

Two traditional strengths of nutrition research have been to identify associations between food intake and health at the population level, and to describe the metabolism and biochemical action of nutrients in healthy individuals. Nutrition research has not excelled at defining the cellular and molecular roles of nutrients in modulating the innate and adaptive immune responses. Such mechanistic information would help nutritionists and physicians make better decisions about the use of such supplements, particularly when their intentions go beyond treating deficiency and enter the realm of preventive and therapeutic medicine.

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## Retinoids and Epigenetic Silencing in Cancer

*An epigenetic event alters the activity of genes without changing their structure. Methylation reactions are important mediators of epigenetic events in cells. Two recent studies have established a mechanistic link between a vitamin A metabolite and epigenetic alterations of its target promoter that could provide insights about the role of vitamin A in cancer prevention.*

**Key words:** vitamin A, cancer, methylation, histones

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Approximately 20% of cancer cases worldwide and 30% in western countries could be attributable to dietary factors.<sup>1,2</sup> Vitamins have been evaluated extensively for

their influence on cancer risk. Certain subgroups of the U.S. population are at risk for vitamin deficiency, including those with poor intake, abnormal losses or metabolism, or insufficient synthesis.<sup>3</sup> Vitamin status and risk for cancer have been assessed in numerous investigations; the resulting evidence supports the efficacy of vitamins in preventing some cancers.<sup>3,4</sup>

Carotenoids and retinoids, which are derivatives of retinol (vitamin A), have received attention as possible cancer-preventing agents. This interest is due to their pro-apoptotic and growth-regulating effects. Provitamin A carotenoids (those that can be converted to vitamin A), in particular  $\beta$ -carotene,  $\alpha$ -carotene, and  $\beta$ -cryptoxanthin, are major sources of vitamin A in foods. Lycopene, lutein, and zeaxanthin have been investigated for cancer-preventing action, but these carotenoids are not converted to vitamin A.

More than 1500 synthetic retinoids have been produced, and their effectiveness as possible cancer chemopreventive agents is currently a subject of intense investigation. These retinoids affect expression and function

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of nuclear retinoid receptors found in normal, premalignant, and cancerous cells. At high dosages, retinoids block in vitro cell transformation, inhibit carcinogenesis in animal tumor models, and reduce premalignant lesions and prevent secondary tumor formation in cancer patients.<sup>5</sup>

Two randomized placebo-controlled trials surprisingly reported that supplementation with  $\beta$ -carotene increased the risk of lung cancer in male smokers and asbestos workers.<sup>6,7</sup> These studies strongly suggest that  $\beta$ -carotene supplementation increases risk of lung cancer in smokers. Evidence linking carotenoids or vitamin A with colorectal or prostate cancer is weak, although somewhat stronger evidence supports a link between carotenoids or vitamin A and bladder and breast cancer.<sup>3</sup> A recent population-based, nested, case-control serum study among women who had donated blood up to 20 years before developing breast cancer reported that in one cohort, total carotenoids and individual carotenoids such as lycopene and  $\beta$ -carotene were significantly associated with reduced risk of breast cancer.<sup>8</sup>

Epidemiologic evidence of an association between vitamin A and carotenoids and cancer thus remains inconclusive, but results from some recent cell culture and animal studies are encouraging. Knowledge of mechanisms underlying the effect of vitamin A and its derivatives on cell-signaling pathways, particularly the effects on retinoid receptor binding or activation, might help clarify whether this vitamin helps to promote or prevent cancer. Two recent studies have provided insight as to how retinol and retinoic acid (RA), an active metabolite of vitamin A, might influence expression of specific genes by induction of DNA methylation and histone modifications at target promoters.<sup>9,10</sup> These epigenetic events alter the activity of genes without chang-

ing their structure, and thereby might influence carcinogenesis (Figure 1). The DNA of CpG islands in the promoter-regulating region of tumor-suppressor genes is commonly hypermethylated in cancer; this hypermethylation subsequently leads to silencing of expression of these specific genes.<sup>11</sup> Modifications of the amino-terminal residues of histones, which are components of the nucleosomes involved in DNA packaging, include phosphorylation, acetylation, and methylation. The implications of how the many possible combinations of histone modifications might alter gene expression are just beginning to be realized,<sup>12</sup> and applications of these studies to nutrition and cancer are meager.

Di Croce et al.<sup>9</sup> were interested in determining the mechanisms underlying the specificity of methylation of tumor-suppressor genes. As noted above, DNA frequently becomes hypermethylated in the promoter of tumor-suppressor genes, but how this occurs is not clear. There is also little information available as to whether individual nutrients can influence DNA methylation of specific gene promoters in cancer, although this subject is beginning to be addressed with the report of Jhaveri et al. that folate depletion induces hypermethylation and silencing of the H-cadherin gene in human nasopharyngeal carcinoma KB cells.<sup>13</sup>

Di Croce et al. studied the PML-RAR protein, which is an oncogenic transcription factor found in acute promyelocytic leukemias (APLs). The APL phenotype depends on the expression of this protein, a fusion protein created when the promyelocytic leukemia (PML) gene on chromosome 15 recombines with the RA receptor  $\alpha$  gene (*RAR $\alpha$* ) on chromosome 17. Treatment with pharmacologic doses of RA induces terminal differentiation of cells that express PML/RAR, and leads to down-regulation of the fusion protein. The PML-RAR protein

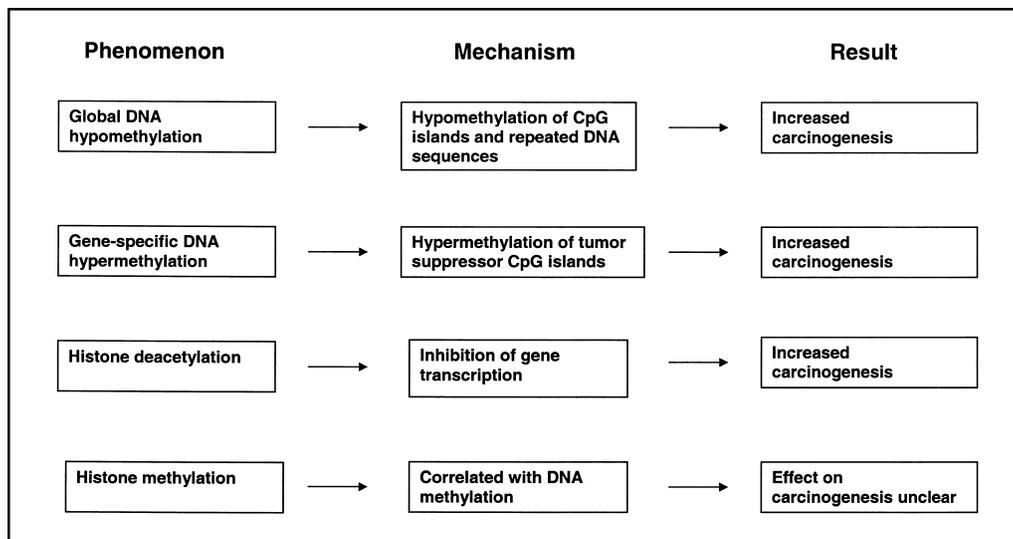


Figure 1. How epigenetic phenomena can influence carcinogenesis.

is a transcriptional regulator of RA target genes. Di Croce et al. focused their studies on one specific target gene, *RARβ2*, because it contains a CpG island in its promoter region that could be subject to methylation, and because *RARβ2* is considered to be a tumor-suppressor gene. The *RARβ2* gene is the biologically active isoform of *RARβ*, and this gene is regulated by the *RARβ2* P2 promoter, which contains a high-affinity RA-responsive element (RARE) near the transcription start site.

