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Site-selective in vivo targeting of cytosine-5 DNA methylation by zinc-finger proteins

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ABSTRACT

Cytosine-5 DNA methylation is a critical signal defining heritable epigenetic states of transcription. As aberrant methylation patterns often accompany disease states, the ability to target cytosine methylation to preselected regions could prove valuable in re-establishing proper gene regulation. We employ the strategy of targeted gene methylation in yeast, which has a naturally unmethylated genome, selectively directing de novo DNA methylation via the fusion of C5 DNA methyltransferases to heterologous DNA-binding proteins. The zinc-finger proteins Zif268 and Zip53 can target DNA methylation by M.CviPI or M.Sssl 5–52 nt from single zinc-finger binding sites. Modification at specific GC (M.CviPI) or CG (M.Sssl) sites is enhanced as much as 20-fold compared with strains expressing either the free enzyme or a fusion protein with the zinc-finger protein moiety unable to bind to DNA. Interestingly, methylation is also selectively targeted as far as 353 nt from the zinc-finger protein binding sites, possibly indicative of looping, nucleosomes or higher-order chromatin structure. These data demonstrate that methylation can be targeted in vivo to a potentially broad range of sequences using specifically engineered zinc-finger proteins. Furthermore, the selective targeting of methylation by zinc-finger proteins demonstrates that binding of distinct classes of factors can be monitored in living cells.

INTRODUCTION

Methylation of the C5 atom of cytosine in DNA (m5C) plays an important role in establishing correct patterns of gene expression in vertebrates, usually through repression of transcription. Mechanistically, one way DNA methylation can lead to transcriptional silencing is by decreasing the binding affinity of a transcriptional activator for its site (1). The introduction of m5C at sites adjacent to a factor binding site can also interfere with its binding (2). Perhaps more importantly, symmetrical methylation of CpG sequences (CG) serves as a signal for the recruitment of a family of methyl-CpG binding domain (MBD) proteins, such as MeCP2 and MBD2 (3). In turn, MBDs, either by themselves or as components of complexes, are known to recruit a variety of co-repressors, such as histone deacetylases (4–7), histone H3 lysine-9 methyltransferases (8) and heterochromatin coating factors like HP1 (9), which can function to establish a local, repressed region of chromatin (10–15). This silencing mechanism is also conserved in plants, as the DNA chromomethyltransferase CMT3, which methylates CNG residues, interacts with HP1 to facilitate heterochromatin formation (8).

While regions of m5C are often associated with hypoacetylation of histones H3 and/or H4 and altered chromatin structure (10–15), recent evidence suggests DNA methylation-and histone deacetylase-independent modes of silencing. First, trichostatin A, a specific inhibitor of histone deacetylation, fails to reactivate transcription from densely methylated DNA (2,11,12,15–17). Additionally, mbd2-null mice are viable and fertile (18) and MeCP2-null mice only display neurological abnormalities (19), questioning their global role in m5C-mediated silencing and cellular differentiation. Moreover, purified MeCP2 itself compacts reconstituted chromatin in the absence of DNA methylation (20).

Although the mechanisms are not yet fully understood, there is a strong correlation between promoter methylation and gene silencing (1,21–23). Moreover, once a methylation state is established, it is maintained heritably after many generations of replication (24) by the maintenance DNA methyltransferase (DMTase), Dnmt1 (25). An exception includes enhancer sequences that can be passively demethylated on replication and subsequent blockage of DMTase access by factor binding (26–30). However, this enhancer-specific loss of DNA methylation does not lead to derepression (26).

Proper regulation of gene expression is essential for normal cellular functions and the avoidance of disease states. DNA methylation, which occurs almost exclusively at CG dinucleotides in non-diseased cells, is localized to precise regions of the genome, usually in transposons and retroviral elements (25). In contrast, CG sites in euchromatic regions, most notably when concentrated in CpG islands, are generally unmethylated and are correlated with transcriptional activity. However, in cancer and other diseases, patterns of DNA methylation are frequently aberrant. For instance, the DNA in
tumor cells is generally hypomethylated relative to that in normal cells (31), which may lead to genomic instability (23). In contrast, a number of tumor-suppressor genes, including BRCA1 and retinoblastoma (Rb), become hypermethylated and transcriptionally inactive (32). The presence of a single methylated CG site in a gene’s promoter is sufficient to repress its activation (21), although higher mC density increases the probability of establishing gene repression (14,33–36). Thus, DNA methylation can be critical in defining the expression state of a gene.

