed, the direct influence of iron pools on epigenetic enzyme activities may become more readily measureable. Until then, *in vitro* assays under different iron conditions may be the only experimentally feasible approach to address this hypothesis.

2. Iron–sulfur center proteins and epigenetic modification. While the LIP dictates what iron is available for intracellular processes, the status of iron-containing proteins themselves also plays a role in the redox regulation of epigenetic processes. In particular, we will focus on the iron–sulfur center proteins, which play major roles in numerous metabolic processes, including redox metabolism, DNA synthesis, and transcription (334). In eukaryotes, the assembly of iron–sulfur centers in proteins is remarkably complex, requiring over 20 known proteins. Notably, mutations or other dysregulation of many of these cluster assembly genes are associated with the pathogenesis of a number of human diseases, highlighting the importance of iron–sulfur proteins for normal cellular function [reviewed in ref. (334)]. As one might surmise from Haber-Weiss chemistry, iron–sulfur proteins are particularly susceptible to oxidative damage secondary to the redox cycling capacity of iron atoms. Multiple pro-oxidant species can interact with the iron atoms in these clusters, including $O_2^{\bullet-}$ and peroxynitrite ($ONOO^-$), promoting the destruction of the iron–sulfur center and inactivation of the associated enzyme activity (34, 77, 88, 89, 111). This phenomenon has been most well studied among aconitases, likely due to their important role in the maintenance of iron homeostasis. Cytoplasmic aconitase is also called iron regulatory protein 1 (IRP1). Apo-IRP1, without an active

iron–sulfur cluster, is an RNA-binding protein that recognizes iron response elements (IREs) in the mRNA transcripts of many iron-responsive genes. Apo-IRP1 can then mediate the degradation or stabilization of these transcripts in a context-dependent fashion [reviewed in ref. (214)]. Formation of an active iron–sulfur cluster in IRP1 occurs when the LIP supports cluster assembly, and effectively abrogates its RNA binding capacity. Collectively, this allows the LIP to directly regulate the translation of various iron responsive genes concomitant with changes in the cytoplasmic free iron concentration. Moreover, this regulatory network can be disrupted in the presence of pro-oxidants through destruction of the iron–sulfur centers in IRP1 (215). This system thus provides a canonical model for the interaction of oxidative stress with iron–sulfur proteins that may potentially be applied elsewhere. One potential analogous system is SDH.

As discussed above, SDH has recently proven to be a key player in epigenetic events due to succinate inhibition of critical epigenetic 2-OG- and Fe(II)-dependent dioxygenases. The three iron–sulfur centers of SDH are part of the SDHB subunit, and they ferry electrons garnered from the oxidation of succinate to promote the reduction of coenzyme Q in the mitochondrial inner membrane (1). While strictly speaking, these centers are not directly involved in the catalytic oxidation of succinate, they are required for overall enzyme functionality because they maintain the oxidized status of the flavin at the catalytic center of SDHA (1). The iron–sulfur centers for SDH are thus a necessary component for appropriate functionality. These centers also render SDH potentially susceptible to oxidative inactivation through redox modulation of the iron atoms. Numerous studies have illustrated that pro-oxidant species such as $O_2^{\bullet -}$ can significantly reduce SDH activity both *in vitro* and *in vivo*, particularly under the noted oxidative stress after hypoxia-reperfusion (250, 348). Notably, the level of inhibition in SDH is substantially less than that of aconitase, which is by far the most pro-oxidant susceptible CAC enzyme (250). Indeed, Pearce *et al.* (243) showed that SDH is relatively resistant to $ONOO^-$-mediated oxidative damage, suggesting that SDH has a more robust iron–sulfur center construction than aconitase. Despite this relative stability in the presence of oxidative stressors, inactivation of SDH secondary to oxidative damage to its iron–sulfur centers may have significant epigenetic ramifications due to downstream effects of succinate-based inhibition of epigenetic enzymes. This mode of redox regulation provides an additional mechanism for metabolic stress to communicate with epigenetic processes—any perturbations that affect the status of SDH could also directly affect the epigenetic status through modification of succinate-sensitive epigenetic enzyme activity, as illustrated in Figure 10.

3. Direct interaction with epigenetic enzyme iron loading. While the LIP dictates the availability of iron as a cofactor for the numerous iron-requiring epigenetic enzymes, there are a number of other factors that can interfere with appropriate iron loading. This has been demonstrated extensively in studies of the HIF signaling axis: many exogenously introduced metals, including cobalt and nickel, produce a "pseudo-hypoxic" phenotype that mimics gene expression patterns seen under true hypoxic conditions (22, 267, 268). There are several postulated mechanisms for this, including that these metals may interfere with adequate maintenance of the LIP through interaction with divalent metal transporter-family proteins (48). An additional hypothesis that is broadly accepted is that these metals may be competing with iron in the active site of the PHD family proteins, leading to inappropriate catalytic function (48, 197). This would promote the stabilization of HIF protein, ultimately allowing it to transcriptionally regulate its target genes. As we have discussed extensively above, the jmjC and Tet1 epigenetic enzymes belong to the same 2-OG and Fe(II)-dependent dioxygenase superfamily as the PHD enzymes, suggesting that they may be susceptible to similar modes of inhibition through metal ions. Indeed, this has proven to be the case in a number of studies focusing on the effects of nickel and cobalt on epigenetic regulation.

Yan *et al.* (332), of the Costa group, initially showed that in some clones of the nickel-treated hamster cell line G12, silencing of the *gpt* transgene was associated with enhanced H3K9me2 signal as measured by chromatin immunoprecipitation (ChIP). The Costa group had previously established that gene silencing through nickel was associated with enhanced histone deacetylation (24), but results from the 2003 study could not be explained solely on the basis of the acetylation of histones or the methylation of DNA, as the clones remained silenced after treatment with both the HDAC inhibitor trichostatin A and the DNA methyltransferase inhibitor 5-aza-deoxycytidine (332). Critically, the jmjC proteins had not at that time been identified as histone demethylases that were sensitive to iron modulation, but reanalysis of these data suggests that they may have played a role in the nickel silencing process. Indeed, a later Costa group publication confirmed this notion through extensive analysis of histone methylation status in the nickel-treated hamster cells. Chen *et al.* (41) established that histone methylation was indeed a key player in the nickel-mediated silencing of the *gpt* transgene, and that it proceeded through a 2-OG- and Fe(II)-dependent dioxygenase mechanism. Notably, this work was accomplished concomitant with the independent identification of jmjC proteins as 2-OG and Fe(II)-dependent histone demethylases, and so represented a critical finding that supported their existence. Importantly, Chen *et al.* additionally demonstrated that the activity of the putative demethylases was nickel-inhibitable through an activity assay, which strongly supported the hypothesis that nickel was competing with iron for loading into the responsible enzyme (41). Later work by the Costa group established that treatment with both cobalt and chromium compounds could recapitulate similar results through inhibition of the jmjC demethylases, though specific modes of inactivation varied among the three metals. The methylation alterations secondary to chromate treatment, for example, could be reversed by pretreatment with ascorbate, which is a critical factor that maintains iron in the Fe(II) redox state. This suggested that chromate may be depleting ascorbate pools rather than directly competing with iron for loading into jmjC proteins, but this would ultimately have a similar effect on histone methylation status (292). In contrast, experiments with cobalt chloride in a controlled experimental system indicated that cobalt likely competed with iron for loading into jmjC proteins in a fashion similar to nickel (177). Studies have recently implicated that arsenite has as another potential player for modulation histone methylation, though the specific mechanism remains unknown and is likely complex (351). The Futscher group has been active in this field

through a series of studies investigating exposure to arsenic-containing compounds. Their findings illustrate that arsenic promotes a broad, gradual, and lasting perturbation in DNA methylation patterns that contributes to arsenic-mediated carcinogenesis in urothelial cells (135, 136, 329). Collectively, these results strongly suggest that the direct perturbation of the iron loaded into epigenetic enzymes is a strong stimulus for the modification of cellular epigenetic status. While this is notably different than the other endogenous metabolic processes discussed above, they are, nevertheless, important for consideration of toxicology and the relationship of environmental stimuli to epigenetic events. They also provide evidence for another potential mechanism for the redox regulation of the Fe(II)-dependent epigenetic enzymes.

4. Ascorbate and 2-OG and Fe(II)-dependent dioxygenases. As mentioned above, one of the potential mechanisms for chromate to interfere with the epigenetic regulation is through the depletion of ascorbate, which is an essential cofactor that maintains iron in the appropriate Fe(II) redox state for catalysis. This suggests that global changes that modulate ascorbate may also serve to significantly change the epigenetic landscape. The importance of ascorbate for PHD enzyme activity is well established (76, 118, 149, 199, 235, 318). Given the numerous parallels between the regulation of both PHD and jmjC function that have proven to be experimentally valid, the exploration of ascorbate and its effects on the regulation of the histone demethylases would be scientifically prudent, especially given how ascorbate levels can vary widely among both cell types and anatomic regions under physiologic conditions. Plasma ascorbate ranges from 10 to 160 μM nominally, and concentrations up to 100-fold greater than that can be found in metabolically active tissues such as adrenal gland, pituitary gland, and some specific classes of neuron (113). While the rationale for having increased ascorbate in these tissues may be secondary to the normal oxidative stress that these tissues might be subjected to (*e.g* adrenal glands oxidatively produce the stress hormones epinephrine and norepinephrine), the relationship between these tissues and frequency of epigenetic aberrations should be extensively explored. Cross-referencing the information about ascorbate concentration and tissues that are preferentially targeted by CAC enzyme perturbations would be an excellent starting point in this regard. SDH mutations lead preferentially to a pheochromocytoma-producing syndrome affecting chromaffin cells of the adrenal gland, whereas IDH mutations and the subsequent overproduction of 2-HG predisposes to neurological malignancy. We would hypothesize that these tissues, which normally have extremely high levels of ascorbate, may be synergistically affected by ascorbate depletion in the presence of CAC enzyme mutations. This would, in essence, provide a "two-hit" mechanism for the development of disease, but the critical second hit would be the metabolic hit of ascorbate reduction rather than a genetic one. In this situation, the epigenetic systems already stressed by aberrant overproduction of CAC intermediates might be tipped toward the breaking point by the removal of ascorbate to maintain the ferrous iron in the remaining functional enzymes. Research into ascorbate-mediated control of the PHD enzymes strongly suggests that this may play a role in epigenetic enzyme regulation. Given the increasing interest in high dose ascorbate supplementation as a potential adjuvant agent for chemotherapeutics [including refs. (42, 43, 51, 59, 176, 205, 272, 303), reviewed in ref. (224)], research into potential epigenetic effects could lead to significant developments in disease therapy.

5. Nitric oxide and iron. Nitric oxide (NO) is a powerful transient signaling molecule that is employed in multiple cellular processes. It is remarkably reactive due to an unpaired electron, and can thus interfere with many redox-sensitive processes, including epigenetic regulatory axes, throughout the cell. One of the means by which this takes place is through NO-mediated modulation of both iron status and 2-OG and Fe(II)-dependent dioxygenase enzymes (349). Studies examining the effects of NO on PHD enzyme functionality in the HIF pathway have demonstrated exciting results that could potentially extend to epigenetic processes. Briefly, NO is known to act as a "pseudo-hypoxic" stimulus, in that it promotes the stabilization of HIF protein through inactivation of PHD enzymes under normoxic conditions (13, 14, 203). It is hypothesized that this inactivation is at least in part secondary to NO interference with the iron redox state at the PHD active site, effectively blocking hydroxylation of HIF. However, under other pseudo-hypoxic conditions when NO is given, the opposite effect is seen: where HIF would normally be stabilized due to iron removal through desferrioxamine, NO promotes the proteolytic degradation of HIF (30). These seemingly contradictory outcomes of NO exposure may be explained by NO interaction with iron: in low iron states (with desferrioxamine), NO may liberate enough iron into the LIP through other means to promote PHD activity, whereas with higher LIP, NO may interact preferentially with iron in PHD active sites to suppress activity. While this construct does not explicitly describe an epigenetic system, it is strongly suggestive of mechanisms for NO-mediated interference with epigenetic 2-OG and Fe(II)-dependent dioxygenases. This could be an especially important mode of regulation in the cardiovascular system, as NO is a potent vasodilator that is critical for maintaining appropriate vascular tone.

E. Redox regulation and noncoding RNA

As introduced above, ncRNAs have significant interactions with epigenetic machinery at multiple regulatory levels, influencing both the relative chromatin status of focal genetic loci and the expression of epigenetic enzymes themselves. Critically, more research is implicating redox processes and metabolic phenomena in the regulation of ncRNAs, suggesting an additional mechanism that altered metabolism can interfere with epigenetic processes. Recent research suggests that ROS themselves play a role in regulating Dicer, a critical intermediate enzyme that processes precursor ncRNAs into mature effector molecules. Wiesen and Tomasi (2009) demonstrated that addition of H_2O_2 to cell culture models could significantly reduce Dicer protein levels, suggesting that oxidative stress may alter the profiles of processed ncRNAs (327). Data from multiple groups working individual miRNAs and their response to oxidative stress support the relationship between redox stressors and miRNA expression, though specific mechanisms remain unclear. For example, miR-9 is activated by the ROS-generating *N*-(4-hydroxyphenyl)-retinamide in human retinal pigment epithelial cells (160),

miR-210 is induced by the HIF-pathway in response to hypoxia (44, 71, 158), miR-21 is induced by H_2O_2 in cardiac myocytes and vascular cells (45, 182), and miR-9, miR-125b, and miR-128 are induced by ROS-producing metal sulfates in human brain cells (189). Collectively, this evidence strongly suggests that oxidative stress and redox processes play a definite role in the regulation of endogenous ncRNAs. Given the numerous ways in which ncRNAs may affect epigenetic processes, these data provide another concrete mechanism for redox changes to modulate epigenetic phenomena.

In addition to the direct means that ncRNAs may utilize to promote epigenetic alterations in oxidatively stressed cells, several ncRNAs are known to modulate critical metabolic pathways that also have strong potential to impact epigenetic regulation. One such pathway involves the regulation of iron–sulfur clusters in mitochondria. MiR-210, which is regulated by hypoxia through the HIF axis, strongly targets transcripts of the iron–sulfur cluster scaffold protein (ISCU) (44, 71). ISCU is a component of the complex pathway leading to iron–sulfur cluster biogenesis, and a reduction in ISCU protein results in decreased assembly of functional iron–sulfur clusters as measured by decreases in both aconitase and complex I activity in cell culture models (71). Dysregulation of these proteins has a significant negative effect on mitochondrial metabolism, and enhances both ROS production and glycolytic flux. The authors further note that cancer cells frequently overexpress miR-210, and an analysis of breast cancer patients grouped by ISCU expression status revealed that patients with below-median expression of ISCU experienced significantly higher rates of relapse when compared to patients expressing relatively high ISCU (71). Although the authors made no specific note of epigenetic phenomena in their model system, the data are, nevertheless, remarkably compelling in light of the issues discussed above. It definitively illustrated how iron–sulfur cluster biogenesis could be altered in response to ncRNA stimulus, with far-reaching effects on the production of both ROS and mitochondrial metabolic intermediates. As described above, these factors both play significant roles in the regulation of epigenetic enzymes, through both direct and indirect means. The potential for epigenetic dysregulation secondary to these phenomena is thus substantial, and miR-210 manipulation may prove to be a significant tool for both research and treatment in various disease states.

An additional example of means by which ncRNAs may interact with metabolic pathways is the proline oxidase (POX) regulatory axis. POX is an inner mitochondrial membrane-bound enzyme that oxidizes free proline to the metabolite Δ^1-pyrroline-5-carboxylic acid (P5C), resulting in the donation of electrons to cytochrome c in the ETC (245). P5C is then exported to the cytosol, where it can be reduced by P5C reductase in an NADPH-dependent manner, recycling proline and thus completing the proline cycle. Overall, this serves two main purposes: (a) to transport reducing equivalents from the cytosol to the mitochondrion to generate ATP, and (b) to modulate the production of mitochondrial ROS for further downstream signaling events. The former purpose likely does not play a significant role in overall energy metabolism, but the latter may have significant impact on cell biology. POX is known to be p53 inducible, and multiple groups have shown that it has putative tumor suppressor function through significant pro-apoptotic activity in tumor cells in an ROS-dependent fashion (64, 185, 186, 198, 236, 257). Critically, POX is a target of miR-23B*, which is commonly overexpressed in renal cancer (184). Liu *et al.* (184) demonstrated that ectopic overexpression of miR-23B* could reduce immunoreactive POX, and demonstrated a significant negative correlation between miR-23B* levels and POX expression in human tumor samples. As with miR-210 and its regulation of the ISCU protein, the impact of this ncRNA-mediated mitochondrial perturbation on epigenetic status is potentially large. POX may interfere with multiple mitochondrial processes through regulation of local ROS levels, and many of these have demonstrable linkages to epigenetic phenomena. While the linkages between miR-23B* and POX are relatively newly established, we anticipate that further study would likely reveal significant epigenetic perturbations secondary to POX-mediated metabolic disruption.

F. Direct modulation of HDAC activity by ROS

HDAC enzymes require fewer cofactors to exert their epigenetic effects than many of the other histone modifying enzymes such as the 2-OG and Fe(II)-dependent dioxygenases or the sirtuins. This renders HDACs relatively insensitive to the metabolic fluxes that may have significant direct effects for other epigenetic enzymes. However, recent data illustrate that HDACs may be directly modulated by oxidative damage, limiting their activity under conditions of oxidative stress. Ito *et al.* (131) demonstrated that H_2O_2 administration induced tyrosine nitration of HDAC2, which correlated with a significant increase in acetylated H4 at the IL-8 promoter. Strikingly, this increase was comparable to the acetylation induced by trichostatin A under similar conditions, suggesting that H_2O_2 may be a potent modifier of HDAC2 activity (131). The Rahman laboratory has elaborated on these findings extensively utilizing additional experimental models. Moodie *et al.* (209) extended the studies to illustrate that cigarette smoke could inhibit HDAC activity both in cell cultures and utilizing *in vitro* activity assays. Yang *et al.* (333) further demonstrated that cigarette smoke induces both tyrosine nitration and aldehyde-adduct formation on HDAC1, HDAC2, and HDAC3. More recently, Doyle and Fitzpatrick (65) examined the kinetic effects of cysteine modulation and alkylation of HDAC1–3, characterizing how these redox modifications specifically alter redox activity. Ultimately, these data provide compelling evidence that HDAC enzymes are directly affected by redox signaling pathways. This mode of regulation could have significant downstream effects on global epigenetic phenomena, especially when one considers the strong likelihood that other epigenetic enzymes will be negatively affected as well.

G. Oxygen tension and epigenetic phenomena

Many of the enzymes we have detailed above require oxygen as a substrate for appropriate activity, including the jmjC histone demethylases and the putative Tet1 DNA demethylase. Oxygen availability is thus a critical determinant for epigenetic processes involving these pathways, as it is with the PHD-HIF axis. We acknowledge the implicit importance of this pathway, and our group has previously examined this at greater length. For a more detailed discussion of how oxygen tension may play a role in modulating the epigenetic regulation of gene expression, in terms of both

developmental programming and in cancer, please refer to Hitchler and Domann (119, 120).

V. Toward a Global Model for Redox Epigenetic Maintenance

In the preceding sections, we addressed several key epigenetic modifications and the importance of redox processes for their appropriate maintenance. Importantly, the redox events described here are not insulated from one another; indeed, there are strong interactions between the individual processes that contribute to an overall redox environment within a cell. This is an important concept considering our initial definition of epigenetics as a broad mechanism for the transduction of both genetic and nongenetic events. It also implies that isolated redox perturbations can have significantly broader consequences on epigenetic enzyme functionality *in vivo*, as the ultimate signal that reaches an epigenetic enzyme in a cellular environment may be dictated by multiple redox stimuli that originated from an original insult. For example, if we consider a situation in which a cell is experiencing prolonged oxidative stress, we might imagine that multiple processes are taking place (Fig. 16). One consequence might be a depletion of the cellular GSH pool, which would perturb cysteine and methionine metabolism, leading to a reduction in the SAM available for critical methyltransferase reactions. Under these same conditions, we might expect that other antioxidants may be depleted as well. The depletion of ascorbate in this manner would be associated with aberrant iron redox metabolism both at the level of the epigenetic enzymes themselves, and in the maintenance of the LIP through the action of the IRP regulatory axis. This would limit the effectiveness of iron-requiring epigenetic enzymes. Moreover, oxidative stress could potentially affect the enzymes of the CAC, leading to aberrant concentrations of critical intermediates that also have direct effects on epigenetic enzymes. In such situations, the epigenetic maintenance systems in cells may be affected on multiple levels, and the overall redox environment would contribute to epigenetic instability. Clearly, this would represent a complex network of redox dysregulation, and in such prolonged cases of oxidative stress, cellular viability might be compromised long before epigenetic processes are irreversibly damaged. However, the modes of redox interactions with epigenetic

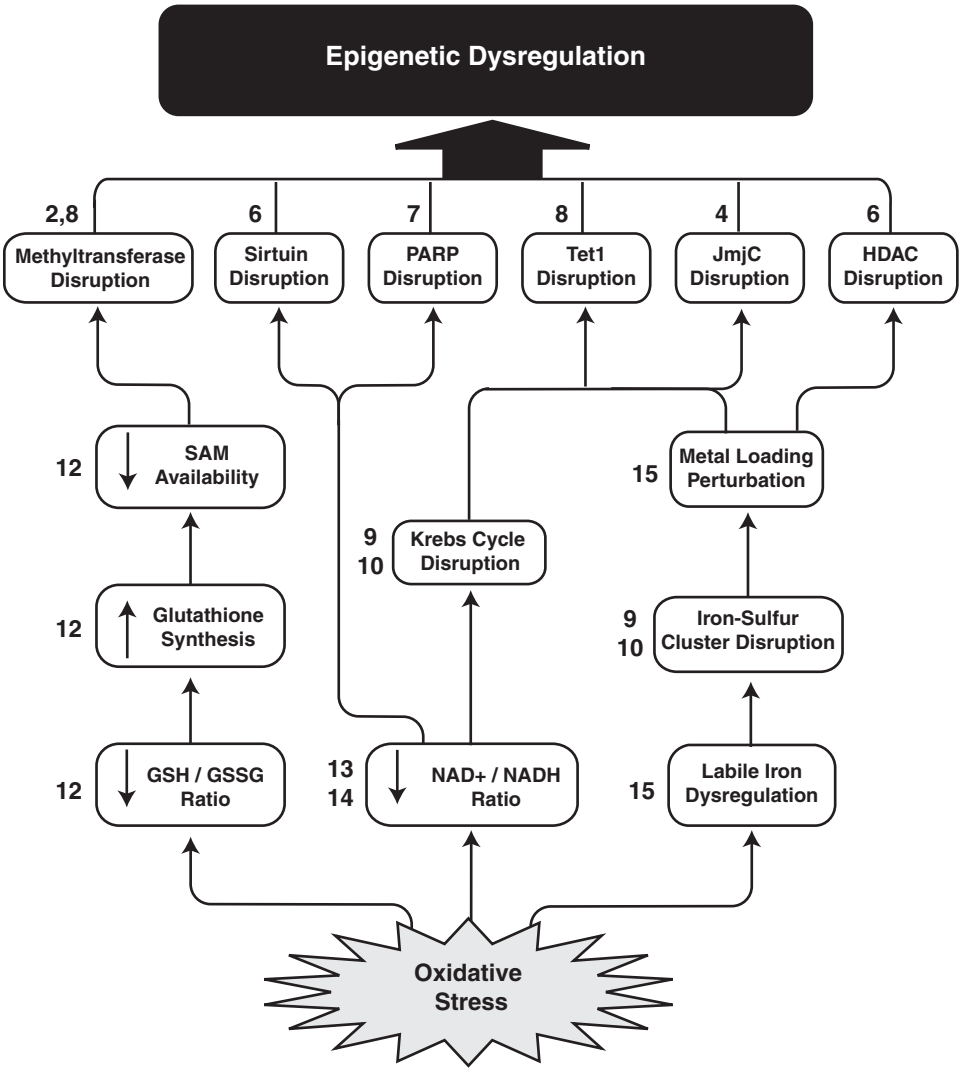

FIG. 16. A global illustration of oxidative stress and epigenetics. Oxidative stress may contribute to epigenetic perturbation through each of the means discussed in this review. The number of ways in which epigenetics may potentially be affected by metabolism strongly suggests that study of the overall epigenetic status will become increasingly important as we move toward better understanding of disease processes. Numbers adjacent to individual sections on the chart indicate the figures that focus more exclusively on each topic. It is also critical to note that some of the arrows indicating the trending directions of individual components may be reversed under certain conditions. For example, some generalized oxidative stress would move the arrow of the NAD^+/NADH ratio up, not down as we have suggested. However, when one considers specific oxidative stressors such as alcohol, we can observe changes in the indicated directions.

processes may also be normal signals that help to drive gene regulatory events. We have previously discussed at length how such paradigms might be employed in the process of development (119), and evidence is mounting that the modes of control that we have discussed here are significant for PHD enzyme regulation, which could directly correlate with the activity of other 2-OG and Fe(II)-dependent dioxygenases (235). Another critical example of known normal linkages between metabolic epigenetics and redox metabolism is the interaction between SIRT1 and the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). Briefly, modulation of SIRT1 activity through the pathways we have described above directly influences the acetylation of PGC-1α. This significantly affects multiple downstream transcriptional events, leading to the alteration of global metabolic patterns through modulation of fatty acid metabolism, glycolysis, and gluconeogenesis. As SIRT1 itself would be sensitive to many of the metabolic changes induced by PGC-1α modulation, this regulatory axis represents a critical closed loop illustrating how metabolism and epigenetic processes may communicate with one another. For a more detailed review of the SIRT1-PGC-1α relationship, please refer to Jeninga, Schoonjans, and Auwerx (134).

Given these concrete examples of metabolism communicating with epigenetic processes under normal circumstances, one might imagine a scenario in which the relative metabolic rate or activity of a particular cell type could directly influence its epigenome. This mode of regulation may ultimately promote a gene expression pattern to allow for appropriate maintenance of a metabolic program. As epigenomic research methods gain increasing sophistication, we will collectively be able to better evaluate some of these putative models.

VI. Metabolic Epigenetics and Disease

To close our discussion, we will examine several diseases with known metabolic and epigenetic components. These representative conditions all illustrate significant potential communication between metabolic perturbations and epigenetic consequences, and highlight the importance of further study of these critical linkages.

A. Cardiovascular disease

The blanket term cardiovascular disease (CVD) refers to a large number of disorders that are collectively associated with significant morbidity and mortality worldwide. The prevalence of CVD has exploded in the past century, and looks to remain a serious public health problem for many years to come. CVD is truly a multifactorial ailment, with numerous known genetic and environmental risk factors, but there is often a discrepancy between documented risk factors and either the severity or the progression of disease among different individuals or populations. Epigenetic mechanisms for gene expression potentially provide the explanation for these divergent cardiovascular outcomes. To that end, a great deal of recent research has focused extensively on how epigenetic phenomena may contribute to CVD.

The most well-characterized factor in CVD that is known to play a role in epigenetic regulation is homocysteine. Hyperhomocysteinemia is an accepted risk factor for CVD, supported by a large amount of research [reviewed extensively in (129, 227, 311)]. High levels of homocysteine promote the development of atherosclerotic lesions by driving aberrant DNA methylation in both vascular smooth muscle and monocytes (335, 336). This occurs through inhibition of methyltransferase enzymes and perturbation of the methionine cycle, as described above. Although these are accepted events in the pathogenesis of CVD, there are a number of other findings that have confounded the issue. One would predict that homocysteine inhibition of DNMTs would promote an overall hypomethylation, but data strongly suggest that hypermethylation predominates at specific loci, including extracellular superoxide dismutase, estrogen receptor α, and endothelial NO synthase (227). Other reports utilizing peripheral blood lymphocytes from patients with coronary artery disease have produced conflicting results, suggesting that global methylation may be increased or decreased in different individuals (35, 278). Collectively, the data support a role for altered methylation secondary to homocysteine overload, but it has been difficult to date to specify how these alterations may synergize with disease pathogenesis.

Some recent research has focused on how histone acetylation may be targeted therapeutically. Mouse models of hypercholesterolemia have demonstrated that HDAC7 plays a role in disease progression through repression of the cholesterol-metabolizing enzyme CYP27A1. Importantly, treatment of these animals with HDAC inhibitors significantly lowered serum cholesterol through enhanced expression of CYP27A1 and subsequently increased production of bile acids (207). Statins, which are commonly utilized anticholesterol pharmaceuticals, have also been implicated in HDAC regulation, though there is some question about the nature of how they interact with gene expression. Underscoring the complex relationship between HDAC regulation and atherosclerosis, Dje N'Guessan *et al.* (62) demonstrated that oxidized LDL reduces HDAC levels and modulates signaling pathways that are partially rescued with statin treatment. This result calls into question the utility of HDAC inhibition in CVD, and highlights a portion of complex communication between metabolic and epigenetic pathways that takes place in CVD.

Collectively, there is significant evidence supporting complex regulatory networks spanning metabolic and epigenetic processes in CVD. While many of the specific interactions remain to be identified, the data so far are promising. Continued research in this field is critically important given the large number of patients who are or will be affected by CVD. Epigenetic therapies to target the underlying mechanisms of CVD will hopefully prove to be valuable treatments in the future.

B. Alzheimer disease

Alzheimer disease (AD) is the most common cause of dementia, and is estimated to affect $\sim$6% in the population over age 65 (29). It is marked by a progressive neurodegeneration that results in the loss of cognitive function and ultimately death. Despite the high number of affected individuals, there currently are not any treatment strategies that directly address the underlying pathologic molecular events. These events include the aberrant processing of amyloid precursor protein into β-amyloid (Aβ) and the hyperphosphorylation of the tau protein, leading to the production of neurofibrillary tangles (29). Both the aggregation of Aβ into extracellular neuritic plaques and the intracellular formation of neurofibrillary

tangles compromise appropriate synaptic signaling and promote neurotoxicity, leading to the progressive loss of cognitive function. A vast research effort has been undertaken to better understand these mechanisms, but a definitive model for AD molecular pathogenesis has proven elusive. This has been in large part due to the many environmental and genetic factors that have been established as players in AD pathophysiology—there are numerous individual risk elements for both familial AD and sporadic AD, but by and large these comprise molecularly distinct mechanisms (46, 343). However, studies highlighting the importance of both epigenetic and metabolic processes in AD pathogenesis have recently been published, suggesting that interplay between these two elements may provide a significant and synergistic driving force toward disease.

Epigenetic events in the development of sporadic AD were initially considered when monozygotic twin studies illustrated longitudinal discordance for disease development (23, 90–92). Additional evidence for epigenetic events in the pathogenesis of AD came from studies examining the effects of known AD risk factors in experimental systems; many of these factors are associated with or directly lead to epigenetic aberrations [reviewed in ref. (46)]. These findings led many groups to pursue both DNA methylation and histone modifications as potential elements in AD pathogenesis. To date, results have been promising, illustrating a strong correlation between epigenetic dysregulation and the development of AD in model systems and in patient samples [reviewed extensively in ref. (46)]. A particularly remarkable recent study combined results from twin studies and molecular epigenetics. Mastroeni *et al.* (195) demonstrated that in a monozygotic twin pair in which one individual suffered from AD and the other did not, cortical neurons from the AD twin had quantitatively less DNA methylation than the control twin. While this remained a correlative study, it, nevertheless, provided strong evidence for an epigenetic role in AD pathogenesis, and represents an exciting finding as epigenetic research moves forward. Recent findings have also suggested that methylation of CpGs preferentially inhibits the repair of hydroxyguanine, leading to a steady accumulation of oxidized DNA base damage over time that contributes to AD pathogenesis (343). Critically, this illustrates how developmental epigenetic processes may ultimately contribute to pathogenesis over a long period, ultimately resulting in clinical disease.

Metabolic events are also known to play roles in AD pathogenesis. Several studies from Mark Smith's laboratory concluded that oxidative stress and mitochondrial dysfunction are likely early events in the development of AD (117, 219, 220). Other investigations have revealed impaired CAC enzyme activity in postmortem examinations of AD brain tissue. Bubber *et al.* (27) showed that both IDH and 2-OG dehydrogenase had significantly reduced activities in AD patient samples, whereas SDH activity was increased by $\sim$44%. Perhaps the best evidence that metabolism is critical for AD pathogenesis is the finding that intact mitochondria are in fact required for Aβ toxicity to develop. Cardoso *et al.* (33) utilized an mtDNA-null ρ0 cell system to effectively demonstrate that Aβ toxicity is mitigated in the absence of functional mitochondria, strongly implicating metabolic events in the processing of Aβ plaques. In addition to these few studies, a significant number of other groups have identified linkages between mitochondrial metabolic function and AD pathogenesis [these are reviewed extensively by Moreira *et al.* in ref. (210)]. Collectively, it appears clear that aberrant metabolism is a hallmark of AD, regardless of the risk factors associated with disease development in individual cases.

Strong linkages between the metabolic and epigenetic events in AD have started to emerge as well. Both CR and supplementation with the sirtuin-activating polyphenol antioxidant resveratrol protocols have shown promise in attenuating the development of AD symptoms in animal models and in small clinical trials (7). This has turned attention to the importance of sirtuin enzymes in the pathogenesis of AD, though the results to date have proven difficult to interpret. Julien *et al.* (140) examined SIRT1 expression levels in patient samples and found that SIRT1 expression correlated negatively with both tau hyperphosphorylation and the duration of cognitive impairment. This result is in agreement with numerous other studies, which have established SIRT1 as a neuroprotective agent (144). In contrast, Green *et al.* (98) demonstrated that treatment with nicotinamide, which competitively inhibits the sirtuins, restored cognitive impairment in a transgenic mouse model of AD. These findings underscore the complexity of the relationship between sirtuins and AD, but also highlight a critical linkage between metabolic processes and epigenetic regulation. Although the sirtuins themselves are involved in significantly more post-translational modification events than just those associated with histones, the sensitivity of sirtuins to metabolic stimuli is well-established, and suggests that other mechanisms may be at play in parallel. The data to date regarding histone methylation and DNA methylation support this hypothesis, as each of these processes has known interactions with metabolic perturbations. Of particular interest in this regard is the finding that CAC enzymes may have altered functionality in AD, given the importance of CAC intermediates in the regulation of the 2-OG and Fe(II)-dependent dioxygenases. We would predict that aberrations in the available pool of intermediates such as succinate or 2-OG would significantly alter the activity of the jmjC histone demethylases, leading to global changes in epigenetic programming. Importantly, this mode of pathogenesis would also be of critical importance for AD, because epigenetic modifications are potentially reversible and thus could be targeted by therapeutic agents. Given the dearth of viable AD treatments currently available, modulation of metabolism-sensitive epigenetic marks *via* either metabolic supplementation or epigenetic modifying agents may prove to be a valuable strategy.

C. Cancer

Cancer is a collection of diseases in which the normal cellular developmental programming is compromised, leading to a malignant population of cells whose growth is no longer mediated strictly by physiologic homeostatic signals. The types of cancer are as phenotypically diverse as the myriad cell types in the human body, but there are several commonalities shared by the vast majority of malignancies. Classical cancer biologists such as Otto Warburg maintained that metabolic aberration was a critical element in the process of carcinogenesis. Warburg made the seminal observation that malignant cells preferentially utilize glycolysis even under aerobic conditions, a finding whose ramifications are still being explored by modern molecular biology (58, 72, 315,

324). Indeed, Warburg maintained that this metabolic change represented the causal step for all carcinogenesis, which would have effectively linked all cancers as diseases of inappropriate metabolic function (323). While molecular biology has since illustrated that there are substantially more elements at play, more and more results are indicating that Warburg's examination of carcinogenesis was remarkably prescient. Many of these results deal with epigenetic processes and the mechanisms by which they are perturbed in cancer.

Epigenetic dysfunction is another of the common hallmarks of malignancy, as an ever-growing list of epigenetically altered genes in cancer attests to. Our laboratory alone has definitively demonstrated the importance of epigenetic regulation of multiple genes in cancer, including maspin (63, 85), desmocollin 3 (228), 14-3-3 sigma (162), and the critical mitochondrial antioxidant enzyme manganese superoxide dismutase (121). Multiple analyses have demonstrated that global epigenetic dysregulation is a ubiquitous finding in essentially all assayed tumors, and there is mounting evidence that epigenetic changes may even precede malignant transformation during carcinogenesis (73). This so-called epigenetic progenitor model, proposed by Feinberg *et al.* (73), holds that a breakdown in epigenetic regulation leads to an expansion of the tumor progenitor cell compartment, which in turn produces a bigger target for subsequent oncogenesis *via* well-characterized means such as tumor suppressor abrogation or oncogene activation. While this remains controversial as an overarching theory of carcinogenesis, it is undeniable that epigenetics plays a major role in the pathogenesis of numerous human cancers. This is best evidenced by the large number of epigenetic modifying agents that are currently being examined as adjuvant chemotherapeutics, including 5-aza-deoxycytidine, a variety of HDAC inhibitors, and new classes of both histone demethylase and methyltransferase inhibitors (96, 169, 287, 313, 328).

The regulatory mechanisms discussed throughout this review clearly indicate that the metabolism-first and epigenetic progenitor theories of carcinogenesis can peacefully coexist. The fact that both metabolism and epigenetics were independently cornerstones of modern cancer research is strongly suggestive of a potential unified model. We have previously reviewed the extensive evidence that suggests that metabolic errors may, in essence, spark the epigenetic switch that contributes to carcinogenesis and cancer progression (120). We also have alluded to examples of how metabolic defects may lead to epigenetic perturbations in established models of carcinogenesis, with respect to the development of malignancies secondary to either germline SDH or IDH mutations (see "Ascorbate and 2-OG and Fe(II)-dependent dioxygenases" for further details). While these models themselves are not perfect examples of the epigenetic progenitor theory because the primary lesions in each cascade were in fact genetic mutations, they, nevertheless, provide the framework for a thought experiment involving the nongenetic manipulation of similar metabolic enzymes. The initial insult in this conceptual model could be toxicologic, physiologic, or even nutritional in nature, with the ultimate outcome being some measurable metabolic perturbation. Given the large number of potential interactions with epigenetic processes through the mechanisms presented above, one might expect significant changes in epigenetic programming secondary to even minor metabolic stressors. Moreover, epigenetic dysregulation through metabolic intermediates may have significant potential for signal amplification, if metabolic genes are among those that become epigenetically altered during the initial insult. Although admittedly simple, the notion that synergism between metabolic dysfunction and an accumulation of epigenetic aberrations could lead to cancer development is one that should be explored given the established importance of each in the current understanding of cancer biology (Fig. 17).

D. Environmental toxicology and epigenetics

Environmental toxicants play a significant role in the modulation of gene expression, and contribute to the incidence of countless human diseases. One of the primary means that these agents may affect disease pathogenesis is through interference with epigenetic processes. Above, we have described several examples of how environmental agents may perturb normal epigenetic phenomena, including how cigarette smoke and heavy metals may directly interfere with HDAC and jmjC functionality, respectively. Others have shown how significant exposures to diesel exhaust modulate gene expression pathways in human airway epithelial cells (32, 249, 298). While these studies did not explicitly focus on epigenetic modification, we hypothesize that epigenetic processes would play a significant role in the modulation of gene expression in those systems. In sum, the collective body of evidence strongly suggests that epigenetic phenomena are distinctly regulated by toxicologic insults. Here, we have chosen to focus more attention on alcohol consumption, which poses a significant health hazard to a large segment of the population.

1. Alcohol. Ethanol consumption represents a critically important public health problem, accounting for $\sim$100,000 deaths and $184.6 billion annually in a 2000 NIAAA estimate. Ethanol exerts its metabolic effects through a variety of processes, involving both ethanol itself and its numerous metabolites (338). The oxidative pathways of ethanol metabolism, which involve alcohol dehydrogenase, cytochrome p450 2E1, catalase, and acetaldehyde dehydrogenase 2, represent the predominant mechanisms for removing ethanol from the body, and can produce deleterious effects on metabolic processes through several means. These include altering the normal redox balance by changing NAD^+/NADH ratios, the production of harmful reactive oxygen species (ROS), and the formation of toxic adducts of the alcohol metabolite acetaldehyde (338). Further, alcoholic liver injury significantly alters the metabolism of both SAM and GSH, through a reduction in both the quantity and activity of SAM biosynthetic enzymes in hepatocytes (10, 66). It is thus abundantly clear that alcohol interferes with normal cellular metabolism. In parallel, there is an emerging body of work suggesting that epigenetic dysregulation is another key element in alcohol-mediated pathogenesis.

Multiple preliminary studies have indicated that ethanol treatment can induce a variety of epigenetic changes in experimental systems. Acetylated H3K9 is demonstrably enriched in an ethanol-dependent manner in both hepatocyte cell culture systems and using *in vivo* animal studies (145, 146, 238). Histone methylation has also been explored as a potential effector of alcohol pathology. Pal-Bhadra *et al.* (234) showed that differential methylation patterns in H3K4 and

H3K9 in ethanol-treated primary rat hepatocytes correlated with distinct changes in gene expression. These findings are unsurprising given the large number of mechanisms by which metabolic processes can interfere with epigenetic ones. As ethanol is directly responsible for the perturbation of several of these pathways, it would be expected to have significant epigenetic consequences such as those that have already been observed. For example, the reduction in the NAD^+/NADH ratio secondary to ethanol metabolism would have significant effects on the sirtuin enzymes, which require NAD^+ for appropriate functionality. This would produce a potential hyperacetylation at loci that rely on sirtuins for normal regulation (including both histones and other proteins), which corresponds well with the current literature. We might also expect that PARP enzymes and polyadenylation of various nuclear substrates would be compromised in ethanol-treated cells, which currently appears to be an under-explored field.

A much broader amount of research has been accomplished examining the effects of ethanol on 1-carbon metabolism in the liver through the regulation of SAM production. SAM levels are significantly reduced in ethanol-treated hepatocytes (10, 66). Ethanol-mediated SAM reduction is believed to be secondary to methionine synthase inactivation, which some have considered the primary molecular defect in chronic ethanol toxicity (12). Many of the mechanisms associated with SAM depletion do not focus explicitly on the epigenetic consequences of reduced substrate for methylation reactions, but instead on the antioxidant capacity of a functional pool of SAM (12, 36, 74). The increasingly clear role of epigenetic modification in the pathogenesis of liver disease cannot be understated in light of this known metabolic stressor, however, and we are confident that further study of these modifications and the synergism with metabolic perturbation will ultimately yield clinically valuable information.

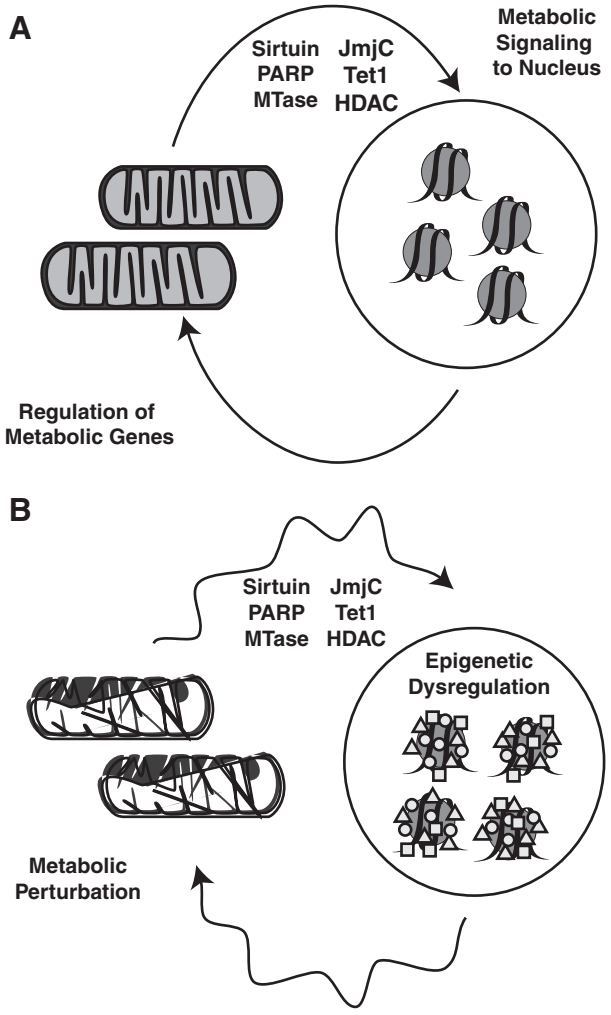

FIG. 17. A model for metabolic-epigenetic synergism in cancer. (A) In the normal condition, intermediates produced through metabolism may influence and maintain appropriate epigenetic regulation of critical metabolic loci in the genome. **(B)** In cancer, there is strong evidence for metabolic perturbation and mitochondrial dysfunction. This may promote deregulation of the epigenetic signals in the nucleus, leading to a synergistic feedback mechanism that promotes increasing metabolic and epigenetic derangement.

VII. Challenges and Future Directions

Epigenetics research is an inherently challenging field secondary to the often transient nature of epigenetic modification. Individual *trans*-activating elements, which include traditional transcription factors, ncRNAs, and, in some cases, histones themselves, are capable of recruiting different subsets of epigenetic modifying enzymes to effectively modulate gene expression. It is also important to recall the idea that epigenetics represents a mechanism for the integration of multiple mechanistic inputs into a coherent gene expression profile. The gestalt of the epigenetic landscape is thus critically important when examining the overall effects of any particular epigenetic modification. Further, the combinatorial possibilities for distinct epigenetic signatures are vast when considering each of the marks that can be made on histones and nucleic acids. The large number of mechanisms that we have described above that metabolism can interact with epigenetic regulation at multiple levels compounds this difficulty be virtually guaranteeing that single epigenetic marks will not be affected independently of other epigenetic marks in experimental system. Collectively, these elements provide substantial technical challenges that hamper our overall understanding of epigenetic phenomena.

One of the ways researchers have begun to address these issues is through the use of genome-wide epigenetic assays. The utility of ChIP for the study of epigenetic marks has been incalculable, and recent high-throughput methods have enabled ChIP-seq to become a viable method. ChIP-seq involves the immunoprecipitation of a particular epigenetic modification followed by the mass sequencing of the associated DNA, generating a genome-wide epigenetic map. This powerful tool, described in greater detail by Peter Park in his 2009 review, should provide a cornerstone for comprehensive epigenomic research both now and into the future (239). Similar commercially available methodologies for the precipitation and analysis of methylated CpG dinucleotides have also been developed, enabling high-throughput screening of methylated DNA (MethylMiner Assay Kit, Invitrogen). These and other similar technologies represent the initial steps toward broader measurement of epigenomic phenomena, rather than isolated epigenetic events. We predict that as these methods

mature, and as high-throughput sequencing technology becomes more efficient, understanding of broad epigenetic phenomena in both health and disease will exponentially grow.

VIII. Conclusions

Metabolism is the process by which organisms harvest energy from various sources to drive the mechanisms that are required for life. In eukaryotes, this includes the epigenetic regulation of gene expression. Given that metabolism is as ancient as life itself, it is of little surprise that nature has found ways to incorporate metabolic inputs into other higher ordered signal transduction pathways. Here, we have demonstrated a variety of means by which metabolic events can communicate directly with gene expression *via* modulation of epigenetic control. Importantly, the mechanisms that we have chosen to discuss here represent but a small fraction of the overall epigenetic machinery, and it is highly probable that as the meaning of other histone and DNA modifications are established, more connections to metabolism will be discovered as well. This will be of particular significance for the study of human disease, as epigenetic mechanisms for pathogenesis are gaining significant support in both research and clinical circles for the diseases discussed here and an ever-increasing number of other conditions.

Although the importance of metabolism and epigenetic interactions in disease is certainly notable, we also must stress that these pathways likely exist as mechanisms of normal homeostatic control. In this way, the various events that we have described in detail likely serve as fine-tuning for epigenetic programming, allowing the relative metabolic activity of the cell to feed back into transcriptional regulation in an effort to maintain homeostasis. A critical point to consider in closing, as we alluded to at the outset of the discussion, is that epigenetics represents a mechanism for signal integration in cells, by combining multiple inputs into a coherent output signal. Ideal model systems for the study of this concept have been difficult to establish due to the extremely broad nature of epigenetic control. The recent development of new high-throughput modalities such as ChIP-Seq promise to revolutionize epigenetic research by allowing evaluation of global changes in epigenetic marks secondary to measurable changes in metabolism. We anticipate that as this technology becomes more available, the metabolic epigenetic concepts that we have presented here will gain significant traction as mechanisms for both physiologic and pathologic events.

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Abbreviations Used

2-HG = 2-hydroxyglutarate
2-OG = 2-oxoglutarate
5-meC = 5-methylcytosine
5-meOHC = 5-hydroxymethylcytosine
Aβ = β-amyloid
AD = Alzheimer disease
AlkB = alkane hydroxylase
ATP = adenosine triphosphate
BER = base excision repair
CAC = citric acid cycle
CGI = CpG island
ChIP = chromatin immunoprecipitation
CR = caloric restriction
CVD = cardiovascular disease
DMT = divalent metal transporter
DNMT = DNA methyltransferase
GSH = glutathione
GSSG = glutathione disulfide
HAT = histone acetyltransferase
HDAC = histone deacetylase
HIF = hypoxia-inducible factor
HMT = histone methyltransferase
IDH = isocitrate dehydrogenase
IRP1 = iron response protein 1
ISCU = iron–sulfur scaffold protein
jmjC = jumonji-domain containing
KDM = lysine demethylase
LIP = labile iron pool
LSD1 = lysine specific demethylase 1
MBD = methyl-binding domain
MSR = methionine sulfoxide reductase
Nam = nicotinamide
ncRNA = noncoding RNA
NO = nitric oxide
$O_2^{\bullet -}$ = superoxide
OAADPr = 2′-O-acetyl-ADP ribose
ONOO = peroxynitrite
PAD4/PADI4 = protein arginine deiminase 4
PARP = poly(ADP-ribose) polymerase
PcG = polycomb group
PGC = primordial germ cell
PGC-1α = peroxisome proliferator-activated receptor γ coactivator 1α
PHD = prolyl hydroxylase domain
SAH = *S*-adenosyl homocysteine
SAM = *S*-adenosyl methionine
SDH = succinate dehydrogenase
Succ = succinate
TTFA = thenoyltrifluoroacetate