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Christopher D. Carvin

*Texas A & M University - College Station*

Rebecca D. Parr

*Stephen F Austin State University, parrrl@sfasu.edu*

Michael P. Kladde

*Texas A & M University - College Station*

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

Christopher D. Carvin, Rebecca D. Parr and Michael P. Kladde\*

Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station, Texas 77843-2128, USA

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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.SssI 5–52 nt from single zinc-factor binding sites. Modification at specific GC (M.CviPI) or CG (M.SssI) 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 ( $m^5C$ ) 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  $m^5C$  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  $m^5C$  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  $m^5C$ -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

\*To whom correspondence should be addressed. Tel: +1 979 862 6677; Fax: +1 979 845 9274; Email: kladde@tamu.edu  
Present address:

Rebecca D. Parr, Department of Pathobiology, Texas A&M University, 4467 TAMU, College Station, TX 77843-4467, USA

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 m<sup>5</sup>C 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 m<sup>5</sup>C has been demonstrated *in vitro* (37,38), however, selective enrichment of m<sup>5</sup>C was not observed *in vivo* (38). Recently, in yeast, using the dinucleotide-specificity 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 m<sup>5</sup>C 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 m<sup>5</sup>C 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 YPH500ΔL (*MATα ade2-101 ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 lys2-Δ1*) (26). Zinc-finger coding sequences were PCR amplified using the primers MKO46, 5'-gcactagttaggccagctgggccATGGCTGATATCGG-ATCTGG-3', and MKO47, 5'-gaataattcgAGCGTTTTCAA-GGTCATGGTGGATCCTAGGCCACCTCCACTCC-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)<sub>2</sub>SGGGGLGST (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'-cagtcgtagtacgAgctaccaccac-3', and MKO73, 5'-gtgggtgtaagcTcgtcactgactg-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 OD<sub>600</sub> 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

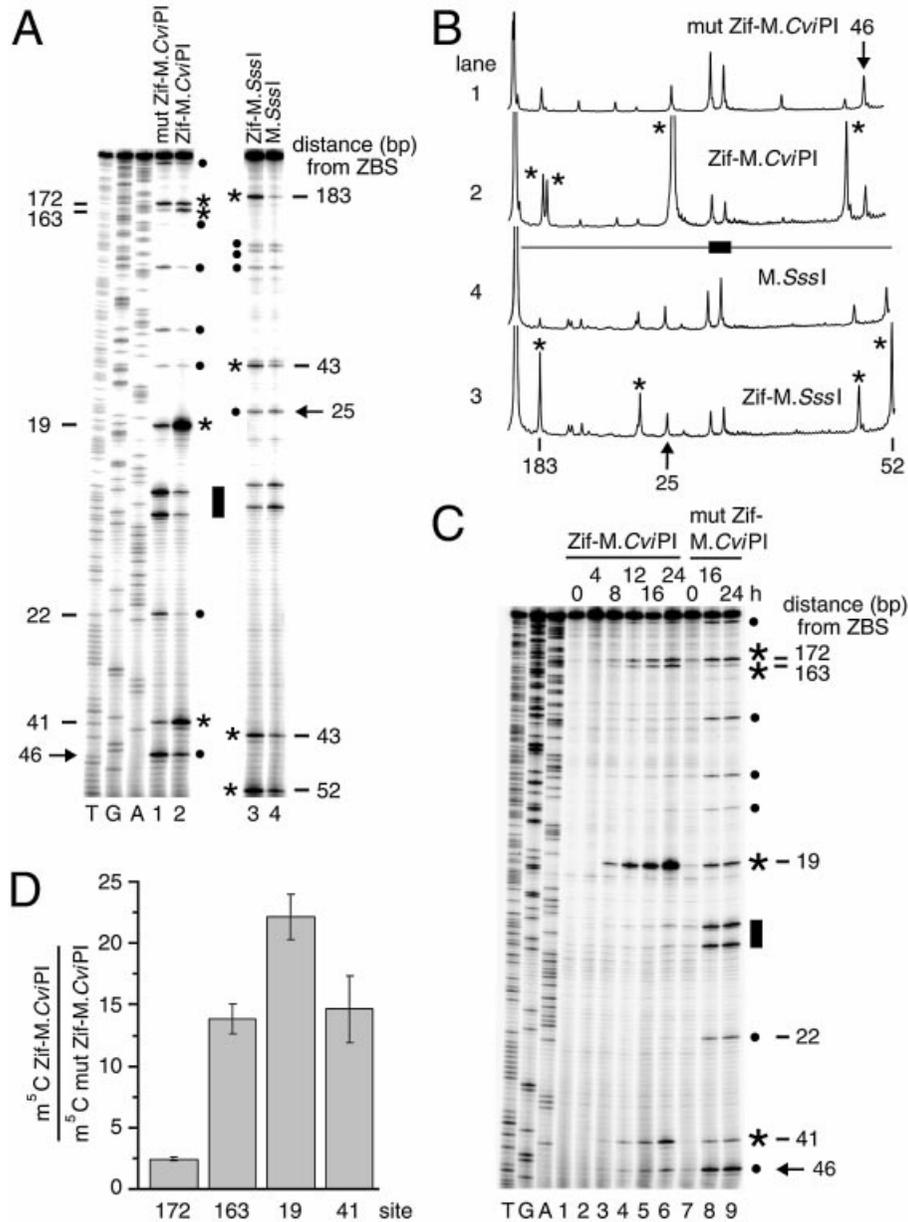
Total genomic DNA was rapidly isolated by the phenol/chloroform lysis method (43) and analyzed by bisulfite genomic sequencing (44,45) as previously modified (26). PCR amplification from bisulfite-treated genomic DNA with the indicated primer pairs was performed with Jumpstart *Taq* DNA polymerase (Sigma) and the resulting products were subjected to primer extension using a <sup>32</sup>P-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

m<sup>5</sup>C has been selectively targeted *in vitro* by fusing C5 DMTases (M.HhaI, 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).

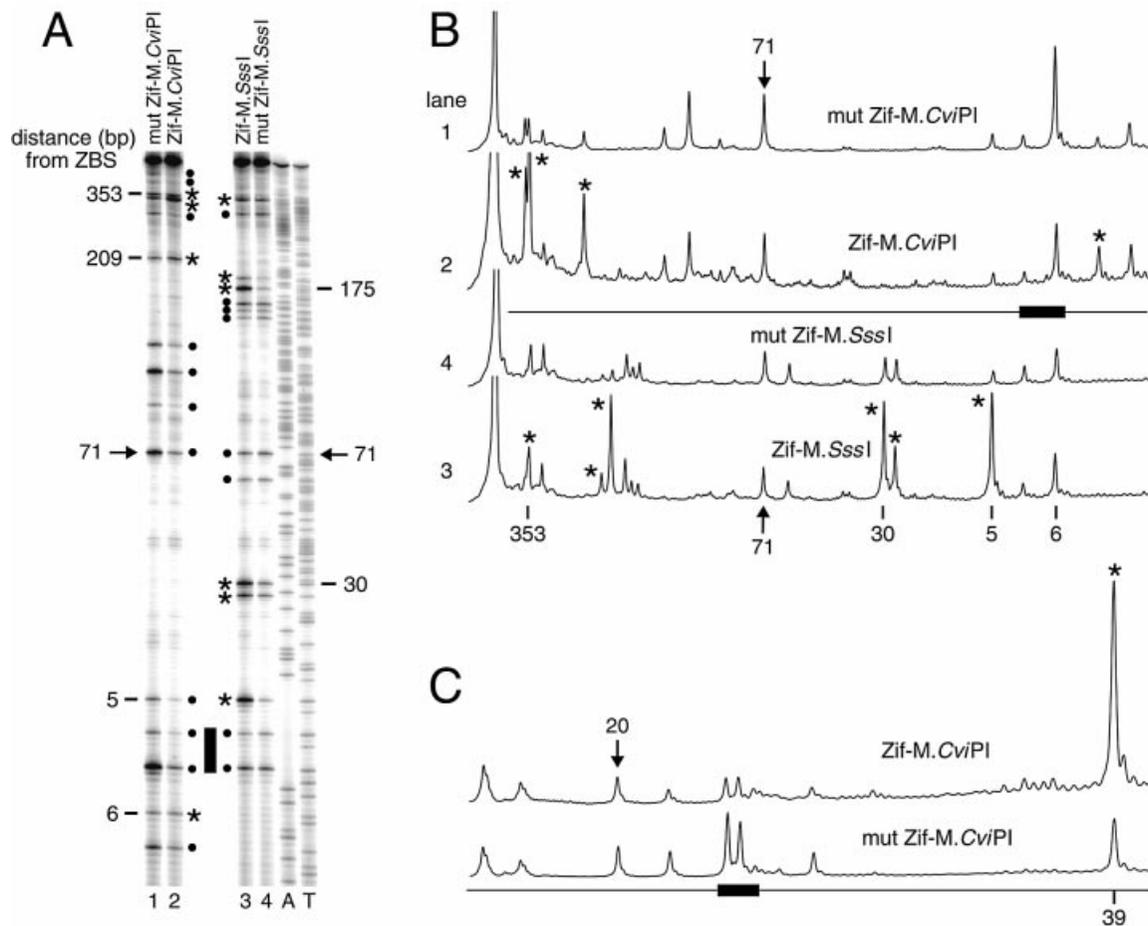
Since chromatin blocks access of DMTases to their target sites (26,27,47–49), our efforts to target m<sup>5</sup>C *in vivo* focus on the use of enzymes that methylate dinucleotide sites. This substantially increases the probability (~20-fold) that DMTase target sites located in accessible, histone-free 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



**Figure 1.** Targeting C5 DMTases near a single Zif268 site. (A) Determination of  $m^5C$  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  $m^5C$  for the indicated sites (normalized to site 46) for Zif–M.CviPI to mut Zif–M.CviPI are given (mean  $\pm$  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).

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′-GCGTGGGCG-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

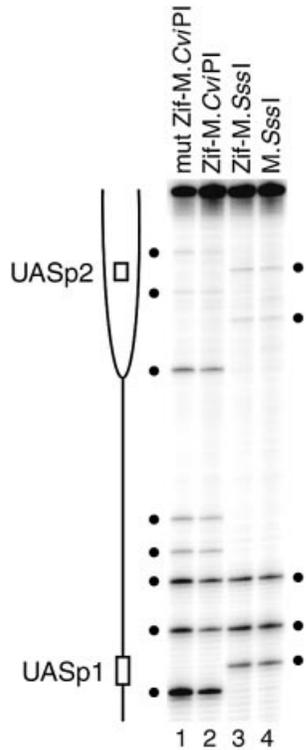


**Figure 2.** Zif268 targets M.CviPI and M.SssI to additional ZBS. (A) Determination of  $m^5C$  levels. A region of *YBR108W* (+1564 to +2163) spanning a single Zif268 site (+2067 to +2075; filled bar) was PCR amplified from the same bisulfite-treated samples analyzed in Figure 1A. Sequencing ladders (A, T) are at the right. Symbols are defined in the caption to Figure 1. (B) Scans of the phosphoimage in (A). The scanned lanes are indicated at the left. (C) Methylation targeted to a third Zif268 site (filled bar) near *YOL019W* (-397 to -389). Only scans of the phosphoimage resulting from the bisulfite genomic sequencing of the region from -509 to +254 are shown.

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

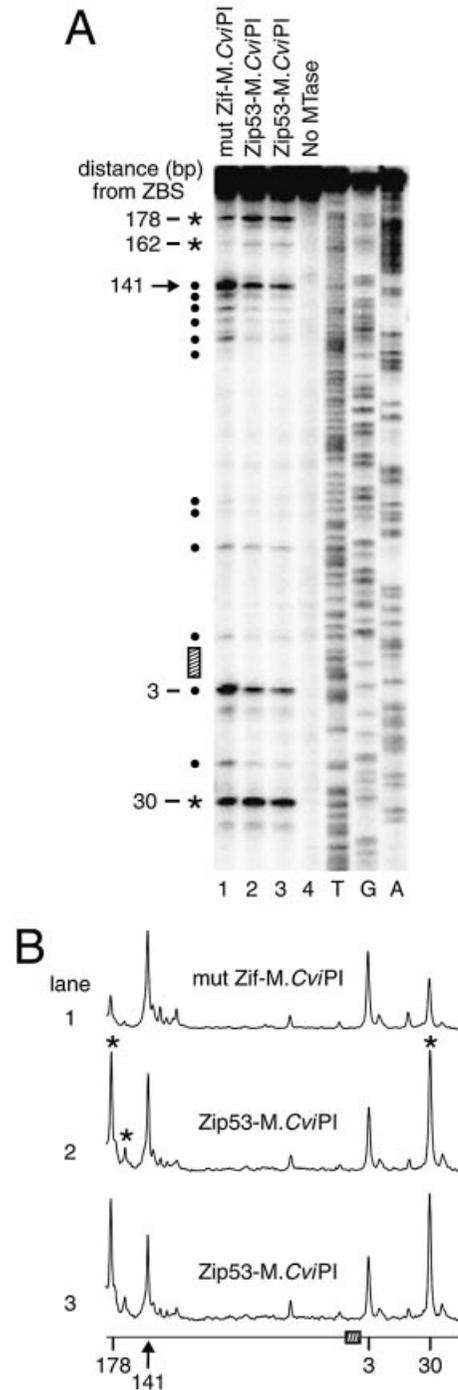


**Figure 3.** Absence of site-selective methylation at unlinked loci. The *PHO5* promoter (–1009 to –205), lacking Zif268 sites, was PCR amplified from the same bisulfite-treated samples analyzed in Figures 1 and 2A to determine levels of m<sup>5</sup>C. The positions of the two known Pho4 transactivator 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 m<sup>5</sup>C accumulates independent of the Zif (or mut Zif) fusion moiety.

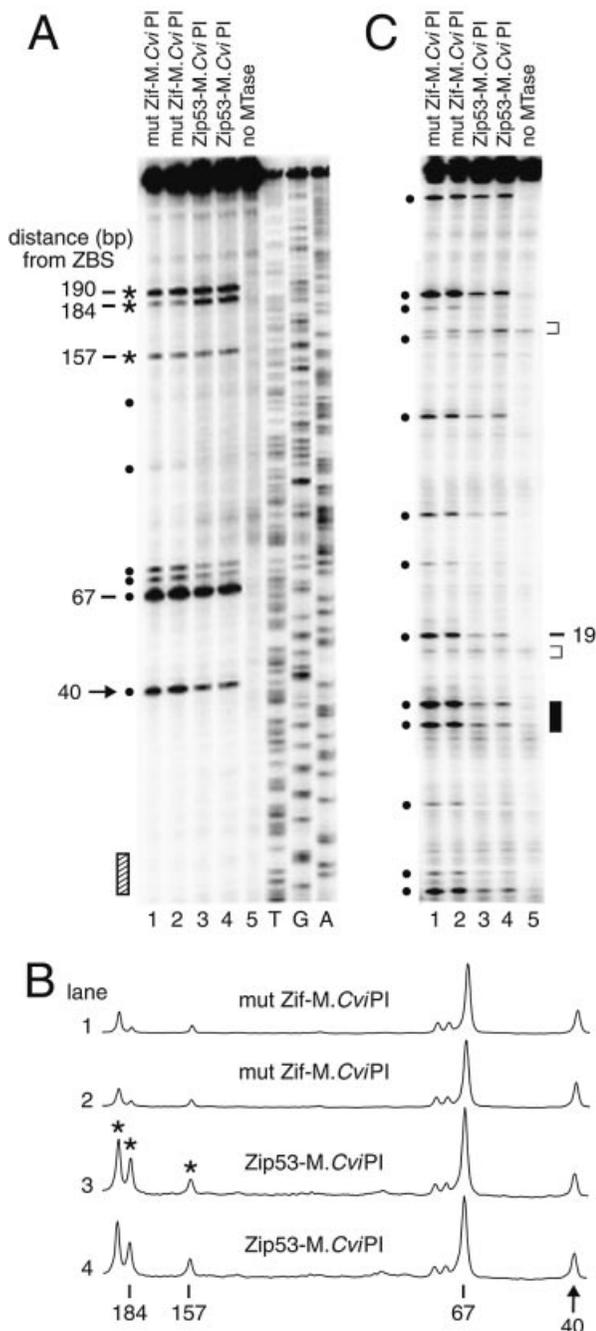
site (–397 to –389 of *YOL019W*), two GC sites are protected against methylation by Zif–M.CviPI bound at the ZBS, and m<sup>5</sup>C is targeted to an additional GC site 39 bp from the ZBS (Fig. 2C). In contrast, the relative levels of CG or GC site methylation at the *PHO5* promoter, which lacks Zif268 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 m<sup>5</sup>C levels near the consensus Zip53 binding site located in the *DED1* coding



**Figure 4.** The engineered zinc-finger protein, Zip53, targets a DMTase to *DED1*. (A) Determination of m<sup>5</sup>C 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).



**Figure 5.** Zip53-mediated targeting of  $m^5C$  to *YLR016C*. (A) Determination of  $m^5C$  levels at *YLR016C* (+418 to +28). Targeted methylation (asterisks), as normalized to site 40 (arrow), is detected at GC sites 157, 184 and 190 bp away from the Zip53 site (hatched bar) in two independent transformants (lanes 3 and 4) relative to mut Zif-M.CviPI (lanes 1 and 2). T, G and A sequencing ladders are at the right. Non-targeted methylation (filled circles). The sample in lane 5 was obtained from a Zip53-M.CviPI transformant harboring a non-functional DMTase. (B) Quantitative scans of the phosphoimage in (A). (C) Determination of  $m^5C$  levels at the *CAR1* locus (+558 to +159) that has a Zif268 binding site (filled bar) but no consensus Zip53 binding site. The PCR products analyzed in lanes 1–4 were amplified from the bisulfite-treated genomic analyzed in lanes 1–4, respectively, in (A). Lane 5 contains a sample from a parental strain that was not transformed with M.CviPI. Non-specific primer extension pauses that do not correspond to GC sites are marked with brackets. Symbols in (B) and (C) are as defined 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  $m^5C$  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  $m^5C$  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  $m^5C$  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  $m^5C$  *in vivo* using two zinc-finger proteins, Zif268 and its artificially engineered derivative Zip53. First, significant targeting of  $m^5C$  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  $m^5C$  was also observed in our previous studies using Pho4 as the DMTase targeting factor (40). The reasons for selective targeting of  $m^5C$  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 sites (26–30,40), the protection against methylation of CG or GC sites next to or within the ZBS provides further evidence of specific ZBS binding by each zinc-finger-DMTase fusion

**Table 1.** Bisulfite genomic sequencing primers

Oligonucleotides for PCR amplification		Figure	<sup>32</sup> P-end-labeled oligonucleotides for primer extension		Figure
Primer <sup>a</sup>	Sequence		Primer	Sequence	
CAR1b1-60	CCATTTaAaaaACTCaaaACAATaTaaaAC	1 and 5C	CAR1b1-60	CCATTTaAaaaACTCaaaACAATaTaaaAC	1 and 5C
CAR1b2-61	TAAtGGAATTAGAGttTtAATGGAtGAG	1 and 5C	YBR108Wa1-58	aCTaTTaaaaaCATCACCCAAACaAC	2A and B
YBR108Wa1-58	aCTaTTaaaaaCATCACCCAAACaAC	2A and B	YLO19Wb1-54	CCaTAaaaAATCAAATTTaCAACAAaTCTaC	2C
YBR108Wa2-59	AAAtTTTtAGAtATGGAtTTGATTtTG	2A and B	PHO5b1-751	TaTTTTCTCATaTAAaCaaAcCaTCaTCT	3
YLO19Wb1-54	CCaTAaaaAATCAAATTTaCAACAAaTCTaC	2C	DED1a1-166	AAaTaACTCTTTaTATTCTTTCATATTaTTT	4
YLO19Wb2-55	ATAGAtTAGtGTTGtTATTGAGTTtAGTTG	2C	YLR016Ca1-1019	CATTCATaAAaATaAaTTCaTAATCaTTATC	5A and B
PHO5b1-922	TTCAATTaCTAAATACAATaTTCCTTaaT	3			
PHO5b2-924	GAAAAtAGGGAtAGAAAtATAAATTTAGTT	3			
DED1a1-166	AAaTaACTCTTTaTATTCTTTCATATTaTTT	4			
DED1a2-165	ttTtAtTTAAGAGGAAAAAtAAGAAGT	4			
YLR016Ca1-1020	TaACaaaAATTCAAAAaTTAACACACTaaAT	5A and B			
YLR016Ca2-1021	GAiTATGTATGAAAtTAGTGATATAAtAG	5A and B			

<sup>a</sup>Pairs of 'a' (a1 and a2) or 'b' (b1 and b2) are PCR amplification primers for the upper and lower DNA strands, respectively, from bisulfite-treated DNA. Nucleotides in lower case represent either G to a or C to t transitions.

protein. Taken together, in addition to demonstrating selective enrichment of m<sup>5</sup>C near ZBS, TAGM provides a highly sensitive means for detecting protein-DNA interactions (40).

The occurrence of targeted m<sup>5</sup>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 near MTase target sites that are >150 bp from the primary Zif268 binding site, despite the fact that high-affinity sites contain two GC sites. Secondly, within 50 bp of each long-range targeted m<sup>5</sup>C site any potential ZBS has a minimum of four to five mismatches from consensus. Since two or more base substitutions result in background levels of binding (42,52-54), it is highly unlikely that any of these sites constitute a significant secondary ZBS. Thirdly, it is improbable that distal secondary ZFP binding sites would be present at all five loci that were analyzed. Finally, Dam MTase can also be targeted at a distance (55,56).

The design of multiple zinc-finger modules with desired specificities is proving a versatile platform for targeting a variety of protein moieties to accessible sites *in vivo* (57). For instance, engineered zinc-finger proteins have been fused to the catalytic domain of R.FokI endonuclease to direct site-specific double-stranded DNA cleavage, and hence homologous recombination, of desired regions (58). Designed zinc-finger proteins have also been used to target the catalytic domains of the histone methyltransferases G9A and SUV39H1 (59) as well as the VP16 activation domain (60-62), leading to repression and activation, respectively, of expression of the endogenous human erythropoietin (*EPO*), vascular endothelial growth factor A (*VEGF-A*) and other mammalian genes (57). This technology has also recently been extended to the regulation of gene expression in plants (63).

The targeting of DMTases by zinc-finger proteins selected to bind specific ZBS could provide an additional way to down-regulate the expression of desired genes. Moreover, since the DNA methylation state of a given promoter is maintained heritably through DNA replication by endogenous cellular

mechanisms, an initial targeting event may be sufficient to establish stable silencing of improperly expressed genes. Therefore, heritable repression could also reduce 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<sup>5</sup>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<sup>5</sup>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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