Therefore, directing DNA methylation to improperly regulated loci could be used to re-establish proper gene expression through silencing. Previously, targeting of mC has been demonstrated in vitro (37,38), however, selective enrichment of mC was not observed in vivo (38). Recently, in yeast, using the dinucleotide-specific DMTase M.CviPI (39) fused to the basic helix–loop–helix activator Pho4, we demonstrated specific targeting of cytosine methylation to promoters containing Pho4 binding sites [targeted gene methylation (TAGM)] (40). Methylation was efficiently targeted to GC sites in nucleosomes that were disrupted on promoter activation, as well as to histone-free regions.

In its present form, the TAGM strategy is limited to known factors that bind to well characterized DNA-binding sites, which are often present in multiple copies throughout the genome. Therefore, we have investigated the ability of zinc-finger proteins, which, in principle, may be selected to recognize one or a small subset of chromosomal regions (41), to target mC in living cells. Whereas preferential targeting of 4 bp specificity MTases was not observed in vivo (38), we now show that, in yeast, both M.CviPI (GC methylation) and M.SssI (CG methylation) can be preferentially targeted by zinc-finger proteins to specific GC or CG sites neighboring their cognate binding sites. The potential to direct mC at ~20-fold increased resolution to a broad range of desired DNA sequences could lead to novel therapeutic approaches.

**MATERIALS AND METHODS**

**Plasmids, yeast strains and growth conditions**

All yeast strains used in this study were derived from the S288C background strain YPH500AL (MATα ade2-101 ura3-52 his3-D200 leu2-Δ1 trp1-Δ63 lys2-Δ1) (26). Zinc-finger coding sequences were PCR amplified using the primers MKO46, 5’-gcaagttaggccagctgcgtggCTGAGCCTGATCCTGGGATCCTAGGCCACCTCCACTCC-3’ and MKO47, 5’-gaaataattcgAGCGCTTTCAAGGTCATGGTGGATCCTAGGCCACCTCCACTCC-3’, and cloned between SfiI and AfeI restriction sites as in-frame fusions to either M.CviPI or M.SssI in pMPK1. The fusion proteins are expressed under the control of the GALI promoter after integration at LYS2 as previously described (26). Each N-terminal zinc-finger protein is separated from the DMTase by a G(SGGGG)5SGGGLGST (GS linker) peptide (37). As a free DMTase control, mutated Zif268 (mut Zif), which contains a single amino acid substitution (H58E) (42) that ablates DNA binding, was constructed by overlap site-directed mutagenesis using the primers MKO72, 5’-cagtcgtagtgactcggAgtacctacccac-3’, and MKO73, 5’-gtgggtgtaacctgAgtcagactg-3’ (mutated residues in upper case).

Cells were pre-grown in yeast extract (Difco)/peptone (Difco)/2% dextrose (YPD) medium and then washed and resuspended at an OD600 of 0.5 in YP/2% galactose (YPG). After resuspension in YPG, cells were incubated at 30°C for 16 h, or for the indicated times (Fig. 1C).

**Bisulfite genomic sequencing**

Total genomic DNA was rapidly isolated by the phenol/chloroform lysis method (43) and analyzed by bisulfite genomic sequencing (44,45) as previously modified (26). PCR amplification from bisulfite-treated genomic DNA with the indicated primer pairs was performed with Jumpstart Taq DNA polymerase (Sigma) and the resulting products were subjected to primer extension using a 32P-labeled oligonucleotide as described previously using final concentrations of 5 μM dATP, dCTP and dTTP (dGTP omitted) as well as 50 μM ddGTP (26) (Figs 1 and 2), or with dNTPs (A, C, T) and ddGTP increased to 50 and 150 μM, respectively (Figs 3–5), as recently reported (40). Product intensities were determined by ImageQuaNT software (Molecular Dynamics) after subtracting the local background average. Absolute frequencies of cytosine methylation were obtained by dividing the intensity of a given band by all summed product intensities in a given lane, including the run-off product at the top of the gel generated by primer extension on templates lacking cytosine residues (i.e. templates not methylated in vivo). Oligonucleotides used for PCR amplification of bisulfite-treated DNA are described in Table 1 using the original naming conventions of Frommer et al. (44).

**RESULTS**

**In vivo targeting of C5 DMTases near single, Zif268 binding sites**

mC has been selectively targeted in vitro by fusing C5 DMTases (M.Hhal, M.HpaII and M.SssI) to zinc-finger DNA-binding factors (37,38). However, attempts to use zinc-finger proteins as targeting entities in vivo have been unsuccessful (38). As a first step toward targeting DNA methylation in vivo using zinc-finger proteins, we tested whether we could increase cytosine methylation levels neighboring zinc-finger protein binding sites (ZBS) in the genetically tractable eukaryote, Saccharomyces cerevisiae. Yeast genomic DNA does not contain detectable levels of endogenous methylated residues (46) (Figs 4A, lane 4 and 5A and C, lane 5), enabling unambiguous detection of de novo DNA methylation. Also, low-level expression of C5 DMTases in yeast has no known effects on gene expression or growth (26,27,47).

Since chromatin blocks access of DMTases to their target sites (26,27,47–49), our efforts to target mC in vivo focus on the use of enzymes that methylate dinucleotide sites. This substantially increases the probability (~20-fold) that DMTase target sites located in accessible, histone-free regions will be modified. Either of two C5 DMTases, M.CviPI (GC specificity) (39) or M.SssI (CG specificity) (50), was tethered to the archetypal zinc-finger protein, Zif268 (51) and expressed as a single-copy, integrated gene under the control of the galactose-inducible GALI promoter. The DNA-binding factor that is fused to the DMTase is designated the targeting factor. As a control, we expressed either the untethered DMTase or a
fusion protein in which the DNA-binding activity of Zif268 was severely impaired (42). Strains expressing these ‘free’ DMTase controls establish the level of non-targeted methylation due to enzyme site preferences and accessibility in protein-free DNA and chromatin (26,27,47±49).

Endogenous yeast Zif268 binding sites (5’-GCGTGGGCGC-3’) were identified by the PatMatch search engine (http://www.yeastgenome.org/). We determined the relative methylation frequencies at multiple GC (M.CviPI) and CG (M.SssI) sites at the CAR1 locus containing a single, consensus binding