An initial experiment was done to demonstrate that PML-RAR actually binds to the *RARβ2* promoter. This was accomplished using chromatin immunoprecipitation (ChIP) analysis (Figure 2) of PR9 cells, which are U937 human promonocytic leukemia cells carrying a vector that can be induced to express the PML-RAR protein. The same cell line carrying an empty inducible vector served as a control. The ChIP assay is used to covalently cross-link histones and other proteins with DNA, in essence “freezing” them in the positions that they occupied on the promoter at a given time after treatment with a nutrient or other agent. The protein-DNA complexes are then immunoprecipitated from the sonicated, fragmented DNA with specific antibodies. Immunoprecipitated histones are analyzed by immunoblot analysis, and

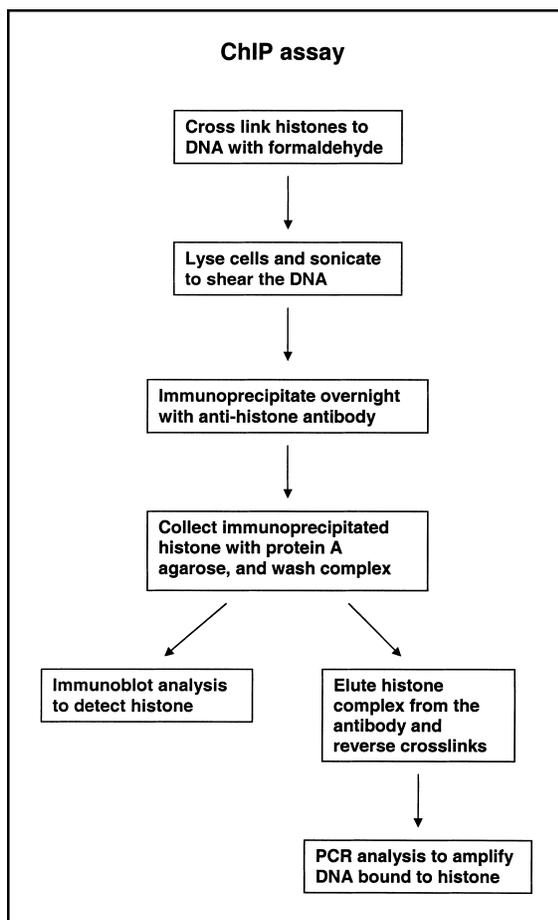
after the cross-linking reaction is reversed, DNA that is bound to the immunoprecipitated histone is amplified by polymerase chain reaction (PCR).

ChIP analysis revealed that the PML-RAR transcription factor was bound to the *RARβ2* promoter in vivo, whether or not its natural ligand (RA) was present. The investigators also used *RARβ2* promoter-luciferase reporter gene constructs to demonstrate that PML-RAR, when transfected into cells that do not express this gene, had a repressive effect on *RARβ2* transcription. These studies established that the PML-RAR oncogene was bound to the *RARβ2* promoter and blocked its transcription.

The methylation of *RARβ2* was next investigated in a CpG-rich region of the promoter near the first exon. Methylation-specific PCR revealed methylation of this region in seven of nine APL samples, but not in normal hematopoietic precursors (CD34<sup>+</sup> cells). When the promoter region of the *RARβ2* reporter construct was methylated in vitro and transfected into cells that did not express PML-RAR, its basal activity was reduced by 85%, which was the same extent of *RARβ2* silencing observed when PML-RAR itself was transfected.

These data are consistent with an effect of PML-RAR on methylation of the *RARβ2* promoter, and point to a possible involvement of DNA methyltransferases (DNMTs) in the action of PML-RAR. ChIP analysis of cells in which PML-RAR was expressed demonstrated that DNMT1 and DNMT3a were specifically enriched at the *RARβ2* promoter, and that these two DNMTs form stable complexes with the *RARβ2* promoter. Mapping of DNMT3a binding sites revealed that DNMTs bind at both PML and RAR protein sites.

In some models of tumorigenesis, activated oncogenes have been shown to block differentiation by stimulating uncontrolled cell growth. In some cases, therefore, induction of differentiation may oppose the action of oncogenes by preventing further proliferation. As noted earlier, treatment of cells that express PML-RAR with high doses of RA leads to induction of differentiation. However, PML-RAR exerts inhibitory effects on differentiation in the absence of RA. These actions of RA are believed to be mediated via RA’s effect on complex formation between PML-RAR and histone deacetylase (HDAC). HDAC recruitment to the promoter by PML-RAR is crucial for repression of both transcription and differentiation, and RA treatment causes dissociation of the PML-RAR/HDAC complex. The RA-induced dissociation of this complex converts PML-RAR into a transcriptional activator, which subsequently results in leukemia cell differentiation. Di Croce et al. observed partial reversal of repression of *RARβ2* transcription by individual treatment with the methylation inhibitor 2'-deoxy-5-azacytidine, or with the HDAC inhibitor tricho-



**Figure 2.** ChIP assay for assessing protein-DNA complexes.

statin A (TSA), but simultaneous treatment with both inhibitors completely released repression of *RARβ2*. This result suggested that PML-RAR represses transcription and blocks differentiation by two distinct mechanisms, one involving recruitment of HDACs and the other involving recruitment of DNMTs.

Does RA only induce dissociation of the PML-RAR/HDAC complex, or does it also block the methylation of the *RARβ2* promoter (Figure 3A)? Di Croce et al.<sup>9</sup> observed that when acute promyelocytic leukemia NB4 cells were treated with pharmacologic doses of 1 μM RA for 48 hours, the methylation levels (as assessed by bisulfite genomic sequencing) were decreased in the *RARβ2* regulatory region extending from base pair -370 in the promoter to base pair +238 in exon 1. Treatment with the methylation inhibitor 2'-deoxy-5-azacytidine led to a similar degree of regulatory region hypomethylation, and the combination of both RA and methylation inhibitor had a somewhat greater effect on hypomethylation than either agent alone. *RARβ2* mRNA, which was not expressed in untreated NB4 cells, was observable after dosing with RA, and was increased even further by combined RA and 2'-deoxy-5-azacytidine treatment. These changes would be expected if silencing of *RARβ2* expression, resulting from promoter hypermethylation, was reversed by demethylation induced by either RA or 2'-deoxy-5-azacytidine.

Di Croce et al. summarized by suggesting that the transcription factor PML-RAR may exert its oncogenic action by recruiting DNMTs to the promoter of tumor-suppressor genes (Figure 3B). This would foster promoter hypermethylation and subsequent recruitment of methyl-binding proteins. In turn, the methyl-binding proteins could interact with HDACs and DNMTs to lock target promoters into a stably silenced chromatin state. Thus, RA would exert a beneficial action by countering oncogene-induced methylation of the *RARβ2* promoter, and preventing silencing of this putative tumor-suppressor gene (Figure 3C).

What was not addressed in the study by Di Croce et al.<sup>9</sup> was the extent to which RA affected the interaction of HDACs with the promoter: only effects of RA on promoter methylation were described. By contrast to the study of Di Croce et al. that used leukemia cells, Sirchia et al.<sup>10</sup> reported that RA treatment of breast cancer cells (which do not express PML-RAR) leads to changes in histone acetylation but not DNA methylation of the *RARβ* P2 promoter. They found, however, that absence of methylation of the *RARβ* P2 promoter was necessary for RA to activate *RARβ2* gene expression. Endogenous *RARβ2* expression was found to be reactivated by RA or chromatin remodeling drugs, as discussed below.

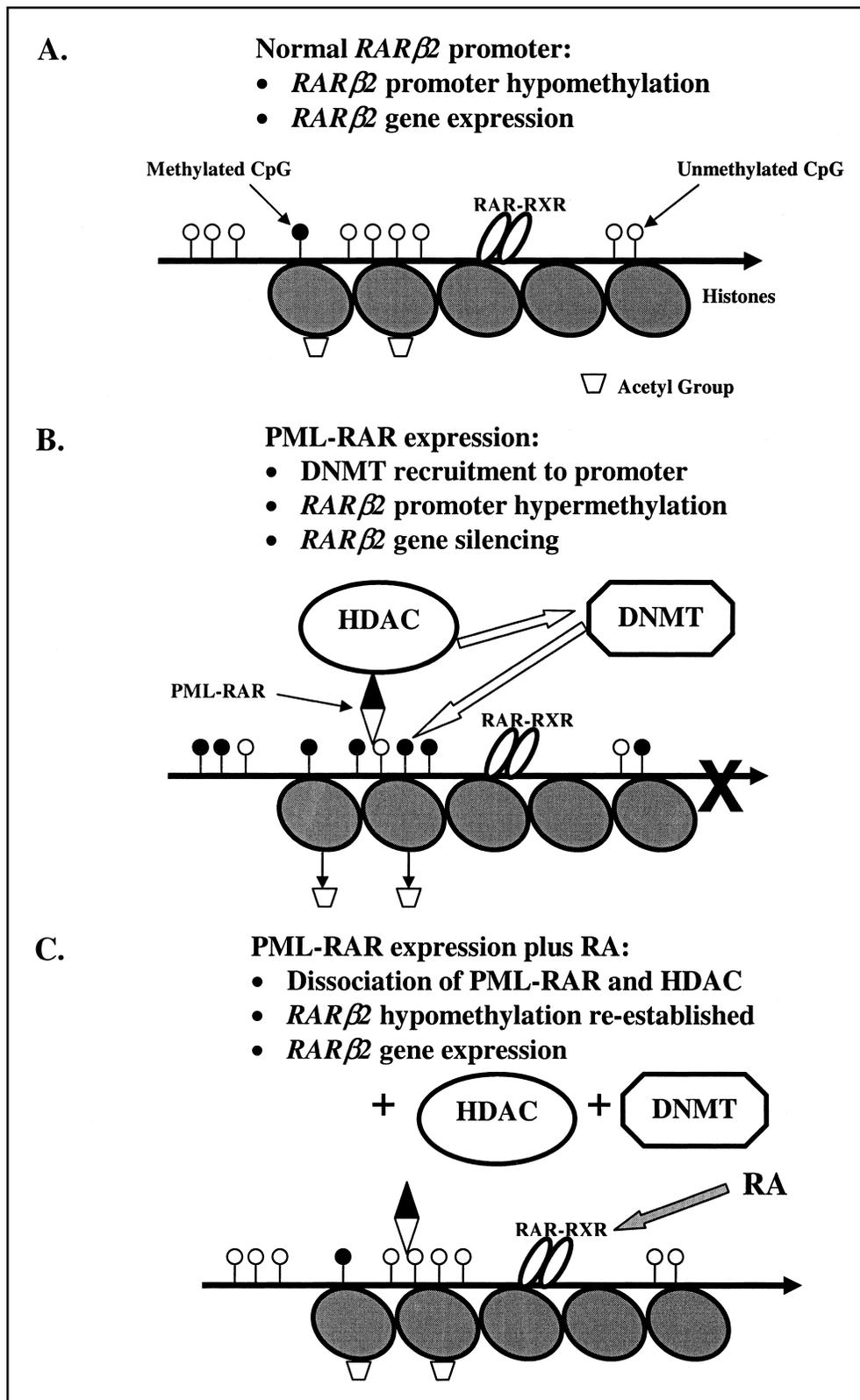
The first experiment by Sirchia et al.<sup>10</sup> was to show that primary breast tumors from patients with ductal

adenocarcinomas had increased *RARβ2* transcription in response to RA therapy only if the *RARβ2* P2 promoter was unmethylated. Similarly in xenograft tumors of breast cancer cell lines, RA treatment induced *RARβ2* expression in T47D breast cancer cells, which contain an unmethylated *RARβ2* P2 promoter, but not in MCF7 breast cancer cells in which the *RARβ2* promoter is methylated. These results would, of course, be expected if promoter CpG island methylation were preventing expression of *RARβ2* in response to RA dosage.

The authors reasoned that DNA methylation, which was preventing the response to RA, might be causing repressive chromatin remodeling at DNA methylation sites in the *RARβ2* P2 promoter because this response to DNA methylation was observed previously.<sup>14</sup> To test whether histone alterations might be involved in response of the *RARβ2* P2 promoter to RA, they evaluated promoter histone acetylation in the same T47D and MCF7 cells used for the xenograft model above. They used a ChIP assay and antibodies to acetyl-histone H3 and acetyl-histone H4 to evaluate acetylation. Pharmacologic doses of RA (1 μM) led to *RARβ2* reactivation and increased promoter histone acetylation only in the T47D cells, which contain an unmethylated promoter, but not in MCF7 cells, whose *RARβ2* P2 promoter is methylated. Reacetylation of histones and reactivation of *RARβ2* expression induced by RA treatment of T47D cells and xenograft tumors was also associated with growth inhibition of the breast cancer cells, a desirable biologic effect of the RA treatment.

The authors also wanted to determine whether agents designed to reacetylate histones (PB, a short fatty acid, and TSA, the previously mentioned HDAC inhibitor) or demethylate DNA (5-aza-2' deoxycytidine) would be able to make methylated, deacetylated cell lines such as MCF7 responsive to RA. The results of this experiment were very informative because they suggested that treatments that reacetylated the histones caused *RARβ2* to become reactivated, even if the promoter was already methylated. Histone reacetylation is thus necessary and sufficient to restore susceptibility of the promoter to RA action. More detailed studies showed that simultaneous dosage with both RA and TSA resulted in greater histone acetylation and *RARβ2* gene expression. To further assess biologic effects, investigators evaluated the influence of RA or TSA (alone or in combination) on growth inhibition of MCF7 cells in vitro and on size of MCF7 xenograft tumors. Although RA alone did not inhibit MCF7 cell growth, the combined treatment of RA plus TSA was more effective than TSA alone.

Sirchia et al. proposed a model of RA action to account for its effects on *RARβ2* expression in breast cancer. They put forward the idea that physiologic con-



**Figure 3.** Retinoic acid prevention of hypermethylation of the *RARβ2* gene promoter. (A) The *RARβ2* promoter is normally unmethylated and acetylated. (B) PML-RAR expression induces promoter methylation and gene silencing. (C) Retinoic acid treatment prevents recruitment of DNMT by PML-RAR. RA = retinoic acid, RAR-RXR = RA receptor, HDAC = histone deacetylase, DNMT = DNA methyltransferase, PML-RAR = oncogenic transcription factor expressed in acute promyelocytic leukemias.

centrations of RA are capable of binding to retinoid receptors in the normal *RARβ2* P2 promoter, resulting in release of HDAC, histone acetylation, expression of *RARβ2*, and growth inhibition. When *RARβ2* P2 is hypoacetylated but unmethylated, as in T47D cells, pharmacologic concentrations of RA are necessary to support adequate histone acetylation. In the worst case scenario of a deacetylated, methylated promoter, pharmacologic RA concentrations and an HDAC inhibitor such as TSA are required for *RARβ2* expression from the P2 promoter. The authors suggest that the extent of *RARβ2* methylation could serve as a predictor of RA responsiveness, and that methylation analysis could be used as a tool to identify breast cancer patients that could benefit from RA therapy.

Both Di Croce et al.<sup>9</sup> and Sirchia et al.<sup>10</sup> described potential benefits for cancer patients treated with the vitamin A metabolite RA. The mechanism that accounts for the reactivation of *RARβ2* expression is, in both instances, an epigenetic one. In one case RA acts by blocking *RARβ2* promoter methylation,<sup>9</sup> however, while in the other it influences histone acetylation.<sup>10</sup> It is likely that the recruitment of methyltransferases to the promoter of the *RARβ2* gene by the oncogenic transcription factor PML-RAR in APL accounts for the effect on promoter methylation in this leukemia. Methylation of the *RARβ2* promoter may in turn bring about further interactions with HDACs. In the case of breast cancer, PML-RAR is not available to support DNMT recruitment and promoter methylation; in this cancer the predominant effect of RA is increased histone acetylation. The new information from both studies is the role that epigenetic changes play in the action of RA. These changes in DNA methylation and histone acetylation may ultimately prove to be major factors responsible for the cancer-preventing effects of RA.

These two reports might prompt the search for other nutrients that mediate epigenetic changes at the promoter of individual genes important in cancer prevention. Folate and vitamin B<sub>12</sub> supply methyl groups for biologic methylation reactions, and might be good candidates to study for their effect on DNA methylation and histone acetylation in cancer. The nutritional status of zinc, selenium, vitamin C, and niacin have also been associated with altered genome-wide DNA methylation.<sup>15</sup> Future studies will hopefully elucidate whether these or

other nutrients play a role in epigenetic changes that lead to neoplasia.

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