Targeting the epigenetic machinery of cancer cells

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Abstract

Cancer is characterised by uncontrolled cell growth and the acquisition of metastatic properties. In most cases, the activation of oncogenes and/or deactivation of tumour suppressor genes lead to uncontrolled cell cycle progression and inactivation of apoptotic mechanisms. Although the underlying mechanisms of carcinogenesis remain unknown, increasing evidence links aberrant regulation of methylation to tumourigenesis. In addition to the methylation of DNA and histones, methylation of non-histone proteins, such as transcription factors, is also implicated in the biology and development of cancer. Because the metabolic cycling of methionine is a key pathway for many of these methylating reactions, strategies to target the epigenetic machinery of cancer cells could result in novel and efficient anti-cancer therapies. The application of these new epigenetic therapies could be of utility to promote E2F1-dependent apoptosis in cancer cells, avoid metastatic pathways and/or sensitise tumour cells to radiotherapy.

Keywords: methionine; antifolates; methylation; epigenetics; cancer therapy.

INTRODUCTION

Human cancer is a heterogeneous disease with respect to molecular alterations, incidence, survival, and response to therapy. The limitations of conventional chemotherapy treatments, especially for patients with advanced cancer, have become apparent, and despite several decades of research, almost half of all patients diagnosed with cancer die of the disease, primarily due to metastases. Surgery remains the most effective therapy. New therapeutic strategies must be identified, and the metabolic abnormalities of cancer cells provide opportunities for alternative therapies.[1-4] Enzymes that regulate the epigenetic status of cells catalyse posttranslational modifications of DNA, histones, and transcription factors, and therefore, these enzymes influence metabolic gene expression. These enzymes require metabolites that are used as cofactors and substrates to carry out reactions. The interface of epigenetics and metabolism constitutes a new avenue of cancer biology and could lead to new insights for the development of anti-cancer therapeutics.[5-8] In this respect, the resistance of cancers to general chemotherapeutics and their ability to evade cellular suicide and resist apoptosis are related to the high activity of the methionine cycle in these cells, which permits the methylation of specific genes and activation of multiple survival pathways.[1,9-11] Many human cancer cell lines and primary tumours have an absolute need for methionine, an essential amino acid, whereas normal cells are relatively resistant to exogenous methionine restriction. Therefore, therapies to block the methionine cycle in cancer cells represent a safe and effective strategy to fight cancer.

Malignant tumours are characterised by a high rate of growth. Tumour cells drain the energy of the host in the form of glucose and amino acids. Methionine is an essential amino acid with at least four major functions (Figure 1).[1] First, methionine participates in protein synthesis. Second, methionine is a precursor of glutathione, a tripeptide that reduces reactive oxygen species, thereby protecting cells from oxidative stress.[12] Third, methionine is required for the formation of polyamines, which have far-ranging effects on nuclear and cell division.[13] Fourth, methionine is the major source of the methyl groups necessary for the methylation of DNA and other molecules.[1] With respect to this last function, the methyl groups required for all biological methylation reactions are derived from dietary methyl donors and from cofactors carrying 1-carbon units. A pathway that is key to many of these reactions is the metabolic cycling of methionine (Figure 1).[14] Briefly, methionine is converted to the methyl cofactor S-adenosylmethionine (SAM). Subsequent to methyl donation, the product S-

adenosylhomocysteine (SAH) becomes homocysteine, which is then either catabolised or re-methylated to methionine. In the context of this review, it is also important to bear in mind the well-established connection of the methionine cycle with two crucial cell metabolites, folic acid and adenosine (Figure 1). Folic acid acts as the fuel for the methionine cycle, which forms N^5-methyl-tetrahydrofolate (N^5-methyl-THF), the co-factor of methionine synthase (MS), the enzyme responsible for methionine synthesis, after transformation by folate cycle enzymes such as dihydrofolate reductase (DHFR), thymine synthase (TS) and 5,10-methylene-tetrahydrofolate reductase (MTHFR). In contrast, adenosine is a product of the methionine cycle and is produced at high concentrations in tumour cells. The efficient intracellular elimination of this product by either adenosine-transforming enzymes, such as adenosine deaminase (ADA), or transport out of the cells by specific adenosine transporters, including the equilibrate nucleoside transporters (ENTs), is of vital importance for cancer cell survival. SAM, the primary biochemical methyl donor, is a co-substrate that reacts with nucleophilic acceptors in association with various methyltransferases.[14] The functionality of the labile methyl group is due to the energy-dependent adenosylation of methionine, which converts the inactive thiomethyl to an active sulphonium group. This reaction is catalysed by methionine adenosyltransferase (MAT), isoforms of which are tissue-specific and differentially regulated according to metabolic conditions. Substrates for SAM-methyl transfer reactions include DNA, RNA, proteins, neurotransmitters, and phospholipids. The products of the methylation reaction are a methylated substrate and SAH (Figure 1). This last product is favoured in equilibrium with adenosine and homocysteine catalysed by SAH hydrolase and inhibits the activities of most SAM-dependent methyltransferases.[15] Thus, the efficient clearing of the reaction products is vital in terms of meeting the methylation demand in the cell.[15]

POTENTIAL BIOLOGICAL FUNCTIONS OF SAM-DEPENDENT METHYLASES

Although the underlying mechanisms of carcinogenesis remain largely unknown, increasing evidence links aberrant regulation of methylation to carcinogenesis.[16-20] DNA methylation is mediated by DNA methyltransferase enzymes (DNMTs), which rely on the methyl donor SAM. Protein methyltransferases (PMTs), which methylate lysine or arginine residues on histones and other proteins, are emerging as an important group of enzymes that play key roles in normal physiology and human diseases.[20,21] Like DNMTs, PMTs utilise SAM as a key methyl donor. In addition to lysine or arginine, other SAM-dependent methyltransferases

also catalyse the carboxyl methylation of proteins. Examples of this class of methylases include leucine carboxyl methyltransferase (LCMT1)[22] and isoprenylcysteine carboxyl methyltransferase (ICMT),[23] which regulate important cellular signalling pathways by modifying the protein phosphatase PP2A[24] or the Ras family of GTP-binding proteins,[25] respectively. Like other posttranslational modifications, protein methylation is also subject to its counter modification, demethylation. The first reported protein demethylase was lysine-specific demethylase 1 (LSD1).[26,27] Here, we will focus on some of the implications of cellular methylases in the biology and physiology of cancer cells and in the response of cancers to therapy.

DNA methylation

Alterations of DNA methylation are an important component of cancer development.[16-19] Hypomethylation arises early during cancer development and is linked to chromosomal instability and loss of imprinting, whereas hypermethylation is associated with promoters and can arise secondary to gene (oncogene and suppressor) silencing.[19] DNA methylation may affect the transcription of genes in two ways. First, the methylation of DNA itself may physically impede the binding of transcriptional proteins to the gene, and second, and likely more important, methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs).[28,29] MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodelling proteins that can modify histones, thereby forming compact, inactive chromatin, which is known as silent chromatin.[30] In mammalian cells, DNA methylation occurs mainly at the fifth position carbon of cytosine within CpG dinucleotides and is carried out by two general classes of enzymatic activities, maintenance methylation and *"de novo"* methylation.[31] Maintenance methylation activity is necessary to preserve DNA methylation after every cellular DNA replication cycle. Without DNMT activity, the replication machinery itself would produce daughter strands that are unmethylated and, over time, would lead to passive demethylation. DNMT1 is the proposed maintenance methyltransferase that is responsible for copying DNA methylation patterns to the daughter strands during DNA replication.[32] Mouse models with both copies of DNMT1 deleted are embryonic lethal at approximately day nine due to the requirement of DNMT1 activity for development in mammalian cells.[33] It is hypothesised that DNMT3a and DNMT3b are the *"de novo"* methyltransferases that establish DNA methylation patterns early in development.[34] Because many tumour suppressor genes are silenced by DNA methylation

during carcinogenesis, there have been attempts to re-express these genes by inhibiting the DNMTs.[29,31] 5-Aza-2'-deoxycytidine (decitabine) is a nucleoside analogue that inhibits DNMTs by trapping them in a covalent complex on DNA by preventing the β-elimination step of catalysis, thus resulting in the enzymes' degradation.[31] However, it is currently unclear whether targeting DNMT1 alone is sufficient to reactivate tumour suppressor genes silenced by DNA methylation.

Methylation of histones

Chromatin undergoes dynamic changes, including massive structural reorganisation, during genetic processes such as DNA replication and cell division, transcription, DNA repair, and recombination. Because histone posttranslational modifications influence the structure and functions of chromatin, histone lysine methylation may control these fundamental biological processes.[35] With the discovery of SUV39H1 as the first histone lysine methyltransferase in 2000,[36] protein lysine methylation has gained tremendous attention. Following this discovery, numerous proteins have been found to possess methyltransferase activity, such as G9a,[37] MLLs,[38] EZH2,[39] SET2,[40] SET7/9 (also known as Set9),[41] DOT1L,[42] MMSET,[43] and SET 8 (also known as PR-Set 7 and SETD8).[44] The functions of these methyltransferases in processes such as genomic stability, DNA damage responses, or epigenetic regulation of the epithelial-mesenchymal transition (EMT) are only starting to become evident. For instance, recent research analysed the functional role of human histone H4-K20 methyltransferase SET8.[45] Histone H4-K20 can be mono-, di-, or tri-methylated, and SET8 catalyses monomethylation.[46] It was established that SET8 was important for proper progression through the cell cycle and that inhibition of SET8 expression by siRNA resulted in the massive accumulation of DNA damage that subsequently activated a Chk1-dependent checkpoint. This led to slower progression through the S phase and decreased proliferation. This report was the first time that a lysine methyltransferase was implicated in protection against genomic instability.[45]

While histone monomethylation is important for genomic stability, dimethylation of histones might play an important role in DNA damage responses.[42,43] The checkpoint mediator 53BP1 is directly recruited to chromatin regions flanking DNA double-strand breaks (DSBs).[47,48] In mammals, this occurs via interaction with histone H4, which is dimethylated at Lys20, or with histone H3 dimethylated at Lys79, with MMSET (also known as NSD2 or WHSC1) and DOT1L as the implicated methylases, respectively (Figure 2A).[42,43] 53BP1

plays an important role in the cellular response to DNA damage by acting as an adaptor in the repair of DNA DSBs. *In vitro* studies have demonstrated that 53BP1 binds more efficiently to H4K20-2Me and that this residue may be the major regulator of ionising radiation (IR)-induced 53BP1 foci formation; however, dimethylation of this residue is regulated in a cell-cycle specific manner with levels peaking during the S-phase and then becoming significantly reduced during G1- and G2/M-phase.[42] This finding suggests that an alternate means of 53BP1 recruitment may be required during these phases of the cell cycle. Interestingly, in contrast to what has been observed in yeast, H3K79-2Me levels in humans remain consistently high and do not appear to fluctuate throughout the cell cycle[49] and thus may provide a means for 53BP1 recruitment in response to IR stress encountered during the G1- and G2/M-phases. Because 53BP1 is a critical regulator of DNA damage signalling and repair to sites of IR-induced DNA damage, knockdown of MMSET or DOT1L led to a reduction of IR-induced 53BP1 foci formation, defects in DNA repair and increased sensitivity to IR.[42,43]

Other recent studies provide insights into the involvement of histone methylases in the epigenetic program of EMT and metastasis.[50,51] The interaction of G9a and Suv39H1 with Snail is critical for Snail-mediated E-cadherin repression in human breast cancer. These findings suggest that both H3K9-2Me and H3K9-3Me play a critical role in silencing the expression of E-cadherin. In one of these reports,[50] the authors found that Snail interacted with G9a and that this interaction was required for G9a and DNMT recruitment to the E-cadherin promoter for DNA methylation (Figure 2B). Knockdown of G9a restored E-cadherin expression by suppressing H3K9-2Me and blocking DNA methylation. This resulted in inhibition of cell migration and invasion *in vitro* and suppression of tumour growth and lung colonisation in *in vivo* models of claudin-low breast cancer (CLBC) metastasis. Together, these results indicated that blocking the binding in Snail-G9a-DNMTs may pave the way for the development of novel therapeutic approaches that target metastatic CLBC.

Methylation of non-histone proteins

In addition to histone methylation, many non-histone proteins have been identified as substrates of PMTs. Among them, the control of several transcription factors, such as p53 and E2F1, by lysine methylation has a marked importance for cancer biology. A pioneering example of the processes regulated by transcription factor methylation is the DNA damage response pathway. Genotoxic stress can induce methylation of p53 in at least four different lysine residues, which mediate the activation or repression of p53-dependent apoptosis.[52-55]

Lysine methylation can affect p53 function in multiple ways, including modulation of its DNA-binding activity, association with the co-activator 53BP1, or increase of p53 protein stability. Set9 promotes cell death via the methylation of p53 at Lys372, which stabilises the protein during DNA damage.[52] p53 is also methylated at Lys370, Lys382, and Lys373 by SMYD2, SET8, and G9a, respectively.[53-55] Similar to histones, this latter modification of p53 is dynamically regulated by LSD1.[56] The above studies point to an active involvement of histone-modifying enzymes in the DNA damage response pathway via direct modulation of p53 activity.

More recent studies indicate that E2F1, a transcription factor with dual and opposite functions in cell life and death,[57] is also dynamically regulated by epigenetic enzymes.[58-60] Thus, the methylation status of this transcription factor has been found to control both its stability and transcriptional activity. Recently, negative crosstalk between methylation and other post-translational modifications of E2F1, such as acetylation and phosphorylation, has been described (Figure 3).[58,59] Thus, E2F1 methylated at Lys185 is prone to ubiquitination and degradation,[58] whereas the demethylation of E2F1 favours its P/CAF-dependent acetylation at lysine residues 117, 120, and 125.[61] Whether acetylated E2F1 binds to the promoter of genes required for the S phase (to allow cell growth) or to the promoters of pro-apoptotic genes (to induce cell death) may depend on its subsequent phosphorylation by specific kinases. In response to severe DNA damage, the hyperacetylated E2F1 protein is stabilised through direct phosphorylation by Chk2 at Ser364 or ATM kinase at Ser31.[62,63] The methylase responsible for E2F1 methylation is Set9, the same enzyme that methylates p53 at Lys372, but with opposite consequences on their respective activities.[58,64] Although Set9-mediated methylation of E2F1 destabilises the protein and impedes E2F1-mediated apoptosis, the methylation of p53 by Set9 stabilises this transcription factor, which promotes apoptosis. The p53 tumour suppressor protein is an essential component of the cell response induced by genotoxic stress, but the *p53* gene is inactivated or mutated in the majority of human tumours. To overcome these obstacles, genes that can compensate or bypass cell death defects regardless of the *p53* status are particularly useful. E2F1 and its pro-apoptotic genes represent such a group of molecules and hence have direct implications as anti-neoplastic therapeutics for cancer lacking p53 activity. Because demethylation of E2F1 is required for its DNA damage-induced accumulation and the activation of its proapoptotic target genes (such as *p73*,

Apaf1 and *Bim*, among others), several demethylating therapies have recently been assayed with the hope of promoting E2F1-mediated apoptosis in cancer cells.[65-69]

TARGETING THE METHIONINE CYCLE WITH CLASSICAL ANTIFOLATES

In parallel with the development of novel inhibitors for specific DNA and protein methylases,[20,31] strategies to block the methionine cycle in cancer cells are being explored as possible anti-cancer therapies.[1,69,70] In addition to effects on nucleotide biosynthesis, treatments with the classical anti-folate methotrexate (MTX) have been linked to a decrease in cellular methylation.[71] In folate-deficient cells, including cells treated with anti-folates, depletion of N^5-methyl-THF blocks the remethylation of homocysteine and drives SAH hydrolase to catalyse the energetically favourable reverse reaction to synthesise SAH,[14] a potent product inhibitor of cellular methyltransferases.[72] According to this mechanism of action, the treatment of cancer cells with an anti-folate would result in a broad and indirect SAH-mediated inhibition of cellular methylases, including DNA and protein methyltransferases. Although MTX is one of the oldest chemotherapeutic drugs, it was not until early this century when the ability of this drug to inhibit the methylation of proteins was demonstrated.[73,74] Carboxyl methylation of Ras is important for proper plasma membrane localisation and function,[75] and therefore, the authors investigated the effects of MTX on the inhibition of ICMT and its possible consequences in Ras protein methylation. After MTX treatment of DKOB8 cells, Ras methylation was decreased by almost 90%, and this hypomethylation was accompanied by a mislocalisation of Ras to the cytosol.[73] Because the Ras signalling pathway plays a central role in the development and progression of human cancer, the results suggested that inhibition of ICMT was a critical component of the anti-proliferative effect of MTX.

In addition to its anti-tumour properties, the hypomethylating effect induced by MTX has also been linked to the resistance of melanomas to this drug.[67,76] Although MTX is the most frequently used anti-folate and is an efficient treatment for several types of cancer, it is not active against melanoma.[77] Experiments from our laboratory and others provided evidence that melanosomes contribute to the refractory properties of melanoma cells by sequestering cytotoxic drugs and increasing melanosome-mediated drug export.[78-81] We have demonstrated that folate receptor α-endocytotic transport of MTX facilitates drug melanosomal sequestration and cellular exportation in melanoma cells, ensuring reduced accumulation of MTX in intracellular compartments.[78] Although MTX is exported within a few hours in

contact with cells, in this short time, MTX is capable of inducing important changes in folate metabolism by initially depleting dihydrofolate and subsequently inducing the expression of folate-dependent enzymes.[76] This produces a lack of folate co-enzymes that blocks the methionine cycle in MTX-treated melanoma cells and induces the demethylation of proteins, such as PP2A and E2F1, with important consequences in the resistance of these cells to MTX.[67,76] On the one hand, by demethylating the PP2A catalytic subunit, MTX inhibits PP2A activity, resulting in Akt2-dependent phosphorylation of myosin Va, which has been proposed as a trigger for melanosome and drug export.[67] On the other hand, demethylation of E2F1 would result in the accumulation of E2F1 in its 'free' state, and in the absence of DNA damage, free E2F1 would be acetylated, leading to the transcription of genes required for S phase (Figure 2). The activation of E2F1 by MTX would allow S phase transition in melanoma cells, and importantly for melanoma survival, cells would recover an operative folate cycle, thereby restoring the original status of the E2F1 system. In the absence of exported MTX, high levels of TS and DHFR would impede the lethal depletion of dTTP and in turn, would produce a nucleotide imbalance that would favour a dTTP excess. Contrary to thymidine depletion, excess thymidine stops cells in S phase by blocking synthesis of DNA, an effect known as ‘thymidine block’.[66,76]

NOVEL ANTIFOLATES AS DEMETHYLATING AGENTS

Based on the observation that classical and non-classical anti-folate compounds possess similar chemical structures to some tea polyphenols, our research group began to explore the hypothesis that tea catechins could inhibit DHFR activity.[82] We observed that ester-bonded gallate catechins isolated from green tea, such as epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG), were potent inhibitors of DHFR activity *in vitro* at concentrations found in the serum and tissues of green tea drinkers and that EGCG could bind to human DHFR in a similar orientation to that observed for a number of structurally characterised DHFR inhibitor complexes.[82] Since this first report describing the inhibition of DHFR by tea polyphenols, studies by various laboratories have reported that EGCG inhibited DHFR from a variety of biological sources.[83-88] Interestingly, tea polyphenols have shown the capacity to disrupt folate metabolism in cancer cells[89,90] and reduce the bioavailability of folate in pregnant woman, which has associated the consumption of tea during early pregnancy with the risk of spina bifida.[91,92] Despite the excellent anti-tumour properties of tea catechins, their low bioavailability is a limitation to treatment. In an attempt to solve such

bioavailability problems, we first synthesised a 3,4,5-trimethoxybenzoyl analogue of ECG [3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin, TMECG].[93] In comparing the anti-proliferative activity of TMECG on several human and mouse cancer cell lines, we noticed that this compound was much more active on melanoma cells than on other epithelial cancer cell lines from breast, lung, and colon cancers.[94] As one of the most striking differences between melanoma and other epithelial cells is the presence of tyrosinase in melanoma, we investigated whether TMECG cytotoxicity against melanoma might be mediated by cellular tyrosinase activation. The results indicated that tyrosinase oxidised TMECG to its corresponding *o*-quinone, which quickly evolved through a series of chemical reactions to a quinone methide (QM), which showed high stability over a wide pH range. The TMECG-QM was found to be a potent irreversible inhibitor of human DHFR, and this highly stable product may be responsible for TMECG's high activity against melanoma cells.[94] Importantly, it was observed that TMECG modulated the expression of genes involved in methionine metabolism, cellular methylation, and glutathione synthesis in melanoma cells.[70]

DRUG COMBINATIONS AS EFFECTIVE ANTI-MELANOMA DEMETHYLATING THERAPIES

Currently, limited therapeutic options exist for patients with metastatic melanomas, and all standard therapies used in the clinic have low efficacy and poor response rates. The observation that TMECG escapes both general and specific mechanisms of drug resistance in melanomas[95] aimed us to develop novel therapeutic strategies against this elusive type of cancer.[68,70] First, we observed that a combined therapy designed to uncouple adenosine metabolism using dipyridamole (DIPY) (an effective inhibitor of both ENTs and ADA) in the presence of TMECG simultaneously and efficiently blocked both the folic and methionine cycles in melanoma cells and resulted in massive cell death.[70] A second strategy was designed to avoid tumour heterogeneity.[68] The failure of current approaches of cancer therapy reflects an underlying problem that has long been recognised,[96] but which is only beginning to be understood at the molecular level: cancer cell population heterogeneity. Advances in understanding the genetics underpinning melanoma progression have been complemented by observations that the Microphthalmia-associated transcription factor (MITF)[97,98] acts as a rheostat in determining sub-population identity.[99-100] In melanomas, reduced MITF expression leads to G1 arrested, invasive cells with stem-like properties,[99,101] including the ability to initiate tumours with high efficiency. Low levels of MITF, driven by transcriptional

repression by the BRN-2 (POU3f2) transcription factor,[102,103] lead to a p27-mediated cell cycle arrest and an invasive stem cell-like phenotype.[99,101] By contrast, elevated MITF leads to activation of differentiation genes driving melanin production such as tyrosinase, and melanosome biogenesis.[104-106] Consequently tumours comprise a mix of MITF-positive and negative melanoma cells.[102] As such, modulation of MITF expression represents one approach towards driving heterogeneous populations of tumour cells to a therapeutically-sensitive phenotype. Although MITF is required for melanoma proliferation[99] and has been described as a lineage addiction oncogene, decreasing MITF expression as proposed[106] would potentially lead to an increase in invasive melanoma cells.[99,101] Therefore, we took the opposite approach to therapy, with the aim of eradicating invasive melanoma cells and at the same time sensitising cells to the tyrosinase-processed anti-folate pro-drug TMECG.[68] The combination of MTX and TMECG led to demethylation of E2F1, depletion of thymidine pools, DSBs, and highly efficient E2F1-mediated apoptosis in culture and *in vivo*. As a result, we described a potent anti-melanoma therapy that was highly effective in pre-clinical trials irrespective of *BRAF* or *p53* status and that validated the concept of directed phenotype switching as a therapeutic option.[68]

DISRUPTING THE EPIGENETIC MACHINERY IN CANCER CELLS

TMECG was shown to be active exclusively in melanoma;[94] however, its catechin epimer derivative, 3-*O*-(3,4,5-trimethoxybenzoyl)-(−)-catechin (TMCG), showed substantial anti-proliferative activity in other epithelial cancer cell lines, including those from breast cancer.[107] DIPY prevents triple-negative breast-cancer progression,[108] and the potential of a TMCG/DIPY combination as a valuable epigenetic therapy against breast cancer was evaluated.[69] This pioneer study analysed the implications of several epigenetic mechanisms and their molecular connections in the reactivation of RAS-association domain family 1 isoform A (*RASSF1A*), a tumour suppressor gene pathway that can regulate proliferation, induce apoptosis, and bind to and stabilise microtubules.[109] Because the gene remains intact but dormant in most tumours,[110-112] reactivation by promoter demethylation presents an attractive approach to therapy.[109] The authors observed that by simultaneous modulation of DNA and E2F1 methylation the TMCG/DIPY combination acted as an epigenetic treatment that reactivated *RASSF1A* expression and induced apoptosis in breast cancer cells (Figure 4A).[69] TMCG/DIPY combination induced RASSF1A-dependent apoptosis in breast cancer cells by targeting several methylation-controlled processes (Figure 4B).[69] After its

stabilisation by acetylation and phosphorylation, E2F1 controls the expression of many pro-apoptotic genes, such as *p73*, *Apaf1, HIC1,* and *RASSF1A*.. In fact, in addition to *RASSF1A*, the promoters of two other tumour suppressor genes and well-characterised E2F1 targets,[62,113] *HIC1* and *p73*, were also found to be highly demethylated after the treatment. The RASSF1A tumour suppressor protein has been observed to function in a coordinated manner with the product of another E2F1 target gene, *p73*, to elicit apoptosis through the pro-apoptotic mammalian STE20-like kinases MST2 pathway.[114] Indications that the MST1/2 pathways might be involved in RASSF1A-mediated apoptosis have also recently been described by Guo et al.[115] These authors showed that the RASSF1A protein functions in MST kinase pathways to provide and preserve the phosphorylated/active state of MST1 and MST2 by preventing dephosphorylation of these kinases by the protein phosphatase PP2A. This discovery illustrates the great complexity in cell apoptotic pathways. Intriguingly, PP2A, a phosphatase that prevents MST1/2-induced apoptosis by competing with RASSF1A, is also controlled by carboxyl methylation of its protein. Because LCMT1, the methylase responsible for PPA methylation, is specifically dependent of SAM, an increase in cellular SAH levels after the treatment of breast cancer cells with TMCG/DIPY may also result in the inhibition of PP2A assembly. Thus, the absence of PP2A activity in TMCG/DIPY-treated cells would facilitate MST kinases-RASSF1A-mediated apoptosis.[115] Because the death pathway induced by this combination does not depend on functional p53,[69] this strategy for simultaneously targeting DNA and protein methylation may also be useful for the treatment of breast tumours harbouring *p53* mutations.

FUTURE PERSPECTIVES

Epigenetics and metabolism are highly interconnected in a reciprocal fashion.[5] Because epigenetic factors contribute to the origin and development of cancer, chemical agents or natural compounds that are direct or indirect regulators of the epigenome constitute an excellent approach in cancer prophylaxis and potentially in tumour therapy. Because of the importance of methylation for a variety of cellular functions, intercellular SAM concentrations and the SAM/SAH ratio have been reported to contribute to variations in biological processes ranging from the epigenetic regulation of gene expression to the control of DNA damage response pathways. All of these experimental results accumulated during the last decade suggest that the methionine cycle may become a valuable therapeutic target. Although the specific inhibition of DNA or protein methylases primarily resulted in specific

unmethylated products, therapies based on the inhibition of the methionine cycle would result in the indirect inhibition of both DNA and protein methylation. The plethora of genes and pathways affected by DNA and protein methylation could make this global strategy remarkably desirable. The effects of hypomethylation therapy would then be the sum of multiple effects on cellular physiology, and it is likely that the net effect would be favourable therapeutically. Indeed, this non-specificity can be viewed as advantageous where multiple defects are corrected simultaneously. Therefore, it may be possible that the application of new therapies to target the epigenetic machinery of cancer cells could be of utility to promote E2F1-dependent apoptosis in p53-defiant tumours, avoid metastatic pathways in cancer cells and/or sensitise tumour cells to radiation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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LEGEND TO FIGURES

Figure 1. The methionine cycle and its connections with several metabolic and survival cell pathways. Abbreviations: ADA, adenosine deaminase; AHYC, S-adenosylhomocysteine hydrolase; COMT, catechol-*O*-methyltransferase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DNMT, DNA methyltransferase; ENT, equilibrative nucleoside transporter; GCS, γ-glutamylcysteine synthetase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TS, thymine synthase. Adenosine is efficiently metabolised by specific enzymes (such as ADA and adenosine kinase) before use in purine nucleotide synthesis, which is particularly necessary for DNA synthesis in highly proliferating cells. Excess adenosine can be transported out of the cells by ENTs, which are bi-directional transporters that allow adenosine release and uptake by facilitating diffusion along its concentration gradient. However, in the presence of an anti-folate compound, adenosine accumulation may represent a severe problem for the cell. In folate-deficient cells, the resulting accumulation of homocysteine drives AHYC to catalyse the energetically favourable reverse reaction and synthesise SAH, a potent product inhibitor of cellular methyltransferases.

Figure 2. Involvement of histone methylases in DNA damage responses and in the EMT epigenetic program. (A) Upon the generation of DNA damage, MMSET and DOT1L, respectively, dimethylate Lys20 on histone H4 (H4K29) and Lys79 on histone H3 (H3K79) near sites of DNA double-strand breaks (DSBs). Then, the checkpoint mediator 53BP1 is directly recruited to chromatin regions flanking DSBs. (B) Model illustrating the interaction of Snail with G9a and DNMTs leading to E-cadherin promoter methylation and EMT induction.[50]

Figure 3. Regulation of E2F1 by post-translational modifications. (A) Schematic representation of the E2F1 protein. Residues susceptible to methylation (Lys185), acetylation (Lys117, Lys120, and Lys125), and phosphorylation (Ser31 and Ser364) are shown. (B) E2F1 is regulated by several posttranslational modifications, including methylation (Me), acetylation (Ac) and phosphorylation (P).[58] E2F1 is reversibly methylated by the enzymatic actions of LSD1 and Set9.

Figure 4. Reactivation of the tumour suppressor *RASSF1A* and induction of RASSF1A-dependent apoptosis in breast cancer cells by simultaneous targeting of DNA and protein methylation. (A) Inhibition of DNMTs by the TMCG/DIPY combination may result in the remodelling of chromatin in the region of the RASSF1A promoter into a transcriptionally permissive state, which may permit the binding of transactivated E2F1 (and other transcription factors) and synthesis of the RASSF1A transcript. (B) Several methylation-controlled processes coordinately participate in RASSF1A-mediated apoptosis in breast cancer cells. Demethylation of promoters of tumour suppressor genes would facilitate the binding of transcription factors, while demethylation of E2F1 would favour its stabilisation and its selective binding to promoters of proapototic genes. Both processes would result in the expression of several proapoptotic transcripts and proteins. Lastly, demethylation of PP2A would facilitate MST kinases-RASSF1A-mediated apoptosis. PP2A is a trimeric serine/threonine phosphatase that contains a regulatory subunit B, which is recruited by a C-A dimer composed of the catalytic subunit C and a structural subunit A.[116] Recruitment occurs when C is carboxyl-methylated on the terminal Leu309, which results in the assembly of the active PP2A trimer. Thus, changes in PP2A methylation can modulate the specificity and activity of PP2A in cells.

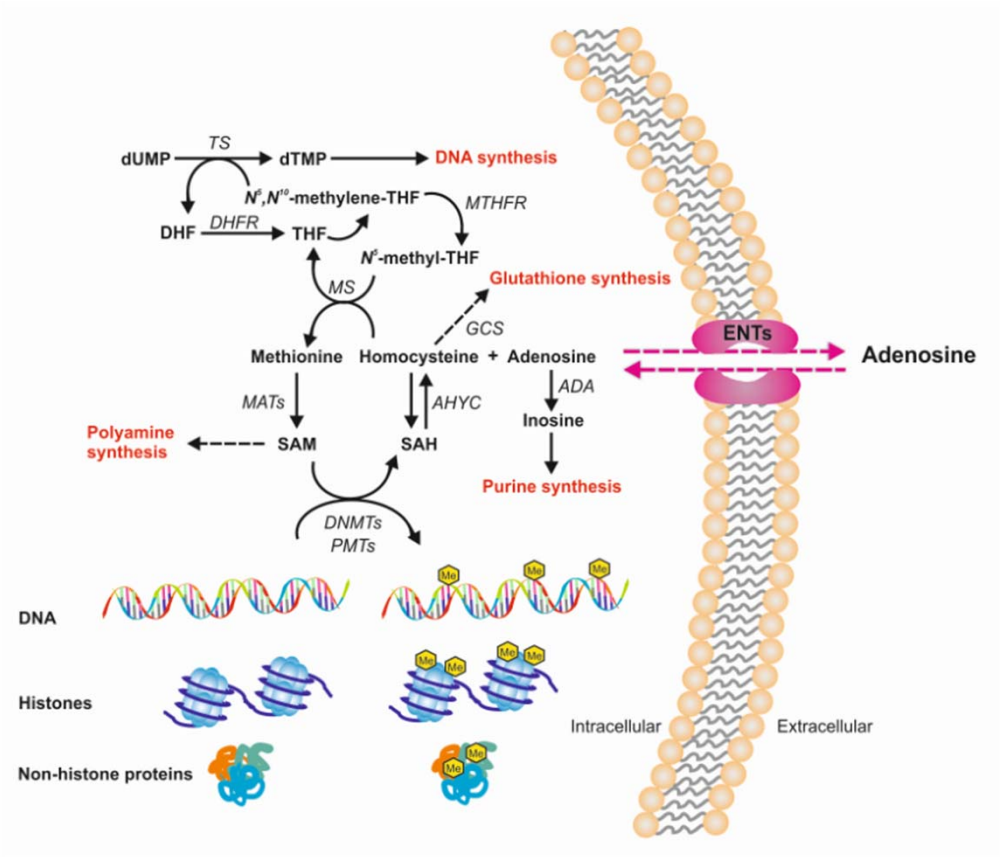


Figure 1. Montenegro et al.

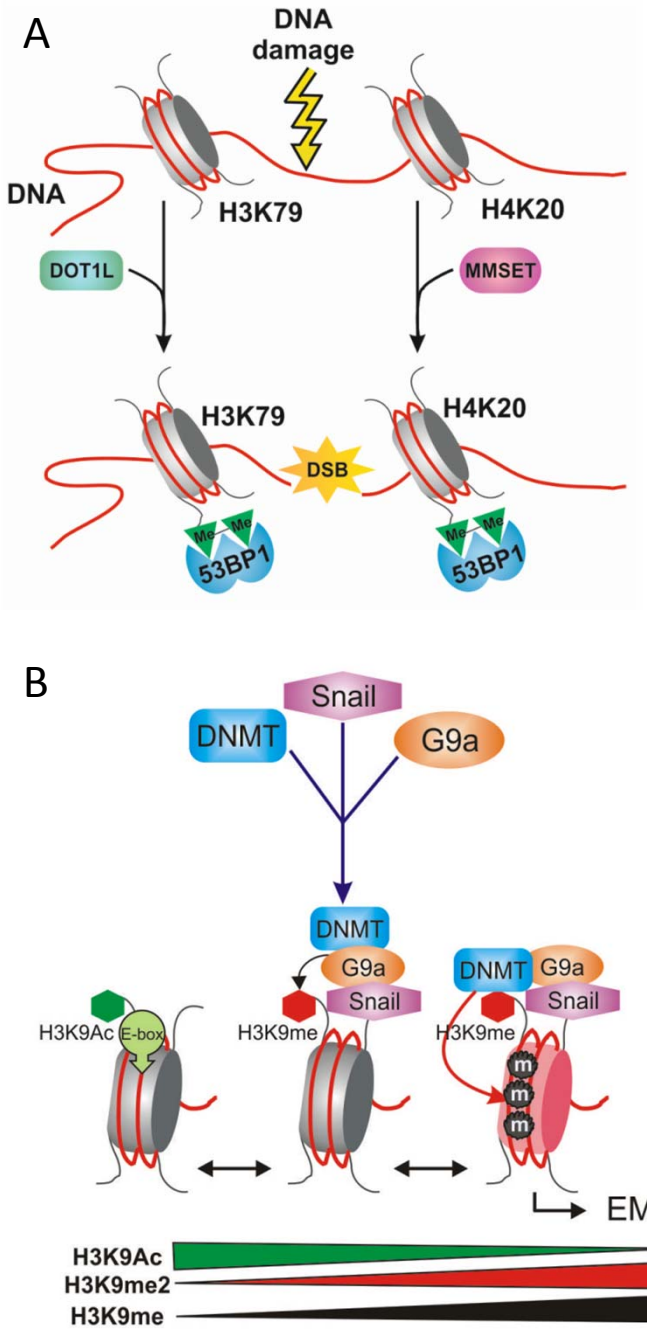


Figure 2. Montenegro et al.

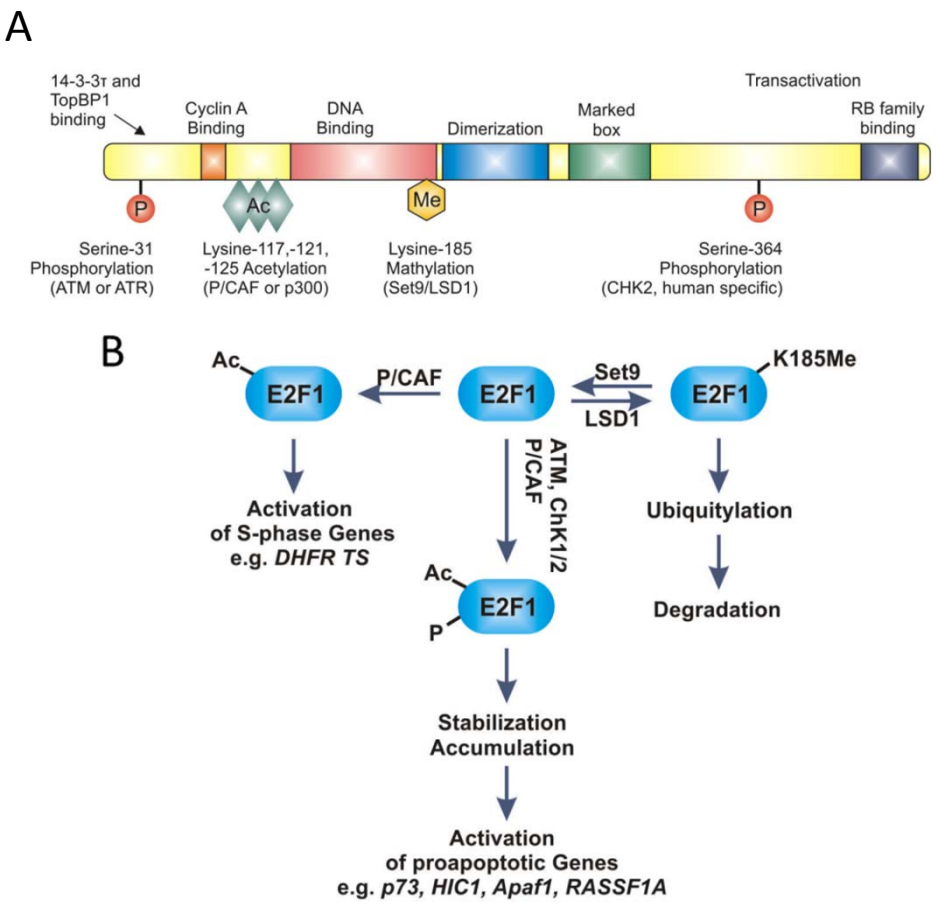
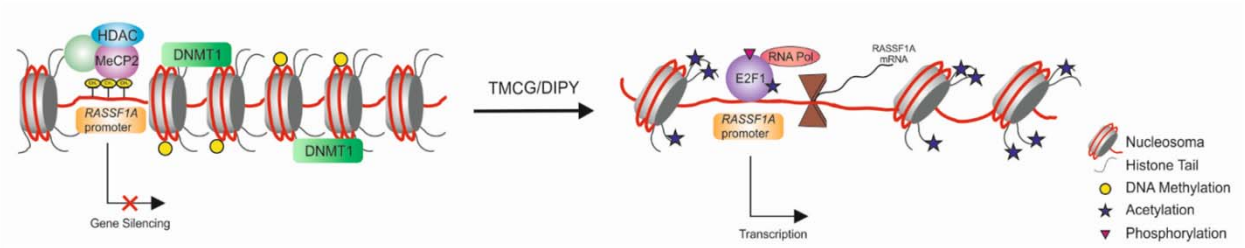


Figure 3. Montenegro et al.

A



B

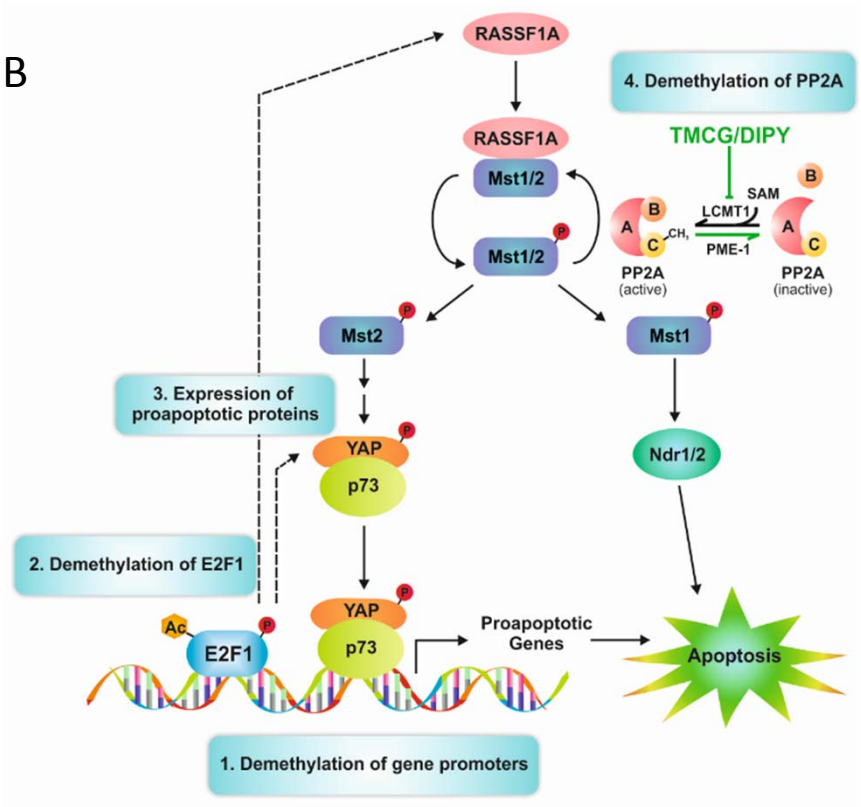


Figure 4. Montenegro et al.