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**Figure 1.** Targeting C5 DMTases near a single Zif268 site. (A) Determination of mC levels targeted by Zif268±DMTase fusions. Genomic DNA isolated from strains expressing wild-type Zif268±M.CviPI (Zif±M.CviPI, lane 2), Zif268±M.SssI (Zif±M.SssI, lane 3), or ‘free’ DMTase controls, a mutated Zif268 fused to M.CviPI (mut Zif±M.CviPI, lane 1) or M.SssI by itself (lane 4), was analyzed by modified bisulfite genomic sequencing (26) of CAR1 from +558 to +159. Distances (bp) of a subset of sites from the proximal edge of the Zif268 ZBS (filled bar; +438 to +446) are indicated at left and right of the gel. Sites of non-targeted methylation (filled circles). Sites 46 (M.CviPI strains) and 25 (M.SssI strains) (arrows) were chosen for normalization to enable lane-to-lane comparisons [see (B)]. Each DMTase was preferentially targeted to several CG and GC sites (asterisks) by Zif binding as compared with its respective control (compare lanes 1 with 2 and 3 with 4). For site 19, 41% (of all summed intensities) of the templates in the population are methylated. Lanes T, G and A (left) contain sequencing reactions with ddATP, ddCTP and ddTTP, respectively. (B) Quantitative scans of the phosphoimage in (A). See (A) for definitions of symbols. (C) Time course of targeting M.CviPI by Zif268. Expression of Zif±M.CviPI (lanes 1–6) and Zif±M.SssI (lanes 7–9) from the GAL1 promoter was initiated by transferring cells from YPD (dextrose) to YPG (galactose) medium. Genomic DNA was isolated at the indicated times and analyzed as in (A). Symbols are defined in (A). (D) Quantification of preferential targeting of M.CviPI by Zif268. Ratios of mC for the indicated sites (normalized to site 46) for Zif±M.CviPI to mut Zif±M.CviPI are given (mean ± standard error; n = 3). Similar values are obtained if the ratios for each site are normalized to other sites of non-targeted methylation (A, filled circles) or calculated using absolute frequencies of methylation (see Materials and Methods).
site for Zif268 by bisulfite genomic sequencing (see Materials and Methods) (40). Specific binding by the Zif268 moiety of each fusion protein is supported by protection of multiple CG and GC sites against methylation at the Zif268 site in strains expressing a wild-type Zif268 fusion as compared with its respective free DMTase (Fig. 1A±C, compare lanes 1 with 2 and 3 with 4, filled bar). Ratios of m^5C among several sites in a given lane are similar, identifying sites at which non-targeted methylation occurs (filled circles), which enables normalization for differences in methylation activity between strains. By this criterion, the mut Zif±M.CviPI strain has ~2-fold more methylation activity than cells expressing Zif±M.CviPI. The reason for this activity difference is unclear. DNA methylation increased substantially at several sites (asterisks) in cells expressing Zif±M.CviPI and Zif±M.SssI versus mut Zif±M.CviPI and M.SssI, respectively. Targeted modification sites (asterisks) are readily identifiable by normalizing to sites of non-targeted methylation (filled circles). In the case of Zif±M.CviPI, which targets m^5C more efficiently, 41, 12.4, 2.3 and 2.6% of GC sites 19, 41, 163 and 172, were methylated, respectively. Since the methylation level at each of these sites exceeds that at the normalization site 46 over a time course of Zif±M.CviPI induction, different levels/duration of Zif±M.CviPI expression do not affect the relative efficiency of targeting m^5C (Fig. 1C). The fold increases in m^5C at each targeted site in strains expressing Zif–M.CviPI versus mut Zif–M.CviPI in three independent experiments are presented in Figure 1D.

M.CviPI is targeted most efficiently to a site located 19 bp from the ZBS (Fig. 1D), which correlates well with the optimal distance of 10±40 bp observed when methylating oligonucleotides with other DMTase fusion proteins in vitro (37,38) and in yeast (40). This optimal distance for introducing m^5C is likely related to the length and amino acid sequence of the flexible peptide separating Zif268 and the DMTase (38). However, targeting methylation distal to the consensus ZBS (e.g. sites 163 and 183) is as or more efficient than to some proximal sites (e.g. sites 41, 43 and 52) (Fig. 1A–D). Preferential targeting of M.CviPI and M.SssI also occurs distally, at sites 163–183 nt from the ZBS (Fig. 1A–D). A single, DNA-bound monomer of Zif268 similarly targets both DMTases close to (5–30 bp) and at a considerable distance from (353 bp) a second consensus Zif268 site in YBR108W (+2067 to +2075; Fig. 2A and B). For a third Zif268 binding
site (−397 to −389 of YOLO19W), two GC sites are protected against methylation by Zif±M.CviPI bound at the ZBS, and m5C is targeted to an additional GC site 39 bp from the ZBS (Fig. 2C). In contrast, the relative levels of CG or GC site methylation at the PHO5 promoter, which lacks Zif268 sites, show no significant differences between the wild-type Zif268 fusion and its respective free DMTase control (Fig. 3, compare lanes 1 with 2 and 3 with 4). We conclude that the targeted methylation is due to site-specific DNA binding by Zif268.

**Targeting M.CviPI via phage display-selected Zip53**

The engineered zinc-finger protein Zip53, which specifically binds to a p53 consensus site (5′-GGGACATGT-3′; hereafter Zip53 binding site) (41), was previously fused to M.SssI and used in vitro to target methylation next to its cognate binding site in an oligonucleotide substrate (37). We tested if Zip53 could direct methylation by M.CviPI to regions containing a single Zip53 site in vivo. As above, the Zip53–M.CviPI fusion protein was integrated as a single copy at LYS2 and expressed from the GAL1 promoter. First, we analyzed m5C levels near the consensus Zip53 binding site located in the DED1 coding sequence (−397 to −389 of YOLO19W), two GC sites are protected against methylation by Zif–M.CviPI bound at the ZBS, and m5C is targeted to an additional GC site 39 bp from the ZBS (Fig. 2C). In contrast, the relative levels of CG or GC site methylation at the PHO5 promoter, which lacks Zif268 sites, show no significant differences between the wild-type Zif268 fusion and its respective free DMTase control (Fig. 3, compare lanes 1 with 2 and 3 with 4). We conclude that the targeted methylation is due to site-specific DNA binding by Zif268.

**Figure 3.** Absence of site-selective methylation at unlinked loci. The PHO5 promoter (−1009 to −205), lacking Zif268 sites, was PCR amplified from the same bisulfite-treated samples analyzed in Figures 1 and 2A to determine levels of m5C. The positions of the two known Pho4 transactivator binding sites, UASp1 and UASp2 (open bars), localized to a histone-free, DNase I-hypersensitive region and positioned nucleosome −2 (partial ellipse) (64), respectively, are indicated. GC (lanes 1 and 2) and CG (lanes 3 and 4) sites (filled circles). Note that, relative to the mut Zif–M.CviPI control (lane 1), the lower methylation frequencies in the Zif–M.CviPI strain (lane 2) at each GC site is consistent with the conclusion that it has reduced overall methylation activity. However, the similar ratios of site intensities within lanes 1 and 2 (M.CviPI) as well as within lanes 3 and 4 (M.SssI) demonstrate that m5C accumulates independent of the Zif (or mut Zif) fusion moiety.

**Figure 4.** The engineered zinc-finger protein, Zip53, targets a DMTase to DED1. (A) Determination of m5C levels at DED1 (+475 to +67). Targeted methylation (asterisks), normalized to site 141 (arrow), is detected at GC sites 30, 162 and 178 bp away from the Zip53 binding site (hatched bar) in two Zip53–M.CviPI strains (lanes 2 and 3) that are representative of four independent strains containing the integrated Zip53–M.CviPI fusion gene. Filled circles indicate remaining CG and GC sites of non-targeted methylation (not selectively methylated) on expression of Zip53–M.CviPI. Lanes 1 and 4 contain bisulfite genomic sequencing results from the mut Zif–M.CviPI strain and a Zip53–M.CviPI transformant that contains a non-functional DMTase, respectively. Sequencing ladders (T, G, A) are at the right. (B) Quantitative scans of the phosphoimage in (A). Symbols are defined as in (A).
sequence (+284 to +276; Fig. 4). As expected, since yeast do not have endogenous cytosine DMTases, no modified cytosines are evident in a strain that does not contain a functional copy of M.CviPI (Fig. 4A, lane 4). Normalizing to site 141, relative to the ‘free’ DMTase control (mut Zif–M.CviPI), targeted methylation is detected 30 bp from the DED1 consensus Zip53 site on expression of Zip53–M.CviPI (Fig. 4A, compare lanes 2 and 3 with lane 1). Further, long-range methylation at sites 162 and 178 bp from the consensus ZBS is substantially enhanced. Lastly, there is reproducible, low-level protection of a GC site located 3 bp from the ZBS, indicative of Zip53 binding (Fig. 4A and B).

We also observed long-range targeting of mC from a second consensus Zip53 site located in the YLR016C coding sequence (+298 to +306; Fig. 5A and B). Methylation was enhanced 5.5-fold at site 184, and somewhat less, but significantly (~2.2-fold), at sites 157 and 190 in strains expressing Zip53–M.CviPI relative to mut Zif–M.CviPI. Protection against DNA methylation could not be observed because no GC sites are adjacent to or within the Zip53 binding site. To examine the specificity of the Zip53–DMTase fusion protein, we analyzed mC levels at the CAR1 locus (Fig. 1), which contains a Zif268 site, but no Zip53 site (Fig. 5C). In each lane of the gel in Figure 5C, little to no change exists in the relative methylation levels of 13 GC sites at CAR1. In particular, methylation at site 19 of the CAR1 region, which shows >20-fold enrichment following expression of Zif–M.CviPI (Fig. 1), is not increased in the presence of Zip53–M.CviPI. This result demonstrates that Zip53 specifically binds its site, but not that of Zif268 (the two binding sites have 22% identity). We conclude that, as for Zif268, Zip53 is able to target M.CviPI and thereby significantly increase cytosine methylation at select GC sites near and distal to a cognate ZBS. The use of Zip53 to deliver mC selectively further demonstrates that zinc-finger proteins engineered to recognize pre-determined sequences can be used to introduce de novo methylation essentially to any region of interest.

DISCUSSION

In this report, we demonstrate the ability to target mC in vivo using two zinc-finger proteins, Zif268 and its artificially engineered derivative Zip53. First, significant targeting of mC is observed at select sites both adjacent (5–52 bp) and distal (>150 bp) to a cognate, consensus ZBS (Figs 1, 2, 4 and 5A and B), whereas DNA methylation is not enriched at control loci lacking the ZBS (Figs 3 and 5C). Proximal and distal targeting of mC was also observed in our previous studies using Pho4 as the DMTase targeting factor (40). The reasons for selective targeting of mC to some sites as opposed to others in the same region are not currently understood. At least locally, the length of the peptide linker separating the DMTase and the targeting factor, the helical face of a particular CG or GC site relative to the DNA-bound targeting factor, and accessibility in chromatin each presumably contribute to the preferential targeting. Secondly, since DNA-bound factors impair access of DMTases to their target loci lacking the ZBS (Figs 3 and 5C). In particular, methylation at site 19 of the CAR1 region, which shows ~20-fold enrichment following expression of Zif–M.CviPI (Fig. 1), is not increased in the presence of Zip53–M.CviPI. This result demonstrates that Zip53 specifically binds its site, but not that of Zif268 (the two binding sites have 22% identity). We conclude that, as for Zif268, Zip53 is able to target M.CviPI and thereby significantly increase cytosine methylation at select GC sites near and distal to a cognate ZBS. The use of Zip53 to deliver mC selectively further demonstrates that zinc-finger proteins engineered to recognize pre-determined sequences can be used to introduce de novo methylation essentially to any region of interest.
protein. Taken together, in addition to demonstrating selective enrichment of m\textsuperscript{5}C near ZBS, TAGM provides a highly sensitive means for detecting protein–DNA interactions (40).

The occurrence of targeted m\textsuperscript{5}C beyond distances of 40 nt suggests that two sites well separated in protein-free DNA are juxtaposed by looping, nucleosomes or higher-order chromatin structure (e.g. Fig. 2A, 353 bp away from the ZBS). While it is formally possible that the occurrence of distal methylation is due to binding at a secondary, non-consensus ZBS, we do not believe this to be the case. First, no footprints are observed at all five loci that were analyzed. Finally, Dam MTase can be used to specifically target m\textsuperscript{5}C, thereby reducing the amount of treatment necessary to establish the proper regulation of a particular gene. In addition to providing a potentially powerful therapeutic tool, methylation-mediated repression of specifically targeted genes could yield an alternative to transgenic knockouts for studying loss-of-function phenotypes. Silencing genes through DNA methylation would be particularly valuable in the case of essential genes where tissue-specific knockouts of function are needed. Optimization of targeting factor occupancy at regions of interest will likely increase the efficacy of specific m\textsuperscript{5}C targeting in vivo as well as minimize non-targeted methylation. The experimental system used herein provides a useful assay for pursuing such further investigations. Finally, the ability to target m\textsuperscript{5}C specifically in vivo is likely to prove valuable in basic investigations of the biological roles and mechanistic consequences of DNA methylation.

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