Dnmt1: Structure and Function

Željko M. Svedružić

Medical Biochemistry, PB Rab, Faculty of Medicine, University of Rijeka, Rab, Croatia

Dnmt1, the principal DNA methyltransferase in mammalian cells, is a large and a highly dynamic enzyme with multiple regulatory features that can control DNA methylation in cells. This chapter highlights how insights into Dnmt1 structure and function can advance our understanding of DNA methylation in cells. The allosteric site(s) on Dnmt1 can regulate processes of *de novo* and maintenance DNA methylation in cells. Remaining open questions include which molecules, by what mechanism, bind at the allosteric site(s) in cells? Different phosphorylation sites on Dnmt1 can change its activity or ability to bind DNA target sites. Thirty-one different molecules are currently known to have physical and/or functional interaction with Dnmt1 in cells. The Dnmt1 structure and enzymatic mechanism offer unique insights into those interactions. The interacting molecules are involved in chromatin organization, DNA repair, cell cycle regulation, and apoptosis and also include RNA polymerase II, some RNA-binding proteins, and some specific Dnmt1-inhibitory RNA molecules. Combined insights from studies of different enzymatic features of Dnmt1 offer novel ideas for development of drug candidates, and can be used in selection of promising drug candidates from more than 15 different compounds that have been identified as possible inhibitors of DNA methylation in cells.

Progress in Molecular Biology
and Translational Science, Vol. 101
DOI: 10.1016/B978-0-12-387685-0.00006-8

1877-1173/11 $35.00

I. Introduction

DNA methylation is a fundamental mechanism in functional organization of the human genome. Studies of DNA methylation can help us to tackle some of the key questions in the current biomedical sciences, such as carcinogenesis,[1] host infection by different viruses,[2–7] cell differentiation,[8] autoimmune diseases,[9] different types of mental illness and neurological disorders,[10–12] and environmental toxicology.[13] Unfortunately, effective therapies and preventive treatments targeting DNA methylation are still very underdeveloped. This is in large part due to lack of knowledge about the molecular mechanism of DNA methylation, starting with the principal DNA methyltransferase in human cells, Dnmt1 (DNA methyltransferase 1).

The first evidence for DNA methylation was reported in 1948,[14] and 27 years later, the first attempts to purify mammalian DNA methyltransferase were described.[15] Over the next 15 years, Dnmt1 was found to be a very large and a complex enzyme. Dnmt1 has catalytic preference for hemimethylated sites,[16,17] and its activity can be regulated by a specific group of DNA and RNA molecules.[18] Dnmt1 has high kinetic preference for poly(dI-dC) substrate, and a distinct allosteric site.[16] The molecular biology era of Dnmt1 began with cloning of its cDNA[19] and the full-length gene in 1992.[20] The first complete purification from mammalian cells[21] laid ground for more than a dozen mechanistic studies (reviewed in Ref. 22). Mechanistic studies of Dnmt1 were greatly aided by the first crystal structure of a flipped-out target base by bacterial enzyme M.HhaI[23] and the numerous subsequent mechanistic studies that were inspired by that landmark achievement.

Dnmt1 expression using a baculovirus vector[24–26] allowed preparation and characterization of different fragments of Dnmt1,[27–29] and made purification of Dnmt1 almost routine for many research groups (today, purified Dnmt1 can be also purchased from several commercial sources). The majority of Dnmt1 studies have been focused on the function of its allosteric site,[22,30–32] on selectivity between *de novo* and maintenance methylation,[30,33] and on characterization of different Dnmt1 fragments.[28,29,34,35] Relatively little work has been done on characterization of the catalytic mechanism in the active site,[36,37] on the mechanisms of action for proposed inhibitors of Dnmt1,[38] and on the multiple phosphorylations of Dnmt1.[39,40] A large research effort has been devoted to search for molecules that interact with Dnmt1 in cells. At this writing (December, 2010), 31 different molecules have been found to interact with Dnmt1 (Table I). Many of those interactions have been mapped to different parts of Dnmt1 sequence,[35,72] only a small fraction of those interactions have been functionally characterized. The majority of Dnmt1 studies

TABLE I
Summary of Molecules Currently Known to Interact Physically and/or Functionally with Dnmt1

Core chromatin replication complex	DNA repair, cell cycle control, and regulation of apoptosis	RNA-directed DNA methylation
– Dnmt3a and Dnmt3b[41] – SNF2h-containing chromatin-remodeling complex NoRC[42] – LSH protein (lymphoid-specific helicases) protein related to the SNF2 family of chromatin-remodeling ATPases[43] – PCNA, DNA clamp processivity-promoting factor[44] – UHRF1[45,46] – HP1β, heterochromatin protein 1β isoform (chromobox protein)[47,48] – SUV39H1, histone-lysine *N*-methyltransferase[48] – G9a histone methyltransferase[49] – HDAC1 and HDAC2, histone deacetylase 1/2[50–52] – PML-RAR promyelocytic leukemia-retinoic acid receptor, oncogene transcription factor[53] – RIP140, metabolic repressor, also known as NRIP1 (nuclear receptor interacting protein 1)[51] – CFP1 CXXC finger protein 1 (PHD domain)[54] – MBD2/MBD3, methyl-CpG-binding domain protein[55] – PcG-EZH2 Polycomb-group proteins enhancer of Zeste homolog 2[56]	– PARP-1 (poly(ADP-ribose) polymerase 1) and poly(ADP-ribose)[57,58] – pRb/E2F1, Retinoblastoma tumor suppressor protein, control of G1/S transition and S-phase[29,59,60] – p53, tumor suppressor, regulation of cell cycle and apoptosis[61] – DMAP1, DNA methyltransferase 1-associated protein 1[50,62,63] – RGS6 member of mammalian RGS (regulator of G-protein signaling) proteins[62] – CK1δ/ϵ kinase that phosphorylates Dnmt1[40] – Annexin V, scaffolding proteins that anchors other proteins to the cell membrane and participates in apoptosis[64] – Hsp90, chaperon[65] – p23, cochaperone[66] – SET 7, protein lysine methyltransferase (Chapter by Shannon R. Morey Kinney and Sriharsa Pradhan) – ATK1, serine/threonine protein kinase (Chapter by Shannon R. Morey Kinney and Sriharsa Pradhan)	– MeCP2, methyl-CpG binding domain protein 2 (Rett syndrome)[67,68] – RNA Pol II[69–71] – Specific tRNA and mRNA[18,24]

The interacting molecules are arranged in different classes in an attempt to highlight the various physiological processes that can regulate DNA methylation in cells.

used human[27–29,31,32] and murine enzymes[30,33,34,36,39,73,74]; the two enzymes share 78% sequence identity and many of the fine details in catalytic mechanism.[36]

Today, 35 years after the first reported study of mammalian DNA methyltransferase,[15] the results from different studies of Dnmt1 structure and function have been summarized in a number of excellent review articles. These cover distinct areas, including a general description of different DNA methyltransferases and DNA methylation,[75–77] different Dnmt1 fragments and their expression,[35,77] enzymatic properties and enzymatic assays of Dnmt1,[22] and functional interactions with molecules involved in DNA methylation.[72,78,79] This chapter is written as an extension of the earlier review articles in the desire to motivate use of the knowledge about Dnmt1 structure and function to advance our understanding of DNA methylation in cells. One of the major limiting factors in current DNA methylation research is a poor connection between cell-based, enzyme-based, medicinal chemistry, and pharmacological studies of DNA methylation. In Section II, I will highlight the major points about Dnmt1 structure and enzymatic function with a specific emphasis on development of Dnmt1 inhibitors. In Section II, I will discuss how Dnmt1 structure and its enzymatic mechanism can help us to understand its functional interactions with other molecules involved in DNA methylation in cells. Finally, in Section IV, I will summarize the results of a new Dnmt1 crystal structure, published as this chapter was being completed.

II. The Functional Domains of Dnmt1 and Dnmt1 Inhibitors

The first landmark study of the catalytic mechanism of bacterial DNA methyltransferase M.HhaI[80] was followed by more than a hundred crystallographic, enzyme kinetics, biophysical, and computational studies to reach the current knowledge about this relatively small enzyme. M.HhaI is about five times smaller than Dnmt1; nevertheless, the success of M.HhaI studies greatly advanced our understanding of Dnmt1 and showed how much work is needed to understand large complex enzymes like Dnmt1. Studies of Dnmt1 structure and function can answer many of the key questions in physiology of DNA methylation, such as: (i) design of Dnmt1 inhibitors and activators, (ii) interaction with other molecules, (iii) Dnmt1 phosphorylation and methylation, or (iv) the mechanism that controls the difference between *de novo* and maintenance methylation activity of Dnmt1.

We still do not know to what extent Dnmt1 can function as maintenance or *de novo* methyltransferase in cells.[78,79] This is one of the key questions in DNA methylation research since the changes in methylation patterns can trigger pathological events.[75,81,82] Dnmt1 is often described in the literature as the maintenance methyltransferase, based on the initial studies that reported higher activity on hemimethylated sites relative to unmethylated sites more than 20 years

ago.[16,17] The crucial insights about Dnmt1 mechanism that have accumulated in the past 20 years have not yet been incorporated into the great majority of the current publications that have attempted to describe DNA methylation in cells.[1,72,75,81,83,84] The often quoted large preference for hemimethylated sites can be observed only in the early presteady state that represent only one segment of different features of allosteric regulation of Dnmt1.[22,30] In the case of allosteric regulation with fully methylated DNA, Dnmt1 is almost equally effective as a maintenance and a *de novo* methyltransferase[30]; under those conditions, the catalytic activity of Dnmt1 can be about 10-fold higher than that of the alleged *de novo* methyltransferases Dnmt3a and Dnmt3b.[32,33,36,85,86] Finally, in certain conditions, the allosteric regulation can completely inhibit Dnmt1 activity on any DNA substrate.[74,87] In sum, our ability to understand DNA methylation and Dnmt1 activity in cells directly depends on our ability to understand its allosteric regulation.[22,30] To complete our understanding of allosteric regulation of Dnmt1, we still have to answer several key questions: (i) How many different allosteric sites exist on Dnmt1?[29,34,88] (ii) Is there cooperativity between the different allosteric sites? (iii) How allosteric regulation affects the catalytic activity of Dnmt1 and its ability to bind the substrate DNA?[74] (iv) What is the binding specificity of the allosteric site(s) (i.e., DNA or RNA, unmethylated or premethylated, single stranded or double stranded)?[22]

Studies of Dnmt1 structure and function can also help us to understand its interaction with other molecules, or different phosphorylations that target Dnmt1. As noted above, 31 different molecules were reported to interact with Dnmt1 (Table I), and this number is likely to grow in the future. It is a delusion to think that we can understand 31 different interactions *in situ* in cells if we do not understand the enzymatic properties of purified Dnmt1. Purified Dnmt1 is first needed for description of different functional properties of this unique enzyme, and then the acquired insights can be used to understand the interaction with other molecules or the functional consequences of different phosphorylation events targeting Dnmt1. In essence, the interacting molecules or phosphorylations can (i) stimulate or inhibit Dnmt1 activity, (ii) guide Dnmt1 to methylation sites, (iii) facilitate dissociation from target-DNA sites, and (iv) modulate the ability of Dnmt1 to interact with other molecules. All four of these events can be measured with purified Dnmt1 using currently available methods.[22,28–30,34] The basic principles for building correlations between Dnmt1 activity in cell-based and enzyme-based assays have been described.[22] The studies of interaction between Dnmt1 and retinoblastoma protein (Rb) are one example of an admirable success in studies of Dnmt1 structure and function, its interaction with other molecules in cells, and the physiological significance of those events.[29,59,60]

The third key segment where studies of Dnmt1 structure and function can have a major impact is the development of Dnmt1 inhibitors or activators. Both, Dnmt1 inhibitors and activators, are acutely needed as a research tool for

studies of different physiological processes that depend on DNA methylation, and ultimately in clinics for treatment of cancer, viral infections, mental illness, or autoimmune diseases. Both activators and the inhibitors are attractive, as the pathological processes related to DNA methylation depend on both an increase and a decrease in DNA methylation at specific genomic loci.[81,82] Dnmt1 has a number of regulatory mechanisms and a number of potential target sites that can be exploited in developing inhibitors and activators. Unfortunately, we still do not have a satisfactory inhibitor of Dnmt1. A number of compounds that inhibit or alter DNA methylation in cells have been described in the literature. Based on their structure and/or putative mechanism of action, the inhibitors can be divided to different groups: (i) cytosine analogues,[89] (ii) AdoMet analogues,[22,90] (iii) hydrazines,[91] (iv) phthalimides,[92] (v) polyphenols,[93] (vi) aminobenzoic derivatives,[94,95] (vii) modified oligonucleotides,[87] (viii) antisense oligos,[96] and (ix) disulfide analogues.[97]

A comprehensive analysis of different Dnmt1 inhibitors indicates that, despite the impressive number of inhibitors reported in the literature, all of those compounds are plagued with fundamental problems.[38,93] Many lack reproducibility,[98] some do not target-DNA methyltransferases and affect DNA methylation only indirectly, and many have unacceptable toxicity. Some compounds that target Dnmt1 have weak inhibition potency, so additional mechanistic studies are needed to increase the potency. Finally, some of the inhibitors share structural features with environmental toxins.[13] Very little of the current knowledge regarding the enzymatic mechanism of mammalian DNA methyltransferase has been used in inhibitor development. Some exceptions are studies of procainamide,[94] single-stranded oligonucleotide inhibitors,[87] or cytosine analogues.[89,99] Despite of described limitations, some of the compounds have entered clinical trials, some have even reached late stage clinical trials, while 5-aza-2′-deoxycytidine (Dacogen®) and 5-azacytidine (Vidaza®) have been approved for treatment of myelodysplastic syndrome.[38]

Dnmt1 has about 1620 amino acids; the actual length depends on the species and the expression of tissue-specific exons.[79] The studies of large enzymes like Dnmt1 can greatly benefit if the enzyme can be divided to smaller domains. Historically, Dnmt1 is divided into a smaller catalytic domain and the large N-terminal regulatory domain.[100] The separation into catalytic and the regulatory domains will be used in the next two sections, to highlight key aspects of Dnmt1 structure and function, with emphasis on development of Dnmt1 inhibitors.

A. The Catalytic C-Terminal Domain

Sequence analysis[101] and the crystal structures of bacterial DNA methyltransferases[23] showed that cytosine carbon 5 DNA methyltransferase, such as Dnmt1, needs at least three structural elements for catalytic activity: an

AdoMet-binding domain, a target base-binding cavity, and a target sequence recognition domain. The first two can be identified, based on conserved sequence motifs.[101] In Dnmt1, the AdoMet-binding site and the target base-binding cavity can be found within the C-terminal region after multiple Gly-Lys repeats.[20] The target recognition domain (TRD) is not precisely defined by the conserved sequence motifs, and cannot be assigned with certainty based on the DNA sequence. Historically, catalytic domain is defined as the C-terminal sequence after the Gly-Lys repeats.[100] This "catalytic domain" does not have catalytic activity.[29,34,102] For catalytic activity, Dnmt1 needs to have about 1000 amino acids from the C-terminal region; precisely the human Dnmt1 constructs missing the first 121, 501, 580, or 621 amino acids are still active, but those missing the first 672 amino acids or more are not active.[28,29,35]

Almost everything that is known today about the catalytic mechanism in the active site of cytosine carbon 5 DNA methyltransferases, such as Dnmt1, comes from studies of bacterial enzymes, most notably M.HhaI. Computational studies, enzyme kinetics, and protein crystallography have been used to describe fine details of its DNA binding, AdoMet binding, base flipping, and catalytic mechanism, encompassing more than 100 relevant studies. About 20 different crystal structures of M.HhaI can be used to trace different catalytic steps in atomic detail.[23,103–113] The crystal structures of M.HhaI also gave valuable insights into the mechanisms of inhibition by cytosine analogues.[89,99] In contrast to M.HhaI, only a handful of studies have analyzed the catalytic mechanism in the active site of Dnmt1. There is a general assumption that Dnmt1 and M.HhaI share the same mechanism in the active site based on the conserved sequence motifs,[20,101] ^{3}H exchange reaction,[36,114] and inhibition by 5-fluoro-cytosine[37] and other cytosine analogues.[38,89] Further comparisons between Dnmt1 and M.HhaI can be a productive strategy to advance our understanding of the catalytic mechanism in the active site of Dnmt1, and the mechanisms of inhibition for the inhibitors that target the catalytic domain.

Methylation and ^{3}H exchange reactions with different AdoMet analogues showed that Dnmt1 and M.HhaI share some very fine features in the catalytic steps that take place once the target base is positioned in the active site cavity.[36,114] In particular, M.HhaI and Dnmt1 have very similar catalytic processes at the carbon 5 of the activated target base including the methyltransfer step.[36,114] The observed similarity is encouraging for studies of Dnmt1 inhibitors, as many of the current inhibitors exploit catalytic processes at the carbon 5 of activated target base. The methyltransfer step is the rate-limiting step for both enzymes.[36,114] Interestingly, even though Dnmt1 and M.HhaI share the same rate-limiting step in methylation and ^{3}H exchange reaction, M.HhaI can have more than a 100-fold faster catalytic rate.[36,114] The difference in catalytic rates can be attributed to the difference in the rapid equilibrium between the initial steps that lead to the target base attack, that is, the recognition of the target base, the base-flipping steps, and

formation of the unstable covalent adduct intermediate[36] (base-flipping experiments have never been reported for Dnmt1; however, it is very likely that Dnmt1 like other DNA methyltransferase flips the target base out of DNA helix[23]). Due to such rapid equilibrium, both M.HhaI and Dnmt1 have a unique feature that their catalytic rates can be controlled independently at two levels: (i) the rate-limiting methyltransfer step and (ii) the rapid equilibrium prior to the irreversible rate-limiting step.[22,36,114] Changes in the rapid equilibrium between the initial catalytic steps is the most likely explanation for the surprisingly high kinetic preference for poly(dI-dC) substrates that are unique to Dnmt1 and cannot be observed with M.HhaI.[16,36] Also it appears that the allosteric regulation of Dnmt1 leads to changes in the methylation rates as a result of changes in the rapid equilibrium between the early catalytic steps.[22,36,114]

The conserved sequence motifs defining the active site cavity translate into very fine similarities in the catalytic steps in the active sites of Dnmt1 and M.HhaI. Dnmt1 and M.HhaI also share conserved sequence motifs that define the AdoMet-binding site; however, the two enzymes show a number of significant differences in interaction with AdoMet. Dnmt1 has a relatively high K_m for AdoMet in comparison to the bacterial enzymes, up to 100-fold higher than the value measured with M.HhaI.[32,33,115,116] With Dnmt1, DNA binding does not lead to a large change in AdoMet-binding affinity as in M.HhaI.[28,36,115,116] However with Dnmt1, AdoMet binding at the start of catalysis can control enzyme activity through slow structural changes that appear to be coupled to allosteric inhibition.[22,29,36] Unlike Dnmt1, M.HhaI shows very rapid rates of target base attack in the absence of AdoMet. This is very surprising, as target base attack in the absence of AdoMet will greatly increase the chances of mutagenic deamination.[117–120] The ability to support mutagenic deamination might be an inherent deficiency of the bacterial enzymes, or a physiological adaptation that can be advantageous to bacterial enzymes but not to Dnmt1.[36,114] Briefly, under conditions of AdoMet insufficiency, mutations induced by the bacterial methyltransferase might slow digestion of host DNA by cognate endonucleases by changing occurrences of the recognition sequence. Deamination (affecting one strand) can subsequently be repaired effectively or, if not repaired, there is a good chance that it can result in a tolerable mutation.[121] The double-strand breaks caused by the nucleases are much more difficult to repair, cannot be tolerated, and therefore are much more lethal.[121] In contrast, AdoMet interaction with Dnmt1 has regulatory features that might prevent Dnmt1 from attacking the target base in the absence of the cofactor.[22,36] It remains to be seen if these regulatory features complicate or facilitate design of Dnmt1 inhibitors that target the active site and the AdoMet-binding site.

The comparisons between Dnmt1 and M.HhaI are often oversimplified, with arguments that Dnmt1 has a large preference for hemimethylated DNA (relative to unmethylated DNA) that cannot be observed with M.HhaI.[84,122]

The ability of M.HhaI to discriminate between hemimethylated and unmethylated sites is often forgotten,[84,122] even though it is well documented by kinetic and crystallographic studies.[108,115,116] Dnmt1 has much more complex interactions with DNA molecules than M.HhaI.[22,30] With Dnmt1, the reported difference in catalytic activity between hemimethylated and unmethylated substrates vary between 3- and 40-fold; some reports indicated that the difference may vary as much as 2- to 200-fold.[30,75,88,123] Such wide range can be confusing; to avoid misleading conclusions, it is necessary to understand the mechanism that can lead to such large variability. Briefly, Dnmt1 has at least two DNA-binding sites, the active site and the regulatory allosteric site.[22,28–30,88] Depending on the DNA bound to each site, Dnmt1 can show different catalytic activity.[22,30]

For example, a comparison of Dnmt1 methylation reactions on hemimethylated and unmethylated DNA revealed that in the early presteady state, the measured methylation rates can differ by 30-fold or more.[22,30] The large difference is due to allosteric inhibition that is caused by an excess of unmethylated substrate.[22,36] However, slow conformational changes at the start of catalysis lead to relief from allosteric inhibition,[36] so that the observed difference between the presteady state methylation rates for the two substrate is only about fivefold[22] (a slow relief from allosteric inhibition at the start of catalysis can be seen only if the catalytic rates on given substrates are faster that the rate of relief from allosteric inhibition). With further progress of the reaction to the steady state, the unmethylated and hemimethylated substrates show almost equal rates.[22,30] A decrease in Dnmt1 saturation with AdoMet results in slower relief from allosteric inhibition and therefore elevated selectivity for premethylated substrate.[22] The described features depend on DNA bound at the active site and the allosteric site[22,30,36]; therefore, different experimental setups will show variations as described in detail elsewhere.[22,30,88] In sum, Dnmt1 is a large and somewhat unique enzyme; design and the interpretation of its activity studies is a unique challenge.[22,36] The established enzyme assay and enzyme kinetics textbooks were written primarily for much faster enzymes that target simple small-molecule substrates. Therefore, established approaches for the studies of enzyme activity[124–126] have to be adapted for Dnmt1 studies[22,36] to avoid inconsistencies and confusion in the future activity studies.

A number of Dnmt1 inhibitors can probably be improved by exploiting details of the catalytic mechanism in the active site and the AdoMet-binding site. Initial design and optimization of such inhibitors can take advantage of the conserved features between Dnmt1 and M.HhaI.[99] Cytosine analogues are the best known active site inhibitors.[38,89,99] The cytosine analogues cannot inhibit Dnmt1 directly, but have to be incorporated into DNA, where they trap Dnmt1 in the process of DNA methylation by forming a covalent bond with the

enzyme.[89] This results in a "suicide inhibition," but also excessive DNA damage and high toxicity. The cytosine analogues are most effective at low concentration that represents a balance between desired interference with DNA methyltransferase and acceptable toxicity.[38,99] The cytosine analogues are also valuable tool for cell-based studies of DNA methylation[127,128] and biochemical studies of DNA methyltransferase[37,89] (the cell-based studies that use cytosine analogues to study DNA methylation often do not differentiate between the inhibitor's effects on DNA methylation from the cellular response to DNA damage that is caused by the inhibitor). Different cytosine analogues show different toxicity, pharmacodynamics, and pharmacokinetic properties in clinical and cell-based studies.[38] Interestingly, the rate of inactivation[129–131] has never been reported for any of the analogues, so we cannot correlate the potency and the toxicity for any of the analogues with its ability to form the covalent adduct with the enzyme (the rate of inactivation by 5-fluoro-cytosine is likely to be comparable to the methylation rates, as 5-fluoro-cytosine is methylated only about two times slower than cytosine[132]).

Despite the continuing effort to improve the pharmacology of cytosine analogues, the reality is that none of the analogues in their current form can reach the true potential that inhibitors of DNA methylation can have. The cytosine analogues have a number of desirable features as lead compounds; however, they need to be modified to achieve the suicide inhibition without the need for toxic incorporation into genomic DNA. It is very likely that Dnmt1, like other DNA methyltransferases, flips the target base out of the DNA helix.[23] Thus, we need to design small-molecule lead compounds that can position cytosine analogues in the active site cavity, mimicking the flipped-out base. The base-flipping mechanism has been described in numerous studies. Design of a compound that can mimic the flipped-out base appears to be very challenging, as base flipping is a dynamic process that depends on an equilibrium between networks of competing interactions.[22,133,134] Stivers and colleagues reported small-molecule derivatives of uracyl that can inhibit uracyl-glycosidase,[135] another base-flipping enzyme that depends on dynamic equilibrium between base-flipping and base-restacking process.[133] However, the most favorable IC_{50} value is about 9 μM,[135] indicating that we are still far from design of a potent small-molecule inhibitor that can mimic the flipped-out base. An alternative, to incorporation of cytosine analogues into small molecules that can mimic the flipped-out base, is to attach cytosine analogue to a small molecule that can exploit binding interactions between Dnmt1 and AdoMet. [1,2-dihydropyrimidin-2-one]-5-methylene-(methylsulfonium)-adenosyl is currently in development as a potential lead compound for such class of Dnmt1 inhibitors.[22] Moreover, due to known action mechanism, [1,2-dihydropyrimidin-2-one]-5-methylene-(methylsulfonium)-adenosyl could be continually modified to increase its binding affinity and specificity.[22]

Procaine and procainamide are two other small molecules that have been reported to inhibit Dnmt1.[94] The two inhibitors are attractive due to known pharmacological properties and good tolerance. Procainamide inhibits DNA methylation in cells and purified Dnmt1, and the IC_{50} for purified Dnmt1 is about 10 μM.[94] This IC_{50} value can be a good starting point for a promising lead compound[94]; however, additional optimization and a higher potency are required for possible application. An elaborate mechanistic study suggested that procainamide binds at the AdoMet-binding site.[94] A fortunate situation, for all studies of Dnmt1 inhibitors that target the AdoMet site, is that Dnmt1–AdoMet interaction is the only Dnmt1 measurement that can use steady state approximations and related equations to determine competition and binding affinity.[36] Unlike substrate DNA, AdoMet is always in a large excess relative to Dnmt1, and catalytic rates with poly(dI-dC) substrates are fast enough to assure multiple turnovers and linear reaction profiles, the key requirements for every steady state analysis.[36,124]

RG108 is another small molecule that can inhibit DNA methylation in cells and purified Dnmt1.[93] The reported IC_{50} value for Dnmt1 inhibition in enzyme-based assay is a very impressive 115 nM.[93] However, RG108 has the basic structure of a phthalate, and phthalates are known to be genotoxic.[136] It remains to be seen if the genotoxic effects are a result of interference with DNA methylation only, or if other targets are involved. The initial results with RG108 are encouraging: the cell-based studies did not reveal multiple targets or excessive toxicity.[93] The binding site for RG108 has not been confirmed experimentally, though initial molecular modeling studies indicated that RG108 might target the active site.[93] This is consistent with the observation that both Dnmt1 and the bacterial enzyme M.SssI can be inhibited by RG108, as the two enzymes share conserved sequence motifs in the active site and the AdoMet-binding pocket.[92] Another still untested possibility is that both enzymes are inhibited by RG108 indirectly as a result of inhibitor's intercalation into substrate DNA. RG108 structure has planar conjugated hydrophobic rings that in principle could intercalate into DNA and interfere with all molecules that bind to DNA. In sum, additional mechanistic studies are needed to show how and if RG108 can directly bind and inhibit Dnmt1.

B. The Regulatory N-Terminal Domain

The bacterial enzyme M.HhaI shows that three basic domains and 327 amino acids are enough for cytosine methylation within a GCGC sequence, with preference for hemimethylated sites. Mammalian Dnmt1 has about 1620 amino acids, suggesting that Dnmt1 has evolved far beyond its basic function of DNA methylation. The N-terminal domain of Dnmt1 appears to have three functions: (i) allosteric regulation of the catalytic activity, (ii) multiple phosphorylation and methylation sites (see Chapter by Shannon R. Morey Kinney and Sriharsa Pradhan) that regulate catalytic activity of Dnmt1, and (iii)

orchestration of interactions between Dnmt1 and other molecules that affect DNA methylation (of 31 molecules known to interact with Dnmt1 (Table I), only one of them, cochaperone p23, was found to bind the C-terminal domain[66]). In sum, the N-terminal domain has multiple regulatory mechanisms that control the activity and the specificity of DNA methylation. Interestingly, we still do not have a Dnmt1 inhibitor that targets any of the regulatory sites. One possible exception is a single-stranded DNA oligonucleotide with one 5-methyl-cytosine that has been developed as an allosteric inhibitor of mouse Dnmt1[87] (it remains to be seen if the same oligonucleotide inhibits human Dnmt1). Design of small-molecule inhibitors or activators that target the N-terminal domain is especially attractive. The inhibitors that target the catalytic domain can only inhibit catalytic activity of Dnmt1, the wide spectra of regulatory features in the N-terminal domain indicate a potential for design of Dnmt1 inhibitors and activators, or the compounds that destabilize the Dnmt1–target–DNA complex. Both activators and the inhibitors are attractive, as the pathological processes related to DNA methylation depend on both an increase and a decrease in methylation at specific genomic loci.[81,82]

Today, we think that the N-terminal domain has a loose structure and acts as a collection of interacting and yet somewhat separate subdomains that can even fold independently.[34,137] This thinking is based on the observations that isolated segments of the N-terminal domain can bind DNA,[28,29,34] or interact with proteins that bind to Dnmt1[35] (some of those interactions are listed in Section III of this chapter). The Dnmt1 fragments that can interact with other molecules range in length from 20 to more than 300 amino acids (the protein fragments bigger than 100 amino acids can be expected to form the secondary structure that is close to their native structures[124]). The isolated segments of the N-terminal domain can effectively mimic Dnmt1 in its interaction with other molecules only if they can form a structure that is very close to their native structures. (To remind the reader, a structural change of one hydrogen bond can lead to a decrease in binding energy that can correspond to a decrease in binding affinity of about an order of magnitude[138–140]; some nice experimental examples of that principle are shown in Ref. 141.) The multiple-independent domains connected by flexible linkers will make determination and interpretation of crystal structures of Dnmt1 very challenging. It appears that at this moment, one productive research strategy would be to continue characterizations of the functional fragments of Dnmt1. For example, crystal structure of human Dnmt1 replication foci-targeting sequence is available at 2.3 Å resolution (PDB code: 3epz,[142] 268 amino acids, residues 350–618), while the approximate structure for some parts of Dnmt1 can be deduced from the homology modeling of the conserved structural motifs.[77,143]

Different segments of murine Dnmt1 have been expressed and purified from *Escherichia coli* cells and to some extent functionally characterized.[34] Baculovirus expression was also used to prepare different segments of human

Dnmt1,[28,29,137] human Dnmt1 that had increasingly shorter N-terminal domain,[27–29,137] or full-length human Dnmt1 with specific mutations,[29] or human Dnmt1 missing sections of its sequence.[28] Yeast two-hybrid systems have been used to identify interactions between different fragments of Dnmt1.[102] The studies of enzymatic function of different Dnmt1 fragments have been focused on two major questions: (i) which parts of the N-terminal domain are required for the catalytic activity and (ii) which parts of the N-terminal domain represent the regulatory allosteric site(s). Steady, admirable progress has been achieved in addressing both questions, and the future prospect for additional insights look very promising.

The search for catalytically active Dnmt1 fragments started with the first report of a successful separation of functional fragments of Dnmt1 using partial proteolysis.[100] The proteolysis resulted in admirably clean cleavage of Dnmt1 into a C-terminal fragment that shows catalytic activity, and an N-terminal regulatory domain that binds radioactive zinc ions. The cleaved "catalytic domain" appeared to be free from allosteric inhibition at high substrate concentration, suggesting that the N-terminal domain contains the allosteric site that leads to substrate inhibition.[16,36] The initial success by Bestor inspired numerous studies; however, none of those studies could prepare the active catalytic domain that was described in the original Bestor's study.[27–29,34,137] The human Dnmt1 constructs missing the first 121, 501, or 580 amino acids are still active, and show the ability to discriminate between hemimethylated and unmethylated DNA substrates similar to the full-length enzyme.[29,35] Human Dnmt1 constructs missing the first 621 amino acids are still active, but constructs missing the first 672 amino acids are not.[28,35]

Interestingly, even though the first 580 amino acids are not necessary for activity, this segment has multiple phosphorylation sites that can control catalytic activity of Dnmt1.[39,40,144,145] Possibly, the phosphorylation sites are not directly involved in catalysis, but conformational changes in the flexible protein structure allow the phosphorylated sites to interact with different Dnmt1 parts and cause inhibition. This proposal is supported by the observation that a very specific peptide mimic of the phosphorylation site at Ser515 can inhibit activity of mouse Dnmt1, apparently by altering the interactions between different domains.[39] Dnmt1 phosphorylation at Ser515 is needed for enzyme activity.[39] Additional phosphorylation sites on mouse Dnmt1 are found within amino acid region 1–290 that binds casein kinase 1δ/ϵ (CK1δ/ϵ).[40] The first phosphorylation takes place at Ser146, and then additional phosphorylations spread to the surrounding amino acids.[40] Described phosphorylations appear to inhibit Dnmt1 as a result of destabilization of the Dnmt1–DNA complex.[40] Phosphorylated peptides that can control Dnmt1 activity are potentially interesting as Dnmt1 inhibitors,[39] though additional mechanistic studies are needed to show if there are practically feasible strategies. Interestingly, Dnmt1 missing the first 501 amino acids has

higher activity than the full-length enzyme,[27,32] and the missing phosphorylation sites could be one of the reasons for this. Apart from the phosphorylation sites, the first 580 amino acids contain exons specific to mammalian enzymes, one of the regulatory DNA-binding sites,[28,29,34] exons specific for different tissues and cell types,[77,79,137] and phosphorylation and methylation sites that control Dnmt1 turnover in cells (Chapter by Shannon R. Morey Kinney and Sriharsa Pradhan).

Two studies, using different experimental approaches, showed that Dnmt1 has two DNA-binding sites in the N-terminal domain and one DNA-binding site in the C-terminal domain.[29,34] The first DNA-binding site is located in the human enzyme within the region 261–356, and more specifically amino acids Lys 284, Lys 285, His 286, and Arg 287.[29] The second DNA-binding site is located within the Zn-finger domain, in the amino acid segment 580–697. Interestingly, functional studies suggest that the two DNA-binding sites might function, at least to some degree, independently. Activation by fully methylated DNA can be observed with full-length Dnmt1 and Dnmt1 missing the first 121 amino acids; however, the activation cannot be observed with the constructs missing the first 501 or 580 amino acids.[29] Nevertheless, the constructs missing the first 501 or 580 amino acids can differentiate between hemimethylated and unmethylated substrates,[28,29] which is one of the features that depends on the allosteric inhibition at high substrate concentrations.[36] Thus, the allosteric activation by the fully methylated DNA and the allosteric inhibition by an excess of unmethylated DNA substrate appear to be at least partially independent. Strictly speaking, we do not know if fully methylated DNA activates Dnmt1, or if instead it leads to higher activity by reducing inhibition. The activation by fully methylated DNA is among the strongest evidence of allosteric regulation of Dnmt1,[30] a number of other more subtle evidences have been reported.[22]

The DNA-binding site between amino acids 261 and 356 does not show sequence homology with any of the known DNA-binding proteins; however, the Zn-finger domain has strong similarity to MBD1 protein, a chromatin modification protein that binds to unmethylated CpG sites (MBD proteins are covered in Chapter by Pierre-Antoine Defossez and Irina Stancheva and in Ref. 79). Human Dnmt1 missing the first 580 amino acids shows slightly higher binding affinity for unmethylated substrate relative to hemimethylated and fully methylated DNA, though interestingly the opposite preference is observed in the catalytic activity.[28] This is consistent with the well-known observation that unmethylated DNA can induce Dnmt1 inhibition by binding at the allosteric site.[16,36] The isolated Zn-finger (amino acids 651–697) shows obvious binding preference for unmethylated DNA, though its DNA-binding affinity is about 100-fold lower than the DNA affinity of full-length Dnmt1. This suggests that additional amino acids around the Zn finger also participate in DNA binding. Consistent with

this proposal, full-length Dnmt1 that is missing the Zn-finger domain shows clear evidences of inhibition by increasing concentration of unmethylated substrate and about 10-fold reduced activity.[28] In sum, the accumulated evidence suggests that the Zn-finger domain could be the allosteric site that leads to inhibition by an excess of unmethylated DNA. The inhibition by excess unmethylated substrate was the first textbook evidence for allosteric regulation of Dnmt1.[16,124–126] Although somewhat subtle, the inhibition by excess unmethylated substrate has been reproduced in numerous studies over the last 30 years.[22]

A number of key questions remain open. First, there is a question about positive or negative cooperativity between the two allosteric sites on Dnmt1. Precisely, even though externally added fully methylated DNA can activate Dnmt1 in methylation reactions on unmethylated substrates,[29,30] Dnmt1 is not self-activated by the methylation sites produced in its methylation reaction on unmethylated substrate.[36] The evidence of self-activation in the enzymatic reaction is easy to detect. In the case of product activation, the reaction time profile after the first turnover shows a continual increase in the catalytic rates as the product accumulates with time.[124–126,140] Following the first turnover, the Dnmt1 methylation reactions show linear reaction time profiles and a decrease in catalytic rates.[30,32,33,36,74]

The allosteric decreases in catalytic activity can be rather drastic. In the early presteady state, the methylation reaction on unmethylated DNA can be more than 30-fold slower than that on hemimethylated DNA or on unmethylated DNA in the presence of allosteric regulation by the fully methylated DNA.[30] However, after the first turnover (i.e., the progress from presteady state to the steady state), the rate difference between different reactions drops drastically and the time profiles for all reactions show very similar rates.[22,30,33,36] This rate drop cannot be attributed to any of the common causes such as product inhibition, enzyme inactivation with time, or substrate depletion.[36,74] The whole process represents an outstanding display of allosteric regulation of Dnmt1.

The second group of interesting questions is which molecules, by what mechanism, bind at the allosteric site(s) of Dnmt1 in cells[22]? The answers to these questions are likely the key for understanding of Dnmt1 function in cells. Apart from the allosteric activation by fully methylated DNA, or the allosteric inhibition by unmethylated DNA, a number of other molecules were found to bind and/or inhibit Dnmt1, such as specific RNA and tRNA molecules,[18,24] single-stranded oligonucleotides with one 5-methyl-C site,[87] poly(ADP-ribose),[57] poly(dA)·poly(dT),[18] or poly(dA-dT).[74] It remains to be seen if any of these interactions compete for the same binding sites or cooperate in control of Dnmt1 activity in cells. Apart from their physiological significance, all insights into the mechanism of allosteric regulation could be exploited in development of Dnmt1 inhibitors and activators.

III. Structure and Function of Dnmt1 and Its Interaction with Other Molecules

In cells, DNA is organized in chromatin structures that have multiple functions, including DNA packing and protection from DNA damage,[121,146,147] and organization of dynamic physiological processes such as transcription, replication, repair, and recombination. DNA methyltransferases affect each of these processes by acting within different multimolecular structures. The interacting molecules can control the access of Dnmt1 to the target sites[148] and/or Dnmt1 activity at the potential target sites.[22,149,150] As noted above, about 31 different molecules have been reported to interact with Dnm1 (Table I); this number is likely to grow in the future. The interacting molecules include proteins, RNA molecules, and poly(ADP-ribose). Nice maps of Dnmt1 fragments that interact with other proteins are provided in earlier publications.[35,72] Interestingly, all of the interacting molecules are found to bind the regulatory N-terminal domain, except co-chaperone p23, that was found to bind the catalytic C-terminal domain.[66]

A full list of different molecules that are known to interact with Dnmt1 is summarized in Table I, the reader is also referred to Jafar Sharif and Haruhiko Koseki in this volume, and to some relevant publications.[18,35,47,72,78,79] Here, I want to point out that it is highly unlikely that all of the interacting molecules bind to Dnmt1 at the same time, thus it will be very useful to group the different interactions according to their physiological functions (Table I). It appears that Dnmt1 participates in four different yet functionally integrated interactions: (i) Dnmt1 dimerization; (ii) core chromatin replication complex; (iii) interaction with molecules involved in DNA repair, cell cycle control, and apoptosis; and (iv) interaction with RNA Pol II, RNA-binding proteins, and specific RNA molecules. The function of different Dnmt1 interactions overlaps to some extent, and this overlapping probably explains why inactivation of many of the interacting proteins results in only partial loss of DNA methylation.[84] In the coming paragraphs, I will discuss some examples of how insights in enzymatic properties of Dnmt1 can help us to understand the interaction between Dnmt1 and other molecules in cells.

A. Dnmt1 Dimerization

Dnmt1 dimers can be observed with purified Dnmt1 and in cells overexpressing Dnmt1.[151] Several bacterial enzymes including M.HhaI can also form dimers.[113] Dnmt1 dimers are formed by interaction between the regulatory N-terminal domains (amino acids 310–629). It has been proposed that dimerization increases enzyme specificity for hemimethylated sites, but unfortunately this has not been confirmed in studies of enzyme activity. Activity studies at different concentrations of Dnmt1 can reveal differences in the

enzymatic properties of Dnmt1 monomers and dimers. I have observed that an increase in Dnmt1 concentration leads to partial inhibition of Dnmt1 activity on unmethylated DNA, independent of DNA concentration.[36] If similar inhibition cannot be observed with premethylated substrate, we could argue that Dnmt1 activity studies support the idea that its dimerization leads to increased selectivity for premethylated substrate. In sum, it is still unknown how, and if, dimerization can affect the enzymatic properties of Dnmt1; the experimental tools required to answer those questions in the future studies are available.[22,27,29,30,32,36]

B. Core Chromatin Replication Complex

During the process of DNA methylation in cells, Dnmt1 is a part of a large multiprotein complex that acts as *epigenetic code replication complex*.[47,149,150] The primary function of such complex is preservation of the existing epigenetic organization and chromatin structures. I will highlight some of the functions of the core chromatin replication complex that can directly depend on enzymatic properties of Dnmt1 (see also Chapters by Xiaodong Cheng and Robert M. Blumenthal; Jafar Sharif and Haruhiko Koseki).

It has been suggested that Dnmt1 can methylate DNA wrapped in nucleosomes, and that nucleosomes influence DNA methylation patterns.[152–155] Studies with purified Dnmt1 and mononucleosomes indicate that Dnmt1 activity on nucleosomes is as high as 20–40% of the activity observed with free DNA, while for some DNA sequences nucleosomes are methylated to a similar extent as the free DNA.[154] Using a very similar approach, another group showed that Dnmt1 and Dnmt3 can methylate nucleosomes at rates two times higher than the free DNA.[153] That study also indicated that Dnmt1 binds tightly to nucleosomes and, surprisingly, that the methylation sites can be found in different translational and rotational setting in the nucleosome.[153] In both studies, the bacterial enzyme M.SssI was inhibited by nucleosomes to a much higher degree than Dnmt1.[153,154]

Mapping of DNA methylation sites in *Arabidopsis thaliana* and human genomic DNA showed that nucleosomal DNA is methylated at a higher frequency and with 10 bp periodicity. The methylation sites are presumably facing away from the histone–DNA interface.[152] This prompted the authors to speculate that DNA might be methylated while it is still bound to the nucleosomes and that the rotational setting on nucleosomes surface could guide DNA methyltransferases. However, there are alternative explanations that have not been considered. First, 5-methyl-cytosine makes DNA more rigid especially for compressions in the major groove,[156,157] so that DNA methylation patterns might have evolved in cohort with the nucleosome positioning sequences to adapt to DNA bending and breading in nucleosomes.[158–161] Second, the 10-bp periodicity might not be a result of guided action of DNA methyltransferase,

but an adaptation of methylation sites that can facilitate the methylation readout by the proteins that bind to methylated and hemimethylated sites while DNA is in compact chromatin structures.

To fully appreciate the unique features that Dnmt1 (and Dnmt3 enzymes) needs in order to methylate nucleosomes, we have to briefly describe the general principles of how nucleosomes affect DNA interaction with proteins. In nucleosomes, 147 bp of DNA is wrapped 1.67 times around a histone octamer so that the DNA sites are not freely accessible to DNA-binding proteins.[162–165] DNA sites are only transiently released from the surface of histone octamers[161] in correlation with DNA flexibility and the translational and the rotational setting of each site.[162–164,166] In a truly landmark study, Polach and Widom used about a dozen different restriction enzymes to show that nucleosomes can block enzyme activity by approximately factors of 10, 100, and 1000 relative to free DNA.[165] The progressive decrease in enzyme activity corresponds to different translational settings starting from the loose DNA ends toward the nucleosome dyad. An enzyme's ability to act on nucleosomes is directly proportional to the enzyme concentration, while the rate-limiting step appears to be the enzyme's k_{cat}/K_m ratio rather than nucleosome unwinding.[165] Dnmt1 has exceptionally slow turnover rates in comparison to other enzymes,[36] which would make Dnmt1 relatively inept to act on target sites positioned in nucleosomes.

Some DNA-binding proteins can access DNA sites on nucleosomes since they do not completely encircle the DNA helix upon binding. For example, uracyl-glycosidase binds DNA asymmetrically[167] so it can effectively access DNA repair sites even at the nucleosome dyad axis.[162] However, even the enzymes that bind DNA asymmetrically can be inhibited by different rotational settings of the DNA target site on the nucleosome surface.[162,164] With a molecular mass of 186 kDa, Dnmt1 is about 1.5 times bigger than nucleosomes. For all of us working on nucleosomes, it would be fascinating to understand how, and if, a large and slow enzyme like Dnmt1 can methylate and bind nucleosomes keeping the original rotation setting of the target sites as the initial studies suggested.[152–154] However, if future studies continue to compare total activity of Dnmt1 to that of M.SssI, the authors need to take into account that M.SssI is 100- to 200-fold faster than Dnmt1.[36,114] In the time it takes for Dnmt1 to methylate one of the most accessible sites in nucleosomes, M.SssI can methylate all of the easy and many not-so-easy accessible sites. This can give the false impression that in comparison to free DNA, the nucleosome-bound DNA affects the activity of M.SssI much more than that of Dnmt1. In sum, the key requirement in studies of enzyme activity on nucleosomes is the ability to quantitatively differentiate between the activity on the easily accessible site at the free DNA ends from the activity on the progressively less-accessible sites toward the nucleosome dyad.[161–166] This key requirement was never achieved in any of the Dnmt1 studies.

Independent of the possibility that Dnmt1 can act on nucleosomes, the reported interactions between Dnmt1 and proteins involved in chromatin remodeling indicate that some forms of chromatin relaxation take place before Dnmt1 can access its target sites. Dnmt1 is functionally associated with SNF2h-containing chromatin-remodeling complex NoRC,[42] and with LSH protein (lymphoid-specific helicases), another protein related to the SNF2 family of chromatin-remodeling ATPases.[43] LSH function showed that even inactive Dnmt1 is crucial for its function. Both studies indicated that functional interactions between Dnmt1 and chromatin-remodeling proteins are closely associated with the function of two histone deacetylases HDAC1 and HDAC2. The two deacetylases were also reported to interact directly with Dnmt1.[60] A number of other histone modifying proteins interact with Dnmt1, like histone-lysine *N*-methyltransferase SUV39H1,[48] G9a histone methyltransferase,[49] and heterochromatin-binding protein HP1β.[47,48] A close functional link between DNA methylation and histone modification is driven by the shared function; the main unanswered questions are as follows: (i) What is the precise order of these events? (ii) What type of cooperativity exists between DNA methylation and histone modification events?[45]

Many of the proteins involved in histone modification that interact with Dnmt1 also interact with DNA methyltransferases Dnmt3a and Dnmt3b[47,79] (Dnmt3 enzymes are described in Chapter by Frédéric Chédin). Dnmt1 also interacts with Dnmt3a and Dnmt3b.[41] Studies of the mechanism of interactions between Dnmt1 and Dnmt3 enzymes can offer answers to some of the key questions about the alleged physiological function of these three enzymes, that is, Dnmt1 as the maintenance methyltransferase and Dnmt3a and Dnmt3b as *de novo* methyltransferases. Many of the cell-based studies suggested that the classification of Dnmt1 as a maintenance methyltransferase and Dnmt3 enzymes as *de novo* methyltransferase is not justified, as both Dnmt1 and Dnmt3 enzymes participate in *de novo* methylation via unknown molecular mechanisms.[79]

The enzyme-based studies support this conclusion, as purified Dnmt1 and Dnmt3 enzymes have (within experimental error) equal catalytic rates as *de novo* methyltransferases.[32,33,36,85,86] However, it is very important to notice that in the presence of fully methylated DNA, Dnmt1 can have more than 10 times higher *de novo* catalytic activity than Dnmt3 enzymes. In those conditions, *de novo* catalytic activity of Dnmt1 is almost identical to its activity as a maintenance methyltransferase.[153] The allosteric effects appear to be the main regulator of Dnmt1 activity as *de novo* and maintenance methyltransferase, the remaining important question is "How do different DNA molecules bind at the allosteric site of Dnmt1 in cells?" It has been suggested that catalytic product of Dnmt3 can stimulate catalytic activity of Dnmt1, but the actual mechanism is not known.[168] It is possible that Dnmt3 can increase catalytic activity of Dnmt1 by feeding its methylated DNA directly to the allosteric site on Dnmt1. This proposal is supported by the observation that Dnmt3 binds the regulatory

domain of Dnmt1.[41] Direct feeding of premethylated DNA to the allosteric site on Dnmt1 can be crucial, since in free solution different DNA molecules randomly compete for different DNA-binding sites on Dnmt1, a mere presence of methylated DNA does not lead to activation (as described in Section II.B).

Apart from Dnmt1, the methylated DNA sites are also recognized by other proteins, such as UHRF1 (ubiquitin-like with PHD and ring finger domain 1) and MBD (methyl-CpG-binding domain) proteins (see also Chapters by Jafar Sharif and Haruhiko Koseki; Pierre-Antoine Defossez and Irina Stancheva). The hemimethylated sites are small, so it is impossible for large proteins like Dnmt1, UHRF1, or MBD to bind such sites simultaneously. This raises the question of whether those molecules cooperate to increase the sensitivity of Dnmt1 for hemimethylated sites and maintenance methylation.[83,123,150] Here, I will discuss interaction between Dnmt1 and UHRF1 (the interaction with MBD proteins is described later in this text in the section on RNA-directed DNA methylation). UHRF1 protein is indispensable for Dnmt1 activity in cells (Ref. 45 and Chapter by Jafar Sharif and Haruhiko Koseki). UHRF1 is a large multidomain protein that appears to be the central molecule in the epigenetic code replication complex.[45] UHRF1 can recognize hemimethylated sites and histone methylation, and bind both Dnmt1 and histone methyltransferase G9a.[45,46,169,170] Thus, UHRF1 appears to link histone methylation and DNA methylation.[45]

The known enzymatic features of Dnmt1 can help us to address the debate how, and if, UHRF1 and Dnmt1 cooperate in preserving hemimethylated sites during replication.[83,123,150] Dnmt1 preference for the hemimethylated sites is primarily a result of kinetic preferences that depend on the allosteric regulation.[22,30,74] UHRF1 or any other molecule can affect the specificity of Dnmt1 for hemimethylated sites only if the process affects the allosteric regulation of Dnmt1 (Section II.B and Refs. 22,30). Dnmt1 preference for hemimethylated DNA sites is not a result of preferred binding equilibrium[28,171]; therefore, UHRF1 cannot increase Dnmt1's preference for hemimethylated sites by increasing its binding affinity for such sites.[83] UHRF1 and Dnmt1 are too big to bind hemimethylated sites simultaneously, and the binding affinity of Dnmt1 for hemimethylated site depends only on its interaction energy with that site. Thus, UHRF1 cannot preactivate Dnmt1 by binding at the hemimethylated site prior to Dnmt1[83] since such "preactivation" would be against the basic chemistry principle that the binding equilibrium does not depend on the path by which the equilibrium is achieved.[124,138–140]

C. DNA Repair, Cell Cycle Control, and Regulation of Apoptosis

The actions of multiprotein complex involved in epigenetic code replication have to be closely correlated with cell cycle regulation. The chromatin structures and the proteins involved in chromatin organization are known to block the access of DNA repair proteins to the damage sites.[121] Therefore, it can be expected that

the molecules involved in response to DNA damage can stop DNA methylation or even facilitate dissociation of DNA methyltransferases from the target sites. The actual experiments have shown that the molecules involved in response to DNA damage can stop DNA methylation at the level of Dnmt1 transcription[172,173] or by direct interaction with Dnmt1,[40,57–59] or regulate apoptosis in complex with Dnmt1.[50,60–63] Probably, one of the best understood examples of such regulation is Dnmt1 interaction with the Rb that results in Dnmt1 inhibition and dissociation of Dnmt1–DNA complex.[29,59] The amino acid region 261–356 in the N-terminal domain of Dnmt1 binds to specific pockets on Rb.[29] The interaction between Dnmt1 and Rb protein could compete with the allosteric regulation of Dnmt1 by fully methylated DNA.[29]

Dnmt1 is also inhibited by poly(ADP-ribose) and PARP1.[57,58] The mechanism of Dnmt1 inhibition by poly(ADP-ribose) has not been investigated. It is not known if the interaction leads only to inhibition, or also to destabilization of Dnmt1 binding to its target site.[74] It is unknown if poly(ADP-ribose) binds at the regulatory N-terminal domain, and whether such binding interferes with other molecules that regulate Dnmt1 activity by binding to the regulatory domain.[22,28–30] Dnmt1 can be inhibited by poly(dA)·poly(dT) and poly(dA-dT), but not by poly(A) or poly(dA).[18,74] It remains to be seen if the inhibiting molecules bind at the same site as poly(ADP-ribose). The specificity of the poly (ADP-ribose)-binding site can be explored as a potential target for design of small-molecule Dnmt1 inhibitors as drug candidates. The experimental tools required to answer the questions related to Dnmt1 inhibition by poly(ADP-ribose) have been developed.[22,27,29,30,32,36,74]

Dnmt1 interacts with p53, and this interaction affects expression of the *survivin* gene, an inhibitor of apoptosis.[61] Dnmt1 and p53 appear to be involved in downregulation of protein phosphatases that regulate the cell cycle.[174] DNA repair and cell cycle regulation are two closely related processes; therefore, it is no surprise that Dnmt1 also interacts with some of the kinases involved in cell cycle regulation.[40,144] As noted above, Dnmt1 is phosphorylated at a number of sites in its regulatory N-terminal domain.[39,40,144,145] Decreased DNA-binding affinity is observed after phosphorylation of serine 146 and surrounding sites by the CK1δ/ϵ.[40] In sum, it appears that Dnmt1 interaction with the molecules involved in response to DNA damage leads to inhibition of DNA methylation (by the release of the DNA target sites), cell cycle arrest, and regulation of apoptosis.

D. RNA-Directed DNA Methylation

The interaction with specific RNA molecules was probably the first report of Dnmt1 interaction with other molecules.[18] The original study is almost 30 years old; nevertheless, many of the fine features that were reported by Weissbach and colleagues almost 30 years ago are fully reproducible.[22] Similar

to Weissbach and colleagues, we routinely found during Dnmt1 purification that its activity is strongly inhibited in cell extracts.[24] The inhibition was found to be due to tightly bound RNA molecule(s).[18,24] RNA-directed DNA methylation has been described in plant cells, and a comparative analysis of plant and mammalian cells shows that mammalian cells have counterparts for the molecules involved in RNA-directed DNA methylation.[175] Growing evidence indicates that RNA molecules are involved in control of DNA methylation in mammalian cells, through the mechanism is poorly understood[176–180] (see Chapter by Anton Wutz).

Several studies reported evidence of physical or functional interaction between Dnmt1 and RNA Pol II,[69–71] but the actual mechanism and the consequences of that interaction are not understood. RNA Pol II is involved in synthesis of the noncoding RNA molecules.[181] Dnmt1 interacts with several members from the family of methyl-CpG-binding domain (MBD) proteins[55,79,182] that bind several types of RNA molecules[183] and interact with histone modifying components.[67] Most notable is MeCP2 (Rett syndrome protein[68]); MeCP2 participates in displacement of histone H1[184,185] and its functional interaction with Dnmt1 can be crucial for neuronal development.[68] Methylated DNA sites are too small to bind Dnmt1 and MBD proteins at the same time, so it is unclear how these proteins act together in the recognition of methylated sites. Interestingly, MBD proteins cannot bind methylated DNA sites when they bind RNA molecules,[183] suggesting that RNA molecules might coordinate the access to methylated DNA sites between MBD proteins and Dnmt1. Dnmt3a, another protein that can bind Dnmt1, also binds RNA molecules in cells.[186] Dnmt3a binds the antisense strand of siRNA molecules in cells, acting in cohort with RNA Pol II and histone methylation.[186]

Interestingly, Dnmt1 cannot be inhibited by every RNA molecule, only some mRNA and tRNA molecules can cause inhibition and it appears that some of them have higher potency than others, with poly(G) being the most preferred sequence known to date.[18] It remains to be seen if tRNA molecules are really involved in DNA methylation, or some tRNA molecules just mimic some of the noncoding RNA molecules that participate in DNA methylation. A puzzling link between DNA methylation and tRNA molecules is also an open question in Dnmt2 studies[187,188]. If RNA molecules control Dnmt1 activity by binding to an allosteric site, the sensitivity of allosteric regulation to 5-methylcytosine suggests that an RNA methyltransferase might methylate the regulatory RNA molecules and thus control Dnmt1 and DNA methylation.[22] In sum, the allosteric regulation of Dnmt1, and Dnmt1 inhibition by the RNA molecules, unfairly remains to this day the two least-explored enzymatic features of Dnmt1.[22] The two features are likely to be related, and likely to be the key for our understanding of differences between maintenance and *de novo* DNA methylation, the two processes that can drive the changes in methylation

patterns that lead to diseases.[81,82] The future studies of Dnmt1 interaction with RNA molecules are likely to have a significant impact on our understanding of Dnmt1 action in cells, and on rational design of novel modulators of Dnmt1 activity in cells.[22]

IV. New Crystal Structures of Large C-Terminal Fragment of Mouse and Human Dnmt1

Just as I finished writing the final version of this chapter, new crystal structures of the large Dnmt1 fragment have been published.[189] The first structure shows mouse Dnmt1 (residues 650–1602) in complex with *S*-adenosyl homocysteine and a 19-bp DNA duplex that contains two unmethylated CpG sites separated by 8 bp (3.0 Å resolution; Fig. 1). The second crystal structure shows human Dnmt1 (residues 646–1600) in complex with the same 19-bp DNA and *S*-adenosyl homocysteine (3.6 Å resolution). The new structures complement the existing structure of human Dnmt1 replication foci-targeting

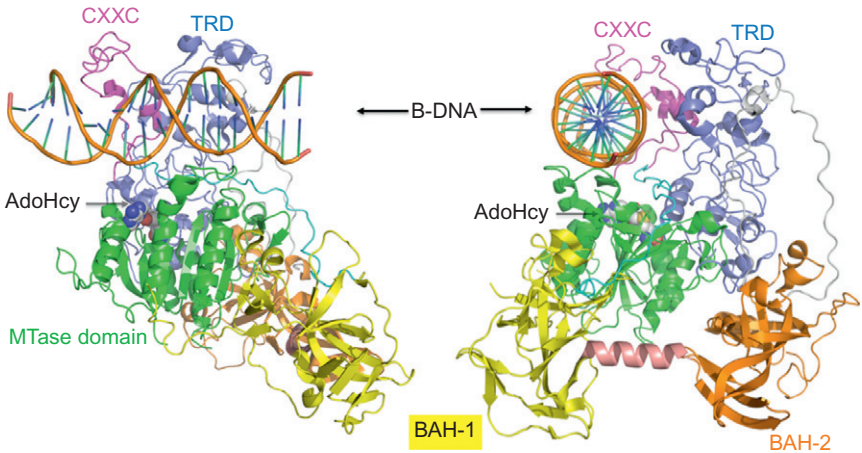

FIG. 1. Structure of mouse Dnmt1. Two orthogonal views of mouse Dnmt1 (residues 650–1602) in complex with unmethylated DNA substrate and cofactor *S*-adenosyl homocysteine (AdoHcy) (PDB 3PT6).[189] The domain structure from N-to-C terminal are Zn-finger CXXC domain (magenta), BAH-1 (yellow), BAH-2 (orange), methyltransferase (green), and target recognition domain (blue).

domain (residues 350–618, PDB code: 3epz[142]). The new structures represent a landmark achievement that will accelerate the future studies of Dnmt1 and design of novel inhibitors or activators.

I will briefly discuss here how the new structural insights add to three decades of Dnmt1 studies that were summarized in the earlier sections. The crystal structures are consistent with Dnmt1 fragments that were prepared following activity studies,[28,29] and confirm predictions that Dnmt1 is composed of multiple subdomains that are connected with flexible regulatory loops. Flexible protein loops are known to develop in protein evolution to facilitate protein–protein interactions, protein phosphorylation, or dynamic structural changes that control enzyme activity.[190,191] Loops from BAH1 and BAH2 domain penetrate into the catalytic domain, suggesting a possible explanation for the earlier observations that separated catalytic domain is inactive,[28,29] but it can become active by binding the other domains.[34] Similar binding sites for penetrating loops might explain Dnmt1 inhibition by phosphorylated peptide with specific amino acid sequence.[39]

The crystal structure confirmed that Dnmt1 and M.HhaI share very similar structure in both the active site and the AdoMet-binding domain,[23] with one major difference. Specifically, the active site cysteine is in a retracted position in Dnmt1. This retracted position might explain why Dnmt1 (unlike M.HhaI) is very slow in attacking its target base in the absence of cofactor, and/or why Dnmt1 shows slow activation (i.e., enzyme hysteresis) at the start of catalysis on unmethylated DNA substrate.[22,36,114] Big differences between Dnmt1 and M.HhaI are observed in the target (DNA methylation site) recognition domain. For both enzymes, the TRDs are composed from flexible loops. The Dnmt1 TRD is about twice the size of the one in M.HhaI, even though its target sequence (CG) is only half the size of the M.HhaI target sequence (GCGC). The loop from the BAH2 domain penetrates the TRD, suggesting that the relatively large domain is an adaptation to regulatory interactions with other parts of Dnmt1. Finally, it appears that (unlike M.HhaI) the TRD of Dnmt1 does not completely encircle the target DNA, and molecular modeling studies might be used to test whether Dnmt1 can dock sideways onto DNA bound to nucleosomes[192] and methylate the target sites facing away from the nucleotide surface.[152–154]

The new structures offer insights into the earlier observations that Dnmt1 is inhibited by unmethylated substrate.[16,22,36,74,87] It appears that the Zn-finger CXXC domain binds DNA first, and this interaction depends on existing DNA methylation. In the case of hemimethylated sites, the Zn-finger CXXC domain will not bind DNA, and the active site should be open for DNA binding and maintenance methylation. In the case of unmethylated substrate, Zn-finger CXXC domain binds the unmethylated CpG site so that the loop between BAH1 and CXXC domain is in position where it can block the DNA binding at the active site, while the TRD is in retracted position bound to the loop from

BAH2 domain. The inhibitory structure must be unstable and dynamic, since Dnmt1 is active on unmethylated substrate. The slow relief from allosteric inhibition that can be observed at the start of catalysis with unmethylated substrate could be a direct consequence of the underlining structural changes.[22,36]

In sum, multiple domains connected by flexible loops, multiple phosphorylation sites,[39,40] and multiple interacting molecules (Table I) indicate that Dnmt1 has a very dynamic structure with many subtle regulatory features. Thus, we always have to bear in mind that the static crystal structures do not reflect many of the catalytic and regulatory features of the active enzyme. Future insights into Dnmt1 function, and design of its inhibitors and activators, will strongly depend on building a correlation between the available structures and the enzyme assays that are designed to capture apparently subtle and yet important regulatory features.[22] I am excited about the possibility that in the next decade, we could expect a burst in enzymatic studies of Dnmt1 that could surpass the burst in M.HhaI studies that came after the first structure with the flipped-out base.[23]

References

1. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010;**31**:27–36.
2. Fernandez AF, Esteller M. Viral epigenomes in human tumorigenesis. *Oncogene* 2010;**29**:1405–20.
3. Bloom DC, Giordani NV, Kwiatkowski DL. Epigenetic regulation of latent HSV-1 gene expression. *Biochim Biophys Acta* 2010;**1799**:246–56.
4. Wei H, Zhou MM. Viral-encoded enzymes that target host chromatin functions. *Biochim Biophys Acta* 2010;**1799**:296–301.
5. Takacs M, Banati F, Koroknai A, Segesdi J, Salamon D, Wolf H, et al. Epigenetic regulation of latent Epstein-Barr virus promoters. *Biochim Biophys Acta* 2010;**1799**:228–35.
6. White MK, Safak M, Khalili K. Regulation of gene expression in primate polyomaviruses. *J Virol* 2009;**83**:10846–56.
7. Hoelzer K, Shackelton LA, Parrish CR. Presence and role of cytosine methylation in DNA viruses of animals. *Nucleic Acids Res* 2008;**36**:2825–37.
8. Sen GL, Reuter JA, Webster DE, Zhu L, Khavari PA. DNMT1 maintains progenitor function in self-renewing somatic tissue. *Nature* 2010;**463**:563–7.
9. Richardson B. DNA methylation and autoimmune disease. *Clin Immunol* 2003;**109**:72–9.
10. Miller G. Epigenetics. The seductive allure of behavioral epigenetics. *Science* 2010;**329**:24–7.
11. Miller G. Epigenetics. A role for epigenetics in cognition. *Science* 2010;**329**:27.
12. van Bokhoven H, Kramer JM. Disruption of the epigenetic code: an emerging mechanism in mental retardation. *Neurobiol Dis* 2010;**39**:3–12.
13. Skinner MK, Manikkam M, Guerrero-Bosagna C. Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends Endocrinol Metab* 2010;**21**:214–22.
14. Weissbach A. A chronicle of DNA methylation (1948-1975). *EXS* 1993;**64**:1–10.
15. Roy PH, Weissbach A. DNA methylase from HeLa cell nuclei. *Nucleic Acids Res* 1975;**2**:1669–84.

16. Pedrali-Noy G, Weissbach A. Mammalian DNA methyltransferases prefer poly(dI-dC) as substrate. *J Biol Chem* 1986;**261**:7600–2.
17. Stein R, Gruenbaum Y, Pollack Y, Razin A, Cedar H. Clonal inheritance of the pattern of DNA methylation in mouse cells. *Proc Natl Acad Sci USA* 1982;**79**:61–5.
18. Bolden A, Ward C, Siedlecki JA, Weissbach A. DNA methylation. Inhibition of de novo and maintenance methylation in vitro by RNA and synthetic polynucleotides. *J Biol Chem* 1984;**259**:12437–43.
19. Bestor TH. Cloning of a mammalian DNA methyltransferase. *Gene* 1988;**74**:9–12.
20. Yen RW, Vertino PM, Nelkin BD, Yu JJ, el-Deiry W, Cumaraswamy A, et al. Isolation and characterization of the cDNA encoding human DNA methyltransferase. *Nucleic Acids Res* 1992;**20**:2287–91.
21. Xu G, Flynn J, Glickman JF, Reich NO. Purification and stabilization of mouse DNA methyltransferase. *Biochem Biophys Res Commun* 1995;**207**:544–51.
22. Svedruzic ZM. Mammalian cytosine DNA methyltransferase Dnmt1: enzymatic mechanism, novel mechanism-based inhibitors, and RNA-directed DNA methylation. *Curr Med Chem* 2008;**15**:92–106.
23. Klimasauskas S, Kumar S, Roberts RJ, Cheng X. HhaI methyltransferase flips its target base out of the DNA helix. *Cell* 1994;**76**:357–69.
24. Glickman JF, Flynn J, Reich NO. Purification and characterization of recombinant baculovirus-expressed mouse DNA methyltransferase. *Biochem Biophys Res Commun* 1997;**230**:280–4.
25. Glickman JF, Reich NO. Baculovirus-mediated high level expression of a mammalian DNA methyltransferase. *Biochem Biophys Res Commun* 1994;**204**:1003–8.
26. Pradhan S, Talbot D, Sha M, Benner J, Hornstra L, Li E, et al. Baculovirus-mediated expression and characterization of the full-length murine DNA methyltransferase. *Nucleic Acids Res* 1997;**25**:4666–73.
27. Bacolla A, Pradhan S, Larson JE, Roberts RJ, Wells RD. Recombinant human DNA (cytosine-5) methyltransferase. III. Allosteric control, reaction order, and influence of plasmid topology and triplet repeat length on methylation of the fragile X CGG.CCG sequence. *J Biol Chem* 2001;**276**:18605–13.
28. Pradhan M, Esteve PO, Chin HG, Samaranayke M, Kim GD, Pradhan S. CXXC domain of human DNMT1 is essential for enzymatic activity. *Biochemistry* 2008;**47**:10000–9.
29. Pradhan S, Esteve PO. Allosteric activator domain of maintenance human DNA (cytosine-5) methyltransferase and its role in methylation spreading. *Biochemistry* 2003;**42**:5321–32.
30. Goyal R, Reinhardt R, Jeltsch A. Accuracy of DNA methylation pattern preservation by the Dnmt1 methyltransferase. *Nucleic Acids Res* 2006;**34**:1182–8.
31. Bacolla A, Pradhan S, Roberts RJ, Wells RD. Recombinant human DNA (cytosine-5) methyltransferase. II. Steady-state kinetics reveal allosteric activation by methylated DNA. *J Biol Chem* 1999;**274**:33011–9.
32. Pradhan S, Bacolla A, Wells RD, Roberts RJ. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *J Biol Chem* 1999;**274**:33002–10.
33. Flynn J, Glickman JF, Reich NO. Murine DNA cytosine-C5 methyltransferase: pre-steady- and steady-state kinetic analysis with regulatory DNA sequences. *Biochemistry* 1996;**35**:7308–15.
34. Fatemi M, Hermann A, Pradhan S, Jeltsch A. The activity of the murine DNA methyltransferase Dnmt1 is controlled by interaction of the catalytic domain with the N-terminal part of the enzyme leading to an allosteric activation of the enzyme after binding to methylated DNA. *J Mol Biol* 2001;**309**:1189–99.

35. Pradhan S, Esteve PO. Mammalian DNA (cytosine-5) methyltransferases and their expression. *Clin Immunol* 2003;**109**:6–16.
36. Svedruzic ZM, Reich NO. DNA cytosine C5 methyltransferase Dnmt1: catalysis-dependent release of allosteric inhibition. *Biochemistry* 2005;**44**:9472–85.
37. Yoder JA, Soman NS, Verdine GL, Bestor TH. DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. *J Mol Biol* 1997;**270**:385–95.
38. Yu N, Wang M. Anticancer drug discovery targeting DNA hypermethylation. *Curr Med Chem* 2008;**15**:1350–75.
39. Goyal R, Rathert P, Laser H, Gowher H, Jeltsch A. Phosphorylation of serine-515 activates the mammalian maintenance methyltransferase Dnmt1. *Epigenetics* 2007;**2**:155–60.
40. Sugiyama Y, Hatano N, Sueyoshi N, Suetake I, Tajima S, Kinoshita E, et al. The DNA-binding activity of mouse DNA methyltransferase 1 is regulated by phosphorylation with casein kinase 1delta/epsilon. *Biochem J* 2010;**427**:489–97.
41. Kim GD, Ni J, Kelesoglu N, Roberts RJ, Pradhan S. Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. *EMBO J* 2002;**21**:4183–95.
42. Zhou Y, Grummt I. The PHD finger/bromodomain of NoRC interacts with acetylated histone H4K16 and is sufficient for rDNA silencing. *Curr Biol* 2005;**15**:1434–8.
43. Myant K, Stancheva I. LSH cooperates with DNA methyltransferases to repress transcription. *Mol Cell Biol* 2008;**28**:215–26.
44. Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF. Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 1997;**277**:1996–2000.
45. Bostick M, Kim JK, Esteve PO, Clark A, Pradhan S, Jacobsen SE. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science* 2007;**317**:1760–4.
46. Sharif J, Muto M, Takebayashi S, Suetake I, Iwamatsu A, Endo TA, et al. The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. *Nature* 2007;**450**:908–12.
47. Cheng X, Blumenthal RM. Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. *Biochemistry* 2010;**49**:2999–3008.
48. Fuks F, Hurd PJ, Deplus R, Kouzarides T. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res* 2003;**31**:2305–12.
49. Esteve PO, Chin HG, Smallwood A, Feehery GR, Gangisetty O, Karpf AR, et al. Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. *Genes Dev* 2006;**20**:3089–103.
50. Rountree MR, Bachman KE, Baylin SB. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat Genet* 2000;**25**:269–77.
51. Kiskinis E, Hallberg M, Christian M, Olofsson M, Dilworth SM, White R, et al. RIP140 directs histone and DNA methylation to silence Ucp1 expression in white adipocytes. *EMBO J* 2007;**26**:4831–40.
52. Robertson KD, Jones PA. DNA methylation: past, present and future directions. *Carcinogenesis* 2000;**21**:461–7.
53. Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M, et al. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* 2002;**295**:1079–82.
54. Butler JS, Lee JH, Skalnik DG. CFP1 interacts with DNMT1 independently of association with the Setd1 Histone H3K4 methyltransferase complexes. *DNA Cell Biol* 2008;**27**:533–43.
55. Tatematsu KI, Yamazaki T, Ishikawa F. MBD2-MBD3 complex binds to hemi-methylated DNA and forms a complex containing DNMT1 at the replication foci in late S phase. *Genes Cells* 2000;**5**:677–88.

56. Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006;**439**:871–4.
57. Reale A, Matteis GD, Galleazzi G, Zampieri M, Caiafa P. Modulation of DNMT1 activity by ADP-ribose polymers. *Oncogene* 2005;**24**:13–9.
58. Zardo G, Reale A, Passananti C, Pradhan S, Buontempo S, De Matteis G, et al. Inhibition of poly(ADP-ribosyl)ation induces DNA hypermethylation: a possible molecular mechanism. *FASEB J* 2002;**16**:1319–21.
59. Pradhan S, Kim GD. The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity. *EMBO J* 2002;**21**:779–88.
60. Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet* 2000;**25**:338–42.
61. Esteve PO, Chin HG, Pradhan S. Human maintenance DNA (cytosine-5)-methyltransferase and p53 modulate expression of p53-repressed promoters. *Proc Natl Acad Sci USA* 2005;**102**:1000–5.
62. Liu Z, Fisher RA. RGS6 interacts with DMAP1 and DNMT1 and inhibits DMAP1 transcriptional repressor activity. *J Biol Chem* 2004;**279**:14120–8.
63. Lee GE, Kim JH, Taylor M, Muller MT. DNA methyltransferase 1-associated protein (DMAP1) is a co-repressor that stimulates DNA methylation globally and locally at sites of double strand break repair. *J Biol Chem* 2010;**285**:37630–40.
64. Ohsawa K, Imai Y, Ito D, Kohsaka S. Molecular cloning and characterization of annexin V-binding proteins with highly hydrophilic peptide structure. *J Neurochem* 1996;**67**:89–97.
65. Zhou Q, Agoston AT, Atadja P, Nelson WG, Davidson NE. Inhibition of histone deacetylases promotes ubiquitin-dependent proteasomal degradation of DNA methyltransferase 1 in human breast cancer cells. *Mol Cancer Res* 2008;**6**:873–83.
66. Zhang X, Verdine GL. Mammalian DNA cytosine-5 methyltransferase interacts with p23 protein. *FEBS Lett* 1996;**392**:179–83.
67. Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem* 2003;**278**:4035–40.
68. Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet* 1999;**23**:185–8.
69. Carty SM, Greenleaf AL. Hyperphosphorylated C-terminal repeat domain-associating proteins in the nuclear proteome link transcription to DNA/chromatin modification and RNA processing. *Mol Cell Proteomics* 2002;**1**:598–610.
70. Tao Y, Xi S, Briones V, Muegge K. Lsh mediated RNA polymerase II stalling at HoxC6 and HoxC8 involves DNA methylation. *PLoS One* 2010;**5**:e9163.
71. Takeshima H, Yamashita S, Shimazu T, Niwa T, Ushijima T. The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands. *Genome Res* 2009;**19**:1974–82.
72. Spada F, Rothbauer U, Zolghadr K, Schermelleh L, Leonhardt H. Regulation of DNA methyltransferase 1. *Adv Enzyme Regul* 2006;**46**:224–34.
73. Flynn J, Reich N. Murine DNA (cytosine-5-)-methyltransferase: steady-state and substrate trapping analyses of the kinetic mechanism. *Biochemistry* 1998;**37**:15162–9.
74. Svedruzic ZM, Reich NO. Mechanism of allosteric regulation of Dnmt1's processivity. *Biochemistry* 2005;**44**:14977–88.
75. Grace Goll M, Bestor TH. Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* 2005;**74**:481–514.
76. Jeltsch A. Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. *Chembiochem* 2002;**3**:274–93.

77. Hermann A, Gowher H, Jeltsch A. Biochemistry and biology of mammalian DNA methyltransferases. *Cell Mol Life Sci* 2004;**61**:2571–87.
78. Kim JK, Samaranayake M, Pradhan S. Epigenetic mechanisms in mammals. *Cell Mol Life Sci* 2009;**66**:596–612.
79. Van Emburgh BO, Robertson KD. DNA methyltransferases and Methyl CpG binding proteins as multifunctional regulators of chromatin structure and development in mammalian cells. In: Tost J, editor. *Epigenetics*. Norfolk, UK: Caister Academic Press; 2008. p. 392.
80. Wu JC, Santi DV. Kinetic and catalytic mechanism of HhaI methyltransferase. *J Biol Chem* 1987;**262**:4778–86.
81. Esteller M. Epigenetics provides a new generation of oncogenes and tumour-suppressor genes. *Br J Cancer* 2006;**94**:179–83.
82. Robertson KD. DNA methylation and human disease. *Nat Rev Genet* 2005;**6**:597–610.
83. Ooi SK, Bestor TH. Cytosine methylation: remaining faithful. *Curr Biol* 2008;**18**:R174–6.
84. Jones PA, Liang G. Rethinking how DNA methylation patterns are maintained. *Nat Rev Genet* 2009;**10**:805–11.
85. Purdy MM, Holz-Schietinger C, Reich NO. Identification of a second DNA binding site in human DNA methyltransferase 3A by substrate inhibition and domain deletion. *Arch Biochem Biophys* 2010;**498**:13–22.
86. Yokochi T, Robertson KD. Preferential methylation of unmethylated DNA by mammalian de novo DNA methyltransferase Dnmt3a. *J Biol Chem* 2002;**277**:11735–45.
87. Flynn J, Fang JY, Mikovits JA, Reich NO. A potent cell-active allosteric inhibitor of murine DNA cytosine C5 methyltransferase. *J Biol Chem* 2003;**278**:8238–43.
88. Jeltsch A. On the enzymatic properties of Dnmt1: specificity, processivity, mechanism of linear diffusion and allosteric regulation of the enzyme. *Epigenetics* 2006;**1**:63–6.
89. Zhou L, Cheng X, Connolly BA, Dickman MJ, Hurd PJ, Hornby DP. Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases. *J Mol Biol* 2002;**321**:591–9.
90. Isakovic L, Saavedra OM, Llewellyn DB, Claridge S, Zhan L, Bernstein N, et al. Constrained (l-)-S-adenosyl-l-homocysteine (SAH) analogues as DNA methyltransferase inhibitors. *Bioorg Med Chem Lett* 2009;**19**:2742–6.
91. Uetrecht J. Current trends in drug-induced autoimmunity. *Autoimmun Rev* 2005;**4**:309–14.
92. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, et al. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res* 2005;**65**:6305–11.
93. Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res* 2006;**66**:2794–800.
94. Lee BH, Yegnasubramanian S, Lin X, Nelson WG. Procainamide is a specific inhibitor of DNA methyltransferase 1. *J Biol Chem* 2005;**280**:40749–56.
95. Castellano S, Kuck D, Sala M, Novellino E, Lyko F, Sbardella G. Constrained analogues of procaine as novel small molecule inhibitors of DNA methyltransferase-1. *J Med Chem* 2008;**51**:2321–5.
96. Stewart DJ, Donehower RC, Eisenhauer EA, Wainman N, Shah AK, Bonfils C, et al. A phase I pharmacokinetic and pharmacodynamic study of the DNA methyltransferase 1 inhibitor MG98 administered twice weekly. *Ann Oncol* 2003;**14**:766–74.
97. Lin J, Haffner MC, Zhang Y, Lee BH, Brennen WN, Britton J, et al. Disulfiram is a DNA demethylating agent and inhibits prostate cancer cell growth. *Prostate* 2011;**71**(4):333–43.
98. Medina-Franco JL, Lopez-Vallejo F, Kuck D, Lyko F. Natural products as DNA methyltransferase inhibitors: a computer-aided discovery approach. *Mol Divers* 2010;**1**: 1–12.
99. Christman JK. 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 2002;**21**:5483–95.

100. Bestor TH. Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J* 1992;**11**:2611–7.
101. Posfai J, Bhagwat AS, Posfai G, Roberts RJ. Predictive motifs derived from cytosine methyltransferases. *Nucleic Acids Res* 1989;**17**:2421–35.
102. Margot JB, Ehrenhofer-Murray AE, Leonhardt H. Interactions within the mammalian DNA methyltransferase family. *BMC Mol Biol* 2003;**4**:7.
103. Youngblood B, Shieh FK, Buller F, Bullock T, Reich NO. S-adenosyl-L-methionine-dependent methyl transfer: observable precatalytic intermediates during DNA cytosine methylation. *Biochemistry* 2007;**46**:8766–75.
104. Shieh FK, Reich NO. AdoMet-dependent methyl-transfer: Glu119 is essential for DNA C5-cytosine methyltransferase M.HhaI. *J Mol Biol* 2007;**373**:1157–68.
105. Shieh FK, Youngblood B, Reich NO. The role of Arg165 towards base flipping, base stabilization and catalysis in M.HhaI. *J Mol Biol* 2006;**362**:516–27.
106. Zhou H, Purdy MM, Dahlquist FW, Reich NO. The recognition pathway for the DNA cytosine methyltransferase M.HhaI. *Biochemistry* 2009;**48**:7807–16.
107. Cheng X. Structure and function of DNA methyltransferases. *Annu Rev Biophys Biomol Struct* 1995;**24**:293–318.
108. O'Gara M, Roberts RJ, Cheng X. A structural basis for the preferential binding of hemimethylated DNA by HhaI DNA methyltransferase. *J Mol Biol* 1996;**263**:597–606.
109. Cheng X, Kumar S, Posfai J, Pflugrath JW, Roberts RJ. Crystal structure of the HhaI DNA methyltransferase complexed with S- adenosyl-L-methionine. *Cell* 1993;**74**:299–307.
110. O'Gara M, Klimasauskas S, Roberts RJ, Cheng X. Enzymatic C5-cytosine methylation of DNA: mechanistic implications of new crystal structures for HhaL methyltransferase-DNA-AdoHcy complexes. *J Mol Biol* 1996;**261**:634–45.
111. Schluckebier G, O'Gara M, Saenger W, Cheng X. Universal catalytic domain structure of AdoMet-dependent methyltransferases. *J Mol Biol* 1995;**247**:16–20.
112. O'Gara M, Zhang X, Roberts RJ, Cheng X. Structure of a binary complex of HhaI methyltransferase with S-adenosyl-L-methionine formed in the presence of a short non-specific DNA oligonucleotide. *J Mol Biol* 1999;**287**:201–9.
113. Dong A, Zhou L, Zhang X, Stickel S, Roberts RJ, Cheng X. Structure of the Q237W mutant of HhaI DNA methyltransferase: an insight into protein-protein interactions. *Biol Chem* 2004;**385**:373–9.
114. Svedruzic ZM, Reich NO. The mechanism of target base attack in DNA cytosine carbon 5 methylation. *Biochemistry* 2004;**43**:11460–73.
115. Lindstrom Jr. WM, Flynn J, Reich NO. Reconciling structure and function in HhaI DNA cytosine-C-5 methyltransferase. *J Biol Chem* 2000;**275**:4912–9.
116. Vilkaitis G, Merkiene E, Serva S, Weinhold E, Klimasauskas S. The mechanism of DNA cytosine-5 methylation. Kinetic and mutational dissection of Hhai methyltransferase. *J Biol Chem* 2001;**276**:20924–34.
117. Zingg JM, Shen JC, Jones PA. Enzyme-mediated cytosine deamination by the bacterial methyltransferase M.MspI. *Biochem J* 1998;**332**:223–30.
118. Zingg JM, Shen JC, Yang AS, Rapoport H, Jones PA. Methylation inhibitors can increase the rate of cytosine deamination by (cytosine-5)-DNA methyltransferase. *Nucleic Acids Res* 1996;**24**:3267–75.
119. Shen JC, Rideout III WM, Jones PA. High frequency mutagenesis by a DNA methyltransferase. *Cell* 1992;**71**:1073–80.
120. Rideout WMI, Coetzee GA, Olumi AF, Jones PA. 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science* 1990;**249**:1288–90.
121. Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, Ellenberger T. *DNA repair and mutagenesis.* 2nd ed. Washington, DC: ASM Press; 2005.

122. D'Aiuto L, Marzulli M, Mohan KN, Borowczyk E, Saporiti F, Vandemark A, et al. Dissection of structure and function of the N-terminal domain of mouse DNMT1 using regional frameshift mutagenesis. *PLoS One* 2010;**5**:e9831.
123. Jeltsch A. Reading and writing DNA methylation. *Nat Struct Mol Biol* 2008;**15**:1003–4.
124. Fersht A. *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding (Hardcover)*. 1st ed. New York, United Media Business; 1998.
125. Motulsky H, Christopoulos A. *Fitting models to biological data using linear and nonlinear regression: a practical guide to curve fitting*. 1st ed. USA: Oxford University Press; 2004.
126. Tipton KF. Enzyme assays. In: Eisenthal R, Danson MJ, editors. *Practical approach*. Oxford, UK: University Press; 2002. p. 282.
127. Luo W, Karpf AR, Deeb KK, Muindi JR, Morrison CD, Johnson CS, et al. Epigenetic regulation of vitamin D 24-hydroxylase/CYP24A1 in human prostate cancer. *Cancer Res* 2010;**70**:5953–62.
128. Yang Q, Tian Y, Ostler KR, Chlenski A, Guerrero LJ, Salwen HR, et al. Epigenetic alterations differ in phenotypically distinct human neuroblastoma cell lines. *BMC Cancer* 2010;**10**:286.
129. Walsh C, Cromartie T, Marcotte P, Spencer R. Suicide substrates for flavoprotein enzymes. *Methods Enzymol* 1978;**53**:437–48.
130. Wang ZX. Kinetics of suicide substrates. *J Theor Biol* 1990;**147**:497–508.
131. Waley SG. Kinetics of suicide substrates. Practical procedures for determining parameters. *Biochem J* 1985;**227**:843–9.
132. Vilkaitis G, Dong A, Weinhold E, Cheng X, Klimasauskas S. Functional roles of the conserved threonine 250 in the target recognition domain of HhaI DNA methyltransferase. *J Biol Chem* 2000;**275**:38722–30.
133. Parker JB, Bianchet MA, Krosky DJ, Friedman JI, Amzel LM, Stivers JT. Enzymatic capture of an extrahelical thymine in the search for uracil in DNA. *Nature* 2007;**449**:433–7.
134. Qi Y, Spong MC, Nam K, Banerjee A, Jiralerspong S, Karplus M, et al. Encounter and extrusion of an intrahelical lesion by a DNA repair enzyme. *Nature* 2009;**462**:762–6.
135. Krosky DJ, Bianchet MA, Seiple L, Chung S, Amzel LM, Stivers JT. Mimicking damaged DNA with a small molecule inhibitor of human UNG2. *Nucleic Acids Res* 2006;**34**:5872–9.
136. Meeker JD, Sathyanarayana S, Swan SH. Phthalates and other additives in plastics: human exposure and associated health outcomes. *Philos Trans R Soc Lond B Biol Sci* 2009;**364**:2097–113.
137. Margot JB, Aguirre-Arteta AM, Di Giacco BV, Pradhan S, Roberts RJ, Cardoso MC, et al. Structure and function of the mouse DNA methyltransferase gene: Dnmt1 shows a tripartite structure. *J Mol Biol* 2000;**297**:293–300.
138. Klotz IM. *Ligand-receptor energetics: a guide for the perplexed.* New York, Wiley; 1997.
139. Haynie DT. *Biological thermodynamics*. 2nd ed. Cambridge, UK: Cambridge University Press; 2008.
140. Cantor C, Schimmel P. *Biophysical chemistry.* New York, United Media Business; 1980.
141. Shearman MS, Beher D, Clarke EE, Lewis HD, Harrison T, Hunt P, et al. L-685, 458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid beta-protein precursor gamma-secretase activity. *Biochemistry* 2000;**39**:8698–704.
142. Walker JR, Avvakumov GV, Xue S, Li Y, Bountra C, Weigelt J, et al. Structure of the replication foci-targeting sequence of human dna cytosine methyltransferase Dnmt1. Structural Genomics Consortium (Sgc), 2008/9/30: PDB 3EPZ.
143. Cheng X, Blumenthal RM. Mammalian DNA methyltransferases: a structural perspective. *Structure* 2008;**16**:341–50.
144. Kameshita I, Sekiguchi M, Hamasaki D, Sugiyama Y, Hatano N, Suetake I, et al. Cyclin-dependent kinase-like 5 binds and phosphorylates DNA methyltransferase 1. *Biochem Biophys Res Commun* 2008;**377**:1162–7.

145. Glickman JF, Pavlovich JG, Reich NO. Peptide mapping of the murine DNA methyltransferase reveals a major phosphorylation site and the start of translation. *J Biol Chem* 1997;**272**:17851–7.
146. Gale JM, Nissen KA, Smerdon MJ. UV-induced formation of pyrimidine dimers in nucleosome core DNA is strongly modulated with a period of 10.3 bases. *Proc Natl Acad Sci USA* 1987;**84**:6644–8.
147. Gale JM, Smerdon MJ. Photofootprint of nucleosome core DNA in intact chromatin having different structural states. *J Mol Biol* 1988;**204**:949–58.
148. Schermelleh L, Haemmer A, Spada F, Rosing N, Meilinger D, Rothbauer U, et al. Dynamics of Dnmt1 interaction with the replication machinery and its role in postreplicative maintenance of DNA methylation. *Nucleic Acids Res* 2007;**35**:4301–12.
149. Bronner C, Chataigneau T, Schini-Kerth VB, Landry Y. The "Epigenetic Code Replication Machinery", ECREM: a promising drugable target of the epigenetic cell memory. *Curr Med Chem* 2007;**14**:2629–41.
150. Bronner C, Fuhrmann G, Chédin F, Macaluso M, Dhe-Paganon S. UHRF1 links the histone code and DNA methylation to ensure faithful epigenetic memory inheritance. *Genet Epigenet* 2009;**2**:29–36.
151. Fellinger K, Rothbauer U, Felle M, Langst G, Leonhardt H. Dimerization of DNA methyltransferase 1 is mediated by its regulatory domain. *J Cell Biochem* 2009;**106**:521–8.
152. Chodavarapu RK, Feng S, Bernatavichute YV, Chen PY, Stroud H, Yu Y, et al. Relationship between nucleosome positioning and DNA methylation. *Nature* 2010;**466**:388–92.
153. Gowher H, Stockdale CJ, Goyal R, Ferreira H, Owen-Hughes T, Jeltsch A. De novo methylation of nucleosomal DNA by the mammalian Dnmt1 and Dnmt3A DNA methyltransferases. *Biochemistry* 2005;**44**:9899–904.
154. Okuwaki M, Verreault A. Maintenance DNA methylation of nucleosome core particles. *J Biol Chem* 2004;**279**:2904–12.
155. Robertson AK, Geiman TM, Sankpal UT, Hager GL, Robertson KD. Effects of chromatin structure on the enzymatic and DNA binding functions of DNA methyltransferases DNMT1 and Dnmt3a in vitro. *Biochem Biophys Res Commun* 2004;**322**:110–8.
156. Choy JS, Wei S, Lee JY, Tan S, Chu S, Lee TH. DNA methylation increases nucleosome compaction and rigidity. *J Am Chem Soc* 2010;**132**:1782–3.
157. Buttinelli M, Minnock A, Panetta G, Waring M, Travers A. The exocyclic groups of DNA modulate the affinity and positioning of the histone octamer. *Proc Natl Acad Sci USA* 1998;**95**:8544–9.
158. Segal E, Widom J. What controls nucleosome positions? *Trends Genet* 2009;**25**:335–43.
159. Kaplan N, Moore IK, Fondufe-Mittendorf Y, Gossett AJ, Tillo D, Field Y, et al. The DNA-encoded nucleosome organization of a eukaryotic genome. *Nature* 2009;**458**:362–6.
160. Segal E, Fondufe-Mittendorf Y, Chen L, Thastrom A, Field Y, Moore IK, et al. A genomic code for nucleosome positioning. *Nature* 2006;**442**:772–8.
161. Li G, Widom J. Nucleosomes facilitate their own invasion. *Nat Struct Mol Biol* 2004;**11**:763–9.
162. Hinz JM, Rodriguez Y, Smerdon MJ. Rotational dynamics of DNA on the nucleosome surface markedly impact accessibility to a DNA repair enzyme. *Proc Natl Acad Sci USA* 2010;**107**:4646–51.
163. Li Q, Wrange O. Translational positioning of a nucleosomal glucocorticoid response element modulates glucocorticoid receptor affinity. *Genes Dev* 1993;**7**:2471–82.
164. Li Q, Wrange O. Accessibility of a glucocorticoid response element in a nucleosome depends on its rotational positioning. *Mol Cell Biol* 1995;**15**:4375–84.
165. Polach KJ, Widom J. Mechanism of protein access to specific DNA sequences in chromatin: a dynamic equilibrium model for gene regulation. *J Mol Biol* 1995;**254**:130–49.

166. Svedruzic ZM, Wang C, Kosmoski JV, Smerdon MJ. Accommodation and repair of a UV photoproduct in DNA at different rotational settings on the nucleosome surface. *J Biol Chem* 2005;**280**:40051–7.
167. Mer G, Bochkarev A, Gupta R, Bochkareva E, Frappier L, Ingles CJ, et al. Structural basis for the recognition of DNA repair proteins UNG2, XPA, and RAD52 by replication factor RPA. *Cell* 2000;**103**:449–56.
168. Fatemi M, Hermann A, Gowher H, Jeltsch A. Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. *Eur J Biochem* 2002;**269**:4981–4.
169. Avvakumov GV, Walker JR, Xue S, Li Y, Duan S, Bronner C, et al. Structural basis for recognition of hemi-methylated DNA by the SRA domain of human UHRF1. *Nature* 2008;**455**:822–5.
170. Kim JK, Esteve PO, Jacobsen SE, Pradhan S. UHRF1 binds G9a and participates in p21 transcriptional regulation in mammalian cells. *Nucleic Acids Res* 2009;**37**:493–505.
171. Flynn J, Azzam R, Reich N. DNA binding discrimination of the murine DNA cytosine-C5 methyltransferase. *J Mol Biol* 1998;**279**:101–16.
172. Shukla V, Coumoul X, Lahusen T, Wang RH, Xu X, Vassilopoulos A, et al. BRCA1 affects global DNA methylation through regulation of DNMT1. *Cell Res* 2010;**20**:1201–15.
173. Tan HH, Porter AG. p21(WAF1) negatively regulates DNMT1 expression in mammalian cells. *Biochem Biophys Res Commun* 2009;**382**:171–6.
174. Le Gac G, Esteve PO, Ferec C, Pradhan S. DNA damage-induced down-regulation of human Cdc25C and Cdc2 is mediated by cooperation between p53 and maintenance DNA (cytosine-5) methyltransferase 1. *J Biol Chem* 2006;**281**:24161–70.
175. Matzke MA, Birchler JA. RNAi-mediated pathways in the nucleus. *Nat Rev Genet* 2005;**6**:24–35.
176. Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Toth KF, et al. A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol Cell* 2008;**31**:785–99.
177. Kanno T, Bucher E, Daxinger L, Huettel B, Bohmdorfer G, Gregor W, et al. A structural-maintenance-of-chromosomes hinge domain-containing protein is required for RNA-directed DNA methylation. *Nat Genet* 2008;**40**:670–5.
178. Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, et al. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev* 2008;**22**:908–17.
179. Tam OH, Aravin AA, Stein P, Girard A, Murchison EP, Cheloufi S, et al. Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* 2008;**453**:534–8.
180. Watanabe T, Totoki Y, Toyoda A, Kaneda M, Kuramochi-Miyagawa S, Obata Y, et al. Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature* 2008;**453**:539–43.
181. Zaratiegui M, Irvine DV, Martienssen RA. Noncoding RNAs and gene silencing. *Cell* 2007;**128**:763–76.
182. Kimura H, Shiota K. Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. *J Biol Chem* 2003;**278**:4806–12.
183. Jeffery L, Nakielny S. Components of the DNA methylation system of chromatin control are RNA-binding proteins. *J Biol Chem* 2004;**279**:49479–87.
184. Nan X, Campoy FJ, Bird A. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* 1997;**88**:471–81.
185. Cross SH, Meehan RR, Nan X, Bird A. A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. *Nat Genet* 1997;**16**:256–9.

186. Weinberg MS, Villeneuve LM, Ehsani A, Amarzguioui M, Aagaard L, Chen ZX, et al. The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *RNA* 2006;**12**:256–62.
187. Goll MG, Kirpekar F, Maggert KA, Yoder JA, Hsieh CL, Zhang X, et al. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science* 2006;**311**:395–8.
188. Jeltsch A, Nellen W, Lyko F. Two substrates are better than one: dual specificities for Dnmt2 methyltransferases. *Trends Biochem Sci* 2006;**31**:306–8.
189. Song J, Rechkoblit O, Bestor TH, Patel DJ. Structure of DNMT1-DNA complex reveals a role for autoinhibition in maintenance DNA methylation. *Science* 2001; **331**(6020): 1036–40.
190. Iakoucheva LM, Radivojac P, Brown CJ, O'Connor TR, Sikes JG, Obradovic Z, et al. The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res* 2004;**32**:1037–49.
191. Dunker AK, Brown CJ, Lawson JD, Iakoucheva LM, Obradovic Z. Intrinsic disorder and protein function. *Biochemistry* 2002;**41**:6573–82.
192. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 1997;**389**:251–60.