DNA base flipping analytical pipeline

Peng Zhang,¹ Florian D. Hastert,¹ Anne K. Ludwig,¹ Kai Breitwieser,¹ Maria Hofstätter,² and M. Cristina Cardoso¹,∗

¹Cell Biology and Epigenetics, Department of Biology, Technische Universität Darmstadt, Germany and ²Max Delbrück Center for Molecular Medicine, Berlin, Germany

*Correspondence address. Technische Universität Darmstadt, Schnittspahnstrasse 10, 64287 Darmstadt, Germany. Tel: +49-6151-16-21882; Fax: +49-6151-16-21880; E-mail: cardoso@bio.tu-darmstadt.de

Abstract

DNA base modifications and mutations are observed in all genomes throughout the kingdoms of life. Proteins involved in their establishment and removal were shown to use a base flipping mechanism to access their substrates. To better understand how proteins flip DNA bases to modify or remove them, we optimized and developed a pipeline of methods to step-by-step detect the process starting with protein–DNA interaction, base flipping itself and the ensuing DNA base modification or excision. As methylcytosine is the best-studied DNA modification, here we focus on the process of writing, modifying and reading this DNA base. Using multicolor electrophoretic mobility shift assays, we show that the methylcytosine modifier Tet1 exhibits little DNA sequence specificity with only a slight preference for methylated CpG containing DNA. A combination of chloroacetaldehyde treatment and high-resolution melting temperature analysis allowed us to detect base flipping induced by the methylcytosine modifier Tet1 as well as the methylcytosine writer M.HpaII. Finally, we show that high-resolution melting temperature analysis can be used to detect the activity of glycosylases, methyltransferases and dioxigenases on DNA substrates. Taken together, this DNA base flipping analytical pipeline (BaFAP) provide a complete toolbox for the fast and sensitive analysis of proteins that bind, flip and modify or excise DNA bases.

Keywords: DNA modifications; base flipping; electrophoretic mobility shift assay; high-resolution DNA melting analysis; base excision; methylcytosine

Introduction

DNA base modifications diversify the genome and regulate gene expression in a spatio-temporal manner. One of the best-studied DNA base modifications is 5-methylcytosine (5mC). Its establishment and maintenance are catalyzed by DNA methyltransferases (Dnmts), which transfer a methyl group from S-adenosylmethionine (SAM) to the fifth position of cytosine [1]. However, cytosine is sheltered inside the DNA double helix and stabilized by base pairing with the base guanine (G). Thus, to access and subsequently modify cytosine, a DNA base flipping mechanism is used by Dnmts. The base flipping phenomenon was first described in 1994 in a ternary complex containing HhaI methyltransferase (M.HhaI) [2], DNA and S-adenosylhomocysteine (SAH). In this complex, cytosine is rotated out from the DNA backbone by 180° and inserted into the catalytic pocket of the M.HhaI enzyme. Since then, more and more Dnmts were described to use a base flipping mechanism to achieve DNA base modifications, including M.HaelIII [3], M.TaqI [4], M.T4Dam [5], M.EcoDam [6], and the murine DNA methyltransferase Dnmt1 [7].

Moreover, 5mC can be modified by Ten-eleven translocation proteins (Tet) to 5-hydroxymethylcytosine [8, 9], 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in an iterative iron and oxoglutarate dependent oxidation reaction [10, 11]. Similar to
other DNA base modifiers, Tet proteins use a base flipping mechanism to modify 5mC to 5hmC [12, 13] and subsequently to 5fC and 5caC [14, 15].

In addition to DNA modifications, mutations of DNA bases that create mismatched base pairing are observed in vivo. Deamination of 5mC for instance [16], which either occurs spontaneously or is catalyzed by APOBEC3A, creates T:C mismatches [17]. To ensure proper genetic and epigenetic heritable information for subsequent cell generations, these mutated DNA bases must be repaired. One enzyme identified to be involved in T:C mismatch repair is Mbd4, a member of the MBD protein family that contains a glycosylase domain [18]. Following binding, the aberrant base is flipped out of the DNA helix and removed by Mbd4. The resulting abasic site is then repaired by enzymes of the base excision repair pathway [19].

To date, several methods are used to detect DNA base flipping, including X-ray crystallography [2] and nuclear magnetic resonance (NMR) spectroscopy [20]. Although X-ray crystallography provides the absolute proof of DNA base flipping, it is very demanding and time-consuming and depends on sophisticated equipment. The above makes it also not the method of choice to analyze the effect of mutations. The 2-aminopurine (2AP), an analog of the base adenine, has been used to detect base flipping in real time [21]. The fluorescence signal of 2AP is highly quenched when it is incorporated into DNA and located inside of the DNA helix. Conformational changes (bending/kinking) of the DNA increase 2AP fluorescence; however, the highest fluorescence intensities were observed when 2AP is flipped out of the DNA helix [22]. Moreover, several chemicals that react with flipped DNA bases have been identified. The resulting reaction products can be subsequently visualized by piperidine-induced strand cleavage and electrophoresis on denaturing gels [23–25]. Chloroacetaldehyde (CAA), for instance, reacts with the unpaired bases A and C to form 1,N6-ethenoadenine and 3,N7-ethenocytosine, respectively [26]. In combination with piperidine treatment, several Dnmts and endonucleases were successfully verified to use a base flipping mechanism to modify DNA bases [24]. Although M.HpaII belongs to the DNA methyltransferase family, several studies failed to detect that it induces base flipping [24], indicating that the current detection methods do not provide sufficient sensitivity and require optimization.

Since double-stranded DNA is mainly stabilized by base pairing between complementary strands, as well as base stacking interactions between adjacent bases [27], any DNA base modification would affect DNA double strand stability. To determine the thermostability of double-stranded DNA, the melting temperature measurement was developed. In particular, high-resolution melting (HRM) temperature analysis was shown to be a highly sensitive method that allows discrimination of a single base mutation within double-stranded DNA [28].

In this study, we developed a pipeline of methods to analyze every step of the DNA base flipping process. We apply these methods to the analysis of the cytosine base modifiers (M.HpaII and Tet1) and a methylcytosine reader and glycosylase (Mbd4). We show that our pipeline of methods is sensitive enough to analyze DNA binding, DNA stability, DNA base flipping and DNA base modification/excision induced by a variety of proteins involved in modifying and reading DNA bases.

Materials and methods
Step-by-step detailed protocols and materials for every assay are included in the Supplementary Data.

DNA preparation
DNA oligonucleotides were purchased from IBA (Germany) or IDT (Integrated DNA Technologies, Germany). To prepare fully-, hemi- or unmethylated double-stranded DNA, same molar of upper (CGUp or MGup, 42-mer) and lower strand (Fill-In-ATT0550 or Fill-In-ATT0647N, 20-mer) were mixed in NEBuffer 2 (50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl2, 1 mM dithiothreitol; NEB, USA), denatured at 95°C for 2 min and annealed by slowly cooling down to 37°C. Then, the primer extension reactions were performed in the presence of 0.05 U/μl Klenow fragment, 1 μM dTTP, dGTP, dATP (PepLab, Germany) and either 0.1 μM dCTP or dmCTP at 37°C for 1 h. The methylation statuses of double-stranded DNA were confirmed by restriction enzyme MspI and HpaII digestion. To prepare double-stranded DNA containing T:C mismatch, same molar of upper (MGup, 42-mer) and lower strand (T:C mismatch DNA, 42-mer) were mixed in NEBuffer 2, denatured at 95°C for 2 min and annealed by slowly cooling down to 37°C.

PCR fragments were amplified from a plasmid containing a MINX sequence [29] using either dCTP or dmCTP as described [30].

Protein purification
GFP-, YFP-, mcherry- or His tagged proteins were purified from Sf9 insect cells as previously described [30]. For GFP and YFP purification, Ni-NTA beads coupled to GFP binding protein (GBP; [31]) were used. For mcherry purification, Ni-NTA beads coupled to RFP binding protein (RBP; [32]) were used. For His-tagged proteins, Talon beads (Clontech Laboratories, USA) were used. M.HpaII methyltransferase was purchased from NEB (cat. no. M0214S).

Electrophoretic mobility shift analysis
Purified proteins were incubated with the different fluorescently labeled oligonucleotides in binding buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 3 mM MgCl2, 2 mM DTT, 4% glycerol, and 0.1% Triton X-100) for 90 min at 37°C for all proteins except Mbd4, which was incubated at 4°C for 30 min. Complexes were analyzed on a nondenaturing 4.5% polyacrylamide gel (acrylamide/bis-acrylamide, 30% solution). Fluorescent signals (protein and DNA) were detected using a fluorescence imager (Amersham Imager 600 RGB) and also a fluorescence plate reader (TECAN Infinite® M200).

The fluorescent signals from the plate reader were plotted as a heatmap using a self-written R script (see Supplementary Data).

HRM temperature analysis
To detect DNA base flipping, we incubated the oligonucleotides with purified proteins at 37°C for 2 h in the presence or absence of 2-CAA. After incubation, Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, cat. no. 11733046) was denatured and added to the sample. HRM temperature analysis was performed in a StepOnePlus™ Real-Time PCR machine (Applied Biosystems). Samples were first denatured at 95°C for 30s, then temperature was decreased to 50°C with a decreasing rate of 2%, followed by increasing the temperature to 90°C with 0.1°C steps.

To detect base modification/excision, we incubated the oligonucleotides with purified proteins at 37°C for 2 h. Then HRM temperature analysis was performed as just described.
Data normalization and visualization were performed using a self-written R script (see Supplementary Data).

Results and discussion

Protein–DNA binding assay

We hypothesized that three steps are involved in DNA base modification: (i) protein–DNA binding, (ii) base flipping, and (iii) base modification itself (Fig. 1). Therefore, we first aimed to understand how proteins interact with DNA, a process that is also critical for basic biological processes such as DNA replication, repair, and transcription in vivo. To better understand these processes, methods allowing accurate detection of protein–DNA interactions are essential. In the past few decades, several methods have been developed to detect protein–DNA interactions [33]. In particular, electrophoretic mobility shift assays (EMSAs) showed to be a robust and sensitive method [34]. Classical EMSAs use radioactively labeled DNA to visualize protein–DNA complexes, whereby proteins in the complex are detected only indirectly (Fig. 2A). However, to better understand the composition of protein–DNA complexes it is very important to directly detect both, protein and DNA signals. Therefore, we optimized multicolor EMSAs, which allows simultaneous detection of differently labeled DNA oligonucleotides and proteins. Because large complexes composed of multiple proteins bound to one DNA molecule cannot easily enter the gel, the fluorescence signals in the gel pockets were also included in our detection (Fig. 2A, right lane 5). Through detection of all complexes and complex components, new potential applications, such as protein competition assays and competitive binding to different DNA species can be implemented (Fig. 2A, right lane 5) [30]. Here, we use multicolor EMSA to directly test the specificity of Tet1CD for differently modified DNA substrates in one reaction, which would have not been possible with classical radioactive EMSAs.

The preparation of fluorescently labeled DNA oligonucleotides, purification of fluorescently tagged proteins (Supplementary Fig. S1) as well as the detailed multicolor EMSAs protocol are described in the “Materials and methods” section. To detect signals of fluorescently labeled proteins and DNA oligonucleotides, we initially used a fluorescence imager with 460 nm, 520 nm, and 630 nm excitation wavelengths, as well as 525BP20, 605BP40, and 705BP40 emission filters. However, since the filter settings were not suitable to discriminate yellow fluorescent protein (YFP) and the mcherry protein signals (Supplementary Fig. S2A), we tested and developed a method to detect any combination of fluorescence signals on the gel using a fluorescence microplate reader, which gives full spectral excitation and emission flexibility. Moreover, this detection way immediately provides intensity values without the need for image analysis, which is a must when using fluorescence imagers. A detailed description on how to image polyacrylamide gels on a fluorescence microplate reader can be found in the “Materials and methods” section (Supplementary Fig. S2B-2F) [30].

Since the catalytic domain of Tet1 (Tet1CD) is sufficient for 5mC to 5hmC conversion [30,35], we were interested in how Tet1CD interacts with DNA. To this end, we incubated purified, mcherry-tagged Tet1CD proteins with ATTO-647N labeled DNA oligonucleotides containing a single fully methylated CpG site for 90 min at 37°C. Protein–DNA complexes and free DNA were then separated on a native polyacrylamide gel. As a control, the methyl-CpG binding domain (MBD) of Mecp2, which was shown to have a preference for methylated CpG dinucleotides [30] was used. As shown in Fig. 2B, both proteins formed complexes with methylated DNA under the given reaction conditions (Fig. 2B, upper row). To further test the specificity of Tet1CD proteins to methylated DNA, we incubated both proteins with methylated DNA in the presence of unmethylated competitor DNA poly(dI:dC). In contrast to the MBD, Tet1CD did not form a complex with methylated DNA in the presence of poly(dI:dC) (Fig. 2B, lower row) indicating that Tet1CD interacts with DNA irrespective of its methylation status.

To further confirm and extend these data, we incubated His-tagged Tet1CD or YFP-tagged MBD proteins with DNA oligonucleotides containing a single fully methylated CpG (ATTO647N labeled) together with either a single unmethylated CpG (ATTO550 labeled) or a non-CpG (ATTO550 labeled). After 90 min of incubation at 37°C, DNA–protein complexes were separated on a native polyacrylamide gel. As shown in Fig. 3, Tet1CD formed complexes with methylated and unmethylated
oligonucleotides corroborating the previous results using poly(dI:dC) as competitor. When compared to MBD proteins, which have a preference for methylated DNA, Tet1CD showed only a slight preference for methylated oligonucleotides. Moreover, Tet1CD exhibited also slightly higher binding affinities to methylated CpG DNA compared to non-CpG DNA (Supplementary Fig. S3). These results indicate that although 5mC is a substrate of Tet, it does not significantly enhance Tet binding to DNA, whereas it does enhance the binding of MBD proteins.

Multicolor fluorescently tagged proteins and fluorescently labeled DNA have already been used to detect protein and DNA interactions. To detect fluorescence intensities, previous studies used fluorescence spectrophotometry analysis [37]. In addition, multicolor EMSAs were also used to measure fluorescence intensity and to determine the specificity of proteins to multiple DNA substrates [38]. However, the simultaneous detection of multiple fluorescence signals is often limited, since excitation and emission filters are not easily exchangeable in commercial fluorescence imagers. Our novel detection method, which is based on a fluorescence multwell plate reader, however, allows detection of most commercially available fluorescent dyes without spectral bleed-through and offers full freedom to choose dyes for protein or DNA labeling.

DNA base flipping detection assay

After protein–DNA complex formation, most DNA base modifiers or excision proteins use a base flipping mechanism to access their DNA substrate (Fig. 1). M.HhaI crystal structures showed that base flipping occurs in both, ternary complexes including DNA, the methyltransferase and its cofactor, as well as in binary complexes lacking its cofactor [20,22], indicating that the cofactor itself is not crucial for the base flipping reaction. Moreover, previous studies showed that M.HhaI induced base flipping can be detected only in the absence of its cofactor by CAA treatment [24], suggesting that the cofactor blocks the reaction of CAA with the flipped base. Until now, CAA has been used in combination with piperidine (which cleaves modified nucleotides such as 3,N4-ethenocytosine) to detect base flipping in various systems due to its high reactivity with unpaired cytosines. The product of the reaction, 3,N4-ethenocytosine, disrupts hydrogen bonds with guanine and decreases DNA double strand stability [39,40] (Fig. 4A). Since HRM analysis is used to measure DNA thermostability, we tested whether a novel combination of CAA treatment and HRM analysis could be used to detect DNA base flipping.

Since CAA reacts with both, adenosine and cytosine in single-stranded DNA, which is produced during HRM analysis, we first tested the effect of different CAA concentrations on the
thermostability of double-stranded DNA under the HRM analysis conditions. Therefore, we incubated hemimethylated DNA with varying amounts of CAA and performed HRM analysis 1h after incubation at 37°C. As shown in Supplementary Fig. S4A, under our assay condition the highest CAA concentration, where most of the double-stranded DNA was still intact, was 50 mM. Next, we incubated hemimethylated DNA with CAA concentrations between 0 mM and 50 mM in the presence or absence of M.HpaII (without the cofactor SAM), respectively. Seventy minutes after incubation at 37°C, the HRM analysis was performed (Supplementary Fig. S4B). We observed the largest melting temperature (Tm) differences between M.HpaII treated and untreated DNA in the presence of 30 mM CAA (Supplementary Fig. S4B). Furthermore, we observed no detectable Tm differences with high CAA concentrations (40 mM and 50 mM) (Supplementary Fig. S4B). However, with low CAA concentrations, the Tm differences correlated with CAA amounts (Supplementary Fig. S4B). Thus, we found 30 mM CAA

Figure 3: Binding specificity of Tet1CD and MBD of Mecp2 to methylated and unmethylated DNA. Purified His-Tet1CD or YFP-MBD was incubated with methylated CpG and unmethylated CpG DNA. After 90 min of incubation at 37°C, free DNA and DNA–protein complexes were separated on a 4.5% native polyacrylamide gel. To quantify the binding, the amounts of unbound DNA (intensities of free DNA bands measured using Image J) were plotted below the gel. Independent experiments were repeated twice. Shown is one representative result. mC:mC, fully methylated CpG containing DNA; C:C, unmethylated CpG containing DNA. Free DNA and protein–DNA complexes are indicated on the gel.
to be the best condition for the detection of cytosine flipping. To further validate this result, we used a control oligonucleotide, which does not contain the M.HpaII recognition motif CCGG, for which M.HpaII showed a slightly lower binding preference than for CCGG containing DNA (Supplementary Fig. S4C). As shown in Fig. 4B, a similar melting temperature was observed for the non-CCGG containing oligonucleotide in the presence of M.HpaII and CAA. In contrast, hemimethylated CCGG containing oligonucleotide showed a decreased melting temperature with M.HpaII incubation. These results indicate that the CAA reaction only takes place when M.HpaII recognizes its substrate.

Previous studies [24] using CAA concentrations >50 mM could not detect M.HpaII-induced DNA base flipping and accordingly claim that CAA disrupts protein–DNA complexes, which might already be the case at our highest tested concentrations (40 mM and 50 mM). In summary, we showed that the combination of CAA treatment and HRM analysis can be used to detect base flipping induced by Dnmts, such as M.HpaII, which until now could not be shown by any other methods.

Next, we assayed Tet1-mediated DNA base flipping using CAA treatment and HRM analysis. We first tested the complex formation ability of Tet1 and DNA in the presence of 30 mM CAA. As shown in Supplementary Fig. S5A, binding of Tet1 to DNA is not affected by the presence of CAA. Therefore, we next incubated hemimethylated DNA with or without Tet1 proteins (without cofactors Fe(II) and 2-oxoglutarate) in the presence of 30 mM or 0 mM of CAA. After 2 h of incubation, the HRM analysis was performed (Fig. 5A). Similar to M.HpaII, the Tm values decreased with Tet1 incubation in the presence of 30 mM CAA.
To further test whether the CAA reaction is specific for flipped 5mC, we incubated a hemimethylated CpG or non-CpG containing DNA with the Tet1 protein in the presence of 30 mM CAA. As shown in Fig. 5B, a decrease $T_m$ value was also observed for non-CpG containing DNA raising the possibility that Tet flips other bases. However, the $T_m$ decrease for non-CpG DNA is smaller than for CpG containing DNA. These results indicate that the combination of CAA and HRM can be used to detect Tet protein-induced base flipping. In summary, the combination of CAA treatment and HRM analysis can be used to detect DNA base flipping induced by M.HpaII and Tet1.

DNA base excision detection assay

As previously mentioned, the glycosylase Mbd4 also uses a DNA base flipping mechanism to excise mismatched DNA bases. The base 2-aminopurine (2AP) has previously been used to detect base flipping because it fluoresces when not base paired. Since Mbd4 excises mismatched DNA bases, we tested whether this simpler method could be used to measure base flipping or base excision. We first tested the fluorescence measurement of 2AP with 320 nm excitation and 370 nm emission wavelengths using a fluorescence micro plate reader. As shown in Supplementary Fig. S6A, 0.4 $\mu$M of single-stranded oligonucleotide containing 2AP showed higher fluorescence intensity than an oligonucleotide containing canonical DNA bases. Then, we further incubated Mbd4 proteins (Supplementary Fig. S6D) with a double-stranded DNA oligonucleotide containing a 2AP mismatch (Supplementary Fig. S6B and Fig. S6C) in the context of a methylated CpG site and measured the 2AP fluorescence intensity (Supplementary Fig. S7A). No increased fluorescence intensity for oligonucleotides containing 2AP was observed in the

Figure 5: Detection of Tet1 mediated DNA base flipping. (A) 0.5 $\mu$M of hemimethylated or non-CCGG containing DNA was incubated with or without 2 $\mu$M of the catalytic domain of Tet1 (Tet1CD) in the presence of 30 mM CAA. CAA reacts with 5mC when it is flipped out of the DNA helix. The reaction product 3,N4-ethenomethylcytosine (C15mC) disrupts hydrogen bonds with base guanine in the complementary strand. The presence of 3,N4-ethenomethylcytosine (C15mC) can be detected with HRM analysis, due to its low $T_m$ contribution to double-stranded DNA. (B) Normalized fluorescent SYBR green intensities (left) and the corresponding derivative of intensities (right) were plotted using RStudio. Independent experiments were repeated two times. Shown is one representative result with three averaged technical replicates. The $P$-values of student’s $t$-test are indicated in the plot. $T_m$: melting temperature. C: cytosine; G: guanine; M:C: hemi methylated DNA; X:X: non-CpG containing DNA.
presence of Mbd4 (Supplementary Fig. S7A). Previous studies showed that Mbd4 preferentially binds to DNA containing a T-G mismatch (Supplementary Fig. S8) which serves as its substrate [18, 41]. To further test whether 2AP could be excised by Mbd4, we performed a Mbd4 glycosylase activity assay by running a denaturing gel using a T-G mismatch containing oligo as a control. As shown in Supplementary Fig. S7B, Mbd4 showed a glycosylase activity for the T-G mismatched oligo but not for the 2AP-G mismatched oligo. Since Mbd4 can excise the mismatched base T and thereby change the melting temperature of the DNA, we performed HRM analysis to test whether it could be used to detect Mbd4-induced base excision. As shown in Fig. 6, a small but significantly decreased Tm was observed for DNA containing mismatched T-G with Mbd4 incubation (Fig. 6A), but not for hemimethylated DNA (Fig. 6B). These results indicate that in addition to denaturing gel analysis, HRM can be used to detect Mbd4-induced base excision.

DNA base modification detection assay
Tet proteins oxidize 5mC to 5hmC in the presence of their cofactors Fe(II) and 2-oxoglutarate [8, 9]. Recent studies have shown that similar to 5mC, 5hmC is a stable epigenetic mark [42]. To better understand the function of 5hmC, several methods have been developed to quantify the amount of 5hmC [43]. The classical method used to measure 5hmC in the past few decades is bisulfite conversion sequencing; however, this method cannot discriminate 5mC from 5hmC [44]. Fortunately, several variants capable of discriminating 5mC from 5hmC [45, 46] and even from 5fC [47] and 5caC [48] have been developed and successfully used to map the different DNA modifications across the genome. Besides sophisticated sequencing methods, restriction enzymes, which specifically digest 5mC/5hmC modified DNA were discovered [49] and antibodies, that specifically recognize DNA base modifications, have been developed. However, all of the above mentioned methods are time-consuming and some of them, like antibody-based approaches, are restricted by their respective detection sensitivity [50].

Previous studies showed that 5mC increases DNA double strand stability by strengthening base stacking interactions [51]. Here, we tested whether modifications of 5mC affect DNA thermostability by HRM analysis. In accordance with previous studies, we found that 5hmC containing DNA exhibits lower Tm values compared to 5mC containing DNA (Supplementary Fig. S9A) [52–54]. As with 5hmC, 5fC, and 5caC containing DNA exhibited lower Tm values (Supplementary Fig. S9A) when compared to methylated DNA and, accordingly, decreased DNA double strand thermostability. The observation that the oxidized derivatives of 5mC decrease DNA thermostability implies that Tet-mediated 5mC oxidation can be detected indirectly by measuring DNA thermostability.

As we have shown above, the HRM analysis can be used to measure DNA double strand thermostability. We first tested the effect of oligonucleotide length (10, 20, and 42 bp) on the melting temperature of unmodified oligonucleotides. We found that 20 and 42 bp oligonucleotides gave melting temperatures >50 °C (Supplementary Fig. S9B). We next assayed Tet1 oxidation using the 42-bp oligonucleotide by HRM analysis. We incubated fully methylated 42 bp oligonucleotides with Tet1 proteins in the presence of their cofactors. As a control, reactions were prepared in duplicates, whereby one sample contained and the other one lacked Tet1. After 2 h of incubation at 37 °C, the HRM analysis was performed. We measured a decreased Tm value of the Tet1 modified versus the unmodified oligonucleotide (Supplementary Fig. S9C). This difference was very small (0.23 °C). As one strategy to enhance this Tm difference, we tested addition of a shorter 20-mer oligonucleotide with the same sequence as the middle part of the 42-bp oligonucleotide before running the HRM analysis (Supplementary Fig. S9D). As shown in Supplementary Fig. S9D, DNA incubated with Tet1 proteins exhibited 0.6 °C lower Tm values than DNA incubated without Tet1 enzymes. Since we showed that Tet oxidation
products decrease Tm values of double-stranded DNA (Supplementary Fig. S9A), we conclude that Tet-mediated 5mC oxidation can be detected by HRM analysis. Considering that Tet proteins produce a mixture of 5mC modifications with variant Tm values (Supplementary Fig. S9A), the expected melting curve would have multiple peaks. To test the behavior of DNA mixtures with different Tm values, we performed HRM analysis with different ratios of unmethylated and fully methylated DNA. As depicted in Supplementary Fig. S9E, only one peak could be detected for each DNA mixture; however, Tm values increased with the amount of methylated DNA. This result indicates that the measured Tm values represent the Tm of a DNA mixture, where the amount of each DNA modification affects the observed Tm. Taken together, we show that HRM analysis is sensitive enough to detect Tet-mediated 5mC oxidation activity.

As mentioned in the introduction, demethylation of one 5mC at symmetrically methylated CpG sites creates T-G mismatches in vivo. Up until now it is not known whether Tet proteins can act on the opposite non-deaminated 5mC. To test this, we incubated DNA oligonucleotides containing a T-G mismatch in the context of a methylated CpG site with Tet1 proteins and performed the HRM analysis. As shown in Supplementary Fig. S9F, we found a 0.3°C decreased Tm value in the presence of Tet1, indicating that Tet1 proteins oxidize 5mC in mismatched CpG sites.

Tet-mediated 5mC oxidation is a Fe(II) and 2-oxoglutarate dependent process. Besides these two cofactors, DTT and ATP were reported to be crucial for Tet1 enzymatic activity [55]. To test whether our assay can be used to detect the effect of cofactors on Tet1 activity, we incubated fully methylated oligonucleotides with Tet1 proteins in oxidation buffer with or without ATP and DTT. After the oxidation reaction, the HRM analysis was performed. As shown in Supplementary Fig. S10A, Tet1 did not alter Tm values of DNA when incubated in oxidation buffer without DTT and ATP. In the presence of ATP and DTT, in contrast, the Tm of DNA was decreased (by 0.6°C) in the presence of Tet1 (Supplementary Fig. S10B), indicating that DTT and ATP are crucial for Tet1-mediated oxidation and HRM analysis can be used to test Tet activity.

Since only one symmetrically methylated CpG is present in the 42-bp oligonucleotide and the decreased Tm after Tet1 incubation is, therefore, small, we next tested whether shorter oligonucleotides would lead to greater Tm differences. We chose a 20-mer long oligonucleotide as this still gave rise to a Tm > 50°C (Supplementary Fig. S9A). As shown in Fig. 7A, the Tm difference between the 20bp DNA with or without Tet is 1°C, which is higher than the 0.6°C for the 42-bp DNA.

To further assess whether the HRM could be used to detect Tet activity in multiple 5mC containing DNA, a 377-bp PCR fragment was incubated with Tet1 and subjected to HRM analysis. This amplicon contains 189 5mC and 42 mCpG sites. As shown in Fig. 7B, although the Tm values vary between reactions possibly due to the varying levels of 5mC oxidation, a large Tm shift was observed (around 3°C) due to Tet activity.

In summary, these results indicate that HRM can be used to test Tet oxidation activity in vitro even with a single (5mC) base resolution.

Prior to 5mC oxidation by Tet enzymes, cytosine methylation is required and performed by Dnmts. The HpaII methyltransferase is one of these enzymes and methylates the internal cytosine in CCGG sequence (Supplementary Fig. S11A). As methylation of cytosine increases DNA Tm values (Supplementary Fig. S9E), we further tested whether the HRM analysis could be used to detect methyltransferase activity. To this end, we incubated M.HpaII and unmethylated CCGG containing oligonucleotide in the presence of its cofactor SAM. Two hours after incubation, the reactions were directly used for HRM analysis. As shown in Supplementary Fig. S11B, a significantly increased Tm (0.3°C) was observed with M.HpaII incubation compared to non-M.HpaII incubated DNA. In addition, similar to Tet oxidation, the Tm difference (1.3°C) is larger when a 20-bp oligonucleotide was used (Fig. 8, compare red line without enzyme with blue line with 2 units of enzyme). These results indicate that the HRM can be used to detect methyltransferase activity in vitro.
Compared to antibody-based assays, the HRM detection of either methyltransferase activity or Tet-mediated 5mC oxidation activity is time saving and highly reproducible. A single modification in DNA is usually difficult to detect by antibodies. HRM analysis, however, is sensitive enough to discriminate even single modifications in DNA oligonucleotides. Altogether, we show that HRM analysis can be used to test Tet-mediated 5mC oxidation and methyltransferase activity in vitro.

Conclusions

Accurate detection of DNA binding, base flipping and modification is crucial to understand the function and regulation of various proteins, such as methycytosine writers, readers, and modifiers. Here, we present methods to step by step detect Tet binding, flipping, and oxidation of 5mC (Fig. 9). Multicolor EMSAs can be used to measure substrate preferences of multiple proteins to multiple oligonucleotides in one reaction. However, the analysis of DNA protein interactions is often limited, since conventional fluorescence imagers contain fixed excitation and emission filters that allow the detection of only some color combinations. Thus, we developed a method for the simultaneous detection of various commercially available fluorescent proteins/dyes on a fluorescence plate reader without spectral bleed-through. As a result, detection optimization allowed us to quantitate the binding of Tet1 proteins to differentially labeled DNA substrates by EMSAs and we found that Tet1CD proteins exhibited little DNA sequence specificity with only a slight preference for methylated CpG containing DNA.

Currently used methods for the detection of base flipping are costly and time consuming. Here, we developed a rapid and sensitive assay, which combines CAA treatment and HRM temperature analysis to assay proteins that use a base flipping mechanism. With this combined method, we could show that Tet1 and also M.HpaII flip its substrate for subsequent modification. The base flipping activity of the latter had so far escaped detection with currently available methods.

For the detection of base excision by Mbd4 glycosylase, denaturing gel analysis has been thus far the method of choice. We show here that HRM analysis can be used to detect a single abasic site and, thus, is an alternative sensitive method to assay glycosylase activity.

Finally, we show that HRM analysis can be used to detect Tet activity under different buffer conditions and can further be extended to methyltransferase activity. Accordingly, this method is particularly suitable to screen different cofactors and inhibitors that facilitate or impede Tet or methyltransferase activity.
In conclusion, all of the above methods are easily performed, highly reproducible, and are suitable for the detection of DNA base modifications, their readers, and modifiers.

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Supplementary data
Supplementary data is available at Biology Methods and Protocols online.

Conflict of interest statement. None declared.

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DNA base flipping analytical pipeline

Peng Zhang\textsuperscript{1}, Florian D. Hastert\textsuperscript{1}, Anne K. Ludwig\textsuperscript{1}, Kai Breitwieser\textsuperscript{1}, Maria Hofstätter\textsuperscript{2} and M. Cristina Cardoso\textsuperscript{1}

\textsuperscript{1}Cell Biology and Epigenetics, Department of Biology, Technische Universität Darmstadt, Germany
\textsuperscript{2}Max Delbrück Center for Molecular Medicine, Berlin, Germany

\textbf{Correspondence to:}
M. Cristina Cardoso
Technische Universität Darmstadt
Schnittspahnstrasse 10
64287 Darmstadt
Germany
cardoso@bio.tu-darmstadt.de
Phone: +49-6151-16-21882
Fax: +49-6151-16-21880
PROTOCOL

OLIGONUCLEOTIDES

- DNA oligonucleotide MG-up (5'-CTC AAC AAC TAA CTA CCA TCG CTX GGA CCA GAA GAG TCA TCA TGG-3', X represents 5mC) (1)
- DNA oligonucleotide CG-up (5'-CTC AAC AAC TAA CTA CCA TCC GGA CCA GAA GAG TCA TCA TGG-3') (1)
- DNA oligonucleotide non-CG-up (5'-CTC AAC AAC TAA CTA CCA TCG CTX GGA CCA GAA GAG TCA TCA TGG-3')
- DNA oligonucleotide Fill-In-550 (5'-ATTO550-CCA TGA TGA CTC TTC TGG TCX GGA TGG TAG TTA GTT GTT GAG-3', X: represents 2-aminopurine) (1)
- DNA oligonucleotide Fill-In-647N (5'-ATTO647N-CCA TGA TGA CTC TTC TGG TCT GGA TGG TAG TTA GTT GTT GAG-3') (1)
- DNA oligonucleotide sense (5'-GGA TGA TGA CTC TTC TGG TCX GGA TGG TAG TTA AGT GTT GAG-3', X represents 5mC, 5hmC, 5fC or 5caC, respectively) (2)
- DNA oligonucleotide antisense (5'-CTC AAC ACT TAA CTA CCA TCG CTX GGA CCA GAA GAG TCA TCA TCC-3', X represents 5mC, 5hmC, 5fC or 5caC, respectively) (2)
- DNA oligonucleotide T:G mismatch-down (5'-CCA TGA TGA CTC TTC TGG TCT GGA TGG TAG TTA GTT GTT GAG-3')
- DNA oligonucleotide T:G mismatch-down-647N (5'-ATTO647N-CCA TGA TGA CTC TTC TGG TCT GGA TGG TAG TTA GTT GTT GAG-3')
- DNA oligonucleotide CG-up (20 bp) (5'-ACT ACC ATC CGG ACC AGA AG-3')
- DNA oligonucleotide MG-up (20 bp) (5'-ACT ACC ATC XGG ACC AGA AG-3', X represents 5mC)
- DNA oligonucleotide CG-down (20 bp) (5'-CTT CTG GTC CGG ATG GTA GT-3')
- DNA oligonucleotide MG-down (20 bp) (5'-CTT CTG GTC XGG ATG GTA GT-3', X represents 5mC)
- MINX forward primer: 5'-CGG TAC CTA ATA CGA CTC ACT ATA GGG AGA-3'
- MINX reverse primer: 5'-CGG TAC CTA ATA CGA CTC ACT ATA-3'

REAGENTS

- 2-Chloroacetaldehyde (CAA) 50% in H2O (Sigma-Aldrich, cat. no. 317276)
- 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma-Aldrich, cat. no. A8456)
- 99.5% formamide (Carl Roth, cat. no. P040.1)
- Acrylamide/Bis-acrylamide, 30% solution (Sigma-Aldrich, cat. no. A3699)
- Adenosine 5'-triphosphate disodium salt hydrate (ATP) (Sigma-Aldrich, cat. no. A2383)
- Ammonium iron(II) sulfate hexahydrate (Sigma-Aldrich, cat. no. 215406)
- Ammonium peroxydisulphate (APS) (Carl Roth, cat. no. 9592)
- Boric acid (Carl Roth, cat. no. 6943.1)
- Bromophenol blue (Carl Roth, cat. no. A512.1)
- CutSmart® Buffer (NEB, cat. no. B7204S)
• D- (+)-Glucose (Sigma-Aldrich, cat. no. G8270)
• D-desthiobiotin (IBA, cat. No. 2-1000-002)
• ddH2O
• Deoxy-5-methylcytidine triphosphate (dmCTP) (NEB, cat. no. N0356S)
• Deoxadenosine triphosphate (dATP) (Carl Roth, cat. no. K035.1)
• Deoxycytidine triphosphate (dCTP) (Carl Roth, cat. no. K038.1)
• Deoxyguanosine triphosphate (dGTP) (Carl Roth, cat. no. K037.1)
• Deoxythymidine triphosphate (dTTP) (Carl Roth, cat. no. K036.1)
• DL-Dithiothreitol (DTT) (Sigma-Aldrich, cat. no. D9779)
• DNA polymerase I, large (Klenow) fragment (NEB, cat. no. M0210S/L)
• E-64 (Sigma-Aldrich, cat. no. E3131)
• EDTA (Merck Millipore, cat no. 324503)
• Ethidium bromide solution (Sigma-Aldrich, cat. no. E1510)
• GeneRuler Ultra Low range DNA ladder, ready-to-use (Thermo Fisher Scientific, cat. no. SM1213)
• GFP-binding protein (3)
• Glycerol (Sigma-Aldrich, cat. no. 49770)
• HEPES PUFFERAN® (Carl Roth, cat. no. 9105)
• HpaII (NEB, cat. no. R0171S/L)
• HpaII methyltransferase (NEB, cat. no. M0214S)
• Imidazole (Sigma-Aldrich, cat. no. I0125)
• L-ascorbic acid (Sigma-Aldrich, cat. no. A4403)
• Magnesium chloride (MgCl2) (Sigma-Aldrich, cat. no. M8266)
• Magnesium chloride hexahydrate (Sigma-Aldrich, cat. no. M9272)
•MspI (NEB, cat. no. R0106S/L)
• N,N,N’,N’-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, cat. no. T9281)
• NEBuffer 1 (NEB, cat. no. B7001S)
• NEBuffer 2 (NEB, cat. no. B7002S)
• NHS-Activated Sepharose 4 Fast Flow (GE Healthcare Life Sciences, cat. no. 17-0906)
• Nonidet™ P 40 (NP-40) Substitute (Sigma-Aldrich, cat. no. 74385)
• Pepstatin A (Sigma-Aldrich, cat. no. P5318)
• Phenylmethylsulfonylfluorid (PMSF) (Sigma-Aldrich, cat. no. P7626)
• Pierce™ 660nm Protein Assay Reagent (Thermo Fischer Scientific, cat. no. 22660)
• Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, cat. no. 11733046)
• Poly(deoxyinosinic-deoxycytidylic) acid sodium salt (poly(dI:dC)) (Sigma-Aldrich, cat. no. P4929)
• Potassium Chloride (KCl) (Sigma-Aldrich, cat. no. P9541)
• Potassium dihydrogen phosphate (KH2PO4) (Carl Roth, cat. no. 3904)
• Proteinase K (Sigma-Aldrich, cat. no. P2308)
• RFP-binding protein (4)
• Sodium Chloride (NaCl) (Carl Roth, cat. no. 9265)
• Sodium dihydrogen phosphate dihydrate (NaH2PO4·2H2O) (Carl Roth, cat. no. T879)
• Sodium hydroxide (NaOH) (Sigma-Aldrich, cat. no. S5881)
• Strep-Tactin® Superflow® 50% suspension (IBA, cat. No. 2-1206-010)
• SYBR® Green II RNA Gel Stain, 10000X concentrate in DMSO (Thermo Fisher Scientific, cat. no. S7564)
• TALON® metal affinity resin (Clontech, cat. No. 635502)
• Tris base (Sigma-Aldrich, cat. no. TRIS-RO)
• Triton-X100 (Sigma-Aldrich, cat. no. T8787)
• Trizma® hydrochloride (Sigma-Aldrich, cat. no. T5941)
• Urea (Merck, cat. no. 1084881000)
• Xylene cyanol FF (Sigma-Aldrich, cat. no. X4126)
• α-Ketoglutaric acid (Sigma-Aldrich, cat. no. K1128)

EQUIPMENT
• Amicon Ultra centrifugal filter units Ultra-15, MWCO 30 kDa (Sigma-Aldrich, cat. no. Z717185)
• ThermoMixer® comfort 1.5 (Eppendorf, 536000011)
• Centrifuge 5810 R, with rotor A-4-62 (Eppendorf, cat. no. 5811 000.320)
• Avanti® J-E centrifuge (Beckman Coulter, cat no. 369001)
• JA-14 Rotor for Avanti® J-E centrifuge (Beckman Coulter, cat. no. 339247)
• VWR Tube rotator (VWR, cat. no. 444-0500)
• TECAN Infinite® M200 (TECAN, cat. no. 30016056)
• Dual Gel Caster for Mini Vertical Units (Hoefer, cat. no. SE245)
• Glass plate 10 cm x 8 cm x 1.8 mm (width x height x thickness) (bsb11 biotech service blu, cat. no. HF8211)
• Glass plate 10 cm x 12 cm x 1.8 mm (width x height x thickness) (bsb11 biotech service blu, cat. no. HF3052)
• Notched glass plate 10 cm x 8 cm x 1.8 mm (width x height x thickness) (bsb11 biotech service blu, cat. no. HF8210)
• Notched glass plate 10 cm x 12 cm x 1.8 mm (width x height x thickness) (bsb11 biotech service blu, cat. no. HF3051)
• T-Spacer 1 mm x 8 cm (bsb11 biotech service blu, cat. no. HT2001)
• T-Spacer 1 mm x 12 cm (bsb11 biotech service blu, cat. no. HT3002)
• Small format comb 10 wells, 1 mm thickness (bsb11 biotech service blu, cat. no. HF711)
• SE250 Mighty Small II Mini Vertical Slab Gel Electrophoresis Unit (Hoefer, cat. no. SE250)
• SE280 Tall Mighty Small Vertical Slab Gel Electrophoresis Unit (Hoefer, cat. no. SE280)
• Hoefer™ Spring Clamps for Mighty Small™ Electrophoresis Units (Fisher Scientific, cat. no. 03-500-484)
• Power Pack 25 (Biometra, cat. no. 846-040-800)
• Conventional refrigerator (4°C)
• Amersham Imager 600 RGB (GE Healthcare Life Sciences, cat. no. 29-0834-61)
• StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, cat. no. 4376600)
• MicroAmp® Optical 8-Tube Strip 0.2 mL (Applied Biosystems, cat. no. 4316567)
• MicroAmp® Optical 8-Cap Strip (Applied Biosystems, cat. no. 4323032)
• SafeSeal tube 1.5 mL (Sarstedt, cat. No. 72.706)
• Tube 50 mL, 114x28 mm, PP (Sarstedt, cat. no. 62.547.254)
• Tube 15 mL, 120x17 mm, PP (Sarstedt, cat. no. 62.554.016)
• 0.20 µM filter (Sarstedt, cat no. 83.1826.001)
• 10 mL syringe HSW NORM-JECT® (HENKE SASS WOLF, 4100.000V0)
• Sterican® Gr. 2, G 21 x 1 1/2” / ø 0,80 x 40 mm, grün (Braun, 4657527)
• Sterican® Gr. 14, G 23 x 1 1/4” / ø 0,60 x 30 mm, blau (Braun, 4657640)

SOLUTIONS

• 20% Glycerol (v/v): Mix 20 mL of 99.5% Glycerol with 80 mL ddH$_2$O. Store at room temperature (RT).
• HEPES, 500 mM (pH 7.9): Dissolve 119.2 g HEPES in 800 mL ddH$_2$O. Adjust pH to 7.9 and fill up to 1 L with ddH$_2$O. Store at RT.
• 1 M Tris–HCl: Dissolve 121.14 g Tris in 800 mL ddH$_2$O. Adjust pH to 8.0 and fill up to 1 L with ddH$_2$O. Store at room temperature.
• 1x PBS: Dissolve 8 g NaCl (137 mM), 0.2 g KCl (2.7 mM), 1.44 g Na$_2$HPO$_4$ (10 mM) and 0.24 g KH$_2$PO$_4$ (1.8 mM) in 800 mL ddH$_2$O and fill up to 1 L. Store at RT.
• EDTA, 500 mM (pH 8.0): Dissolve 18.61 g EDTA in 80 mL ddH$_2$O. Adjust pH to 8.0 and fill up to 100 mL with ddH$_2$O. Store at RT.
• 10% Triton-X100: Dissolve 10 mL Triton-X100 in 90 mL ddH$_2$O. Store at RT.
• 100 mM DTT: Dissolve 154.25 mg DTT in 10 mL ddH$_2$O. Store in 1 mL aliquots at -20°C.
• 100 mM AEBSF: Dissolve 357.41 mg in 10 mL ddH$_2$O. Store in 1 mL aliquots at -20°C.
• 100 mM E64: Dissolve 357.41 mg in 10 mL ddH$_2$O. Store in 1 mL aliquots at -20°C.
• 1 mM Pepstatin A: Dissolve 3.43 mg in 5 mL ddH$_2$O. Store in 1 mL aliquots at -20°C
• 20 mg/mL Proteinase K: Dissolve 100 mg in 5 mL ddH$_2$O. Store in 1 mL aliquots at -20°C
• 2x Binding buffer: Mix 400 µL of 500 mM HEPES (pH 7.9), 20 µL of 500 mM EDTA, 60 µL of 500 mM MgCl$_2$, 400 µL of 99.5% glycerol, 100 µL of 10% Triton-X100. Fill up to 4.8 mL with ddH$_2$O. Add 1mM of DTT and 1mM of AEBSF freshly before each experiment. Store at 4°C.
• 5x TBE buffer (pH 8.3): Add 54 g of Tris base, 27.5 g of boric acid and 20 mL of 500 mM EDTA (pH 8.0) to 800 mL of ddH$_2$O and fill up to 1 L with ddH$_2$O. Store at RT.
• 6x DNA loading dye: Mix 25 mg bromophenol blue, 25 mg xylene cyanol FF and 3 mL of 99.5% glycerol and fill up to 10 mL with ddH$_2$O. Store in 1 mL aliquots at 4°C.
• 2x DNA loading dye (denaturing gel): Mix 0.1 g xylene cyanol, 0.2 g bromophenol blue, 0.2 g SDS, 20 µL of 0.5 M EDTA and 9.5 mL of 99.5% formamide. Store in 1 mL aliquots at -20°C.
• Ethidium bromide staining solution: Mix 10 µL Ethidium bromide with 100 mL ddH$_2$O.
• 2x Mbd4 base flipping buffer: Mix 200 µL of 1 M Tris–HCl, pH 8.0, 40 µL of 500 mM EDTA and fill up to 10 mL with ddH$_2$O. Store at 4°C.
• **M.HpaII storage buffer:** Mix 10 µL 1M Trix-HCl, pH 8.0, 40 µL 5 M NaCl, 10 µL 100 mM DTT, 0.2 µL 500 mM EDTA, 100 µL 2 µg/µL bovine serum albumin (BSA), 500 µL glycerol and 340 µL H2O. Store at -20°C

• **Oxidation buffer 1:** Dissolve 14.7 mg of Fe(NH₄)₂(SO₄)₂·6H₂O to 1 mL of ddH₂O and make a 24-fold dilution. Final concentration is 1.5 mM. Aliquot solutions and store at -80°C.

• **Oxidation buffer 2.1:** Mix 333 µL of 5 M NaCl, 1670 µL of 0.5 M HEPES pH 8.0, 41.5 µL of 1 M DTT, 200 µL of 100 mM ATP, 330 µL of 50 mM α-ketoglutaric acid, 136 µL of 246.4 mM L-ascorbic acid and 2290 µL of ddH₂O. Aliquot solutions and store at -80°C. Avoid repeated freeze and thaw cycles.

• **Oxidation buffer 2.4:** Mix 333 µL of 5 M NaCl, 1670 µL of 0.5 M HEPES pH 8.0, 330 µL of 50 mM α-ketoglutaric acid, 136 µL of 246.4 mM L-ascorbic acid and 2532 µL of ddH₂O. Aliquot solutions and store at -80°C. Avoid repeated freeze and thaw cycles.

• **Talon wash buffer (TWB):** Dissolve 1.95 g (50 mM) NaH₂PO₄, 4.38 g (300 mM) NaCl and 0.17 g (10 mM) Imidazole in 200 mL ddH₂O, adjust the pH to 8.0 with NaOH and fill up to 250 mL with ddH₂O. Store at 4°C.

• **Talon lysis buffer (TLB):** Add fresh 100 µL (0.5%) NP-40 to 20 mL Talon wash buffer.

• **Talon elution buffer (TEB):** Dissolve 1.95 g (50 mM) NaH₂PO₄, 4.38g (300 mM) NaCl and 2.55 g (150 mM) Imidazole in 200 mL ddH₂O, adjust the pH to 8.0 with NaOH and fill up to 250 mL with ddH₂O. Store at 4°C.

• **Strep-beads elution buffer:** Mix 1000 µL of 1 M Tris-HCl pH 8.0, 300µL of 5 M NaCl, 20 µL of 0.5 M EDTA, 21.43 mg desthiobiotin and fill up to 10 mL with ddH₂O.

• **100 mM CAA:** Mix 63 µL CAA solution and 37 µL ddH₂O and make a 100-fold dilution. Store at RT. Avoid light exposure.

• **4 M MgCl₂ pH 4.4:** Dissolve 40.66 g MgCl₂ in ddH₂O and fill up to 50 mL. Filter the solution using a 0.20 µm filter and do not adjust pH. Store at RT.

• **PARP buffer (0.5 M NaCl):** Mix 2.5 mL of 1 M Tris pH 8.0, 10 mL of 5 M NaCl, 0.9 g of glucose, 2 mL of 0.5 M EDTA, 0.2 mL of Tween and 0.2 mL of NP40, then fill up with ddH₂O to 100 mL. Store at RT.

• **PARP buffer (1 M NaCl):** Mix 2.5 mL of 1 M Tris pH 8.0, 20 mL of 5 M NaCl, 0.9 g of glucose, 2 mL of 0.5 M EDTA, 0.2 mL of Tween and 0.2 mL of NP40, then fill up with ddH₂O to 100 mL. Store at RT.

**PLASMIDS AND CELLS**

• **Sf9 cells** (Invitrogen, Paisley PA4 9RF, UK) were cultivated in EX-CELL 420 Insect Serum Free (SAFC) medium as described before (5).

• **pFBDMbd4-2-strep** (Mbd4-Strep, pc1796): GFP tag was replaced by 2-strep tag from pFB-MBD4-C-GFP (5).

• **pFB-MeCP2Y.5** (MBD-YFP, pc1576) was described before (5).

• **pFBDM-HisTet1** (His-Tet1CD, pc2708) was described before (6)

• **pFBDM-chmTet1.1** (mcherry-Tet1CD, pc2859) was described before (6).

**PROCEDURES**

1. Electrophoretic mobility shift assay (EMSA)

1.1 Preparation of DNA oligonucleotides
1.1.1 Fully methylated CpG oligonucleotides preparation

- Mix 1 µL of 100 mM dATPs, 100 mM dGTPs, 100 mM dTTPs and 10 mM dCTP in 46 µL ddH₂O in 1.5 mL tube (dCTP nucleotides mix).
- Mix 10 µL of 100 µM MG-up with 10 µL of 100 µM Fill-In-647N, 10 µL NEBuffer 2 and 20 µL H₂O in 1.5 mL tube.
- Incubate oligo mixture at 95°C for 2 minutes in a ThermoMixer and let slowly cool down to 37°C by switching off the ThermoMixer.

(Note: The cooling-down process takes approximately 50 minutes and should not be accelerated to ensure proper annealing of the oligos.)
- Add 50 µL of dCTP nucleotide mix and 1µL (5 U/µL) DNA polymerase I, large (Klenow) fragment in a total reaction volume of 100 µL. Incubate for 1 h at 37°C. Store at -20°C.

1.1.2 Unmethylated CpG oligonucleotides preparation

- Mix 1 µL of 100 mM dATPs, 100 mM dGTPs, 100 mM dTTPs and 0.1 µL of 100 mM dCTP in 46.9 µL ddH₂O in 1.5 mL tube (dCTP nucleotides mix).
- Mix 10 µL of 100 µM CG-up with 10 µL of 100 µM Fill-In-550, 10 µL NEBuffer 2 and 20 µL H₂O in 1.5 mL tube.
- Incubate oligo mixture at 95°C for 2 minutes in a ThermoMixer and let slowly cool down to 37°C by switching off the ThermoMixer.

(Note: see 1.1.1)
- Add 50 µL of dCTP nucleotide mix and 1µL (5 U/µL) DNA polymerase I, large (Klenow) fragment in a total reaction volume of 100 µL. Incubate for 1 h at 37°C. Store at -20°C.

1.1.3 Non-CpG oligonucleotides preparation

- Mix 1 µL of 100 mM dATPs, 100 mM dGTPs, 100 mM dTTPs and 0.1 µL of 100 mM dCTP in 46.9 µL ddH₂O in 1.5 mL tube (dCTP nucleotides mix).
- Mix 10 µL of 100 µM NonCG-up with 10 µL of 100 µM Fill-In-550, 10 µL NEBuffer 2 and 20 µL H₂O in 1.5 mL tube.
- Incubate oligo mixture at 95°C for 2 minutes in a ThermoMixer and let slowly cool down to 37°C by switching off the ThermoMixer.

(Note: see 1.1.1)
- Add 50 µL of dCTP nucleotide mix and 1µL (5 U/µL) DNA polymerase I, large (Klenow) fragment in a total reaction volume of 100 µL. Incubate for 1 h at 37°C. Store at -20°C.

1.2 Verification of methylation status and size of DNA oligonucleotides

1.2.1 Control digestion

- Mix 0.5 µL of each DNA oligonucleotide with 1x CutSmart® Buffer and 10 U of MspI in a total reaction volume of 20 µL. Incubate for 2 h at 37°C.
- Mix 0.5 µL of each DNA oligonucleotide with 1x CutSmart® Buffer and 10 U of HpaII in a total reaction volume of 20 µL. Incubate for 2 h at 37°C.
- Mix 0.5 µL of each DNA oligonucleotide with 1x CutSmart® Buffer in a total reaction volume of 20 µL. Incubate for 2 h at 37°C.

1.2.2 Native polyacrylamide gel electrophoresis
• Assemble the Dual Gel Caster for Mini Vertical Units according to the manufacturer's instruction. Use notched and unnotched glass plates, as well as T-spacers with a height of 8 cm.

• Per gel, mix 6.7 mL of 30% Acrylamide/Bis-acrylamide with 2 mL 5x TBE, 1.3 mL ddH₂O, 70 µL of 10% APS and 7 µL TEMED.

• Insert the comb, pour the gel and let solidify at RT.

• Assemble the Mighty Small (II) Mini Vertical Electrophoresis Unit (SE250) and fill the upper and lower buffer chamber with 0.5% TBE.

• Mix 20 µL of each sample (undigested, MspI- and HpaII digested) with 3 µL of 6x DNA loading dye and load into the gel.

• Load 5 µL of GeneRuler Ultra Low Range DNA Ladder into the gel.

• Connect Mighty Small (II) Mini Vertical Electrophoresis Unit (SE250) to the Power Pack 25 and let the gel run at 100 V at RT.

• Once the tracking dye (bromophenol blue, see 6X DNA loading dye) has reached the bottom of the gel, turn off the power supply, disconnect the leads and remove the gel.

• Stain gel for 15 minutes in ethidium bromide staining solution (see Reagent Setup).

• Rinse gel for 5 minutes in ddH₂O.

• Detect the ethidium bromide signal on a fluorescent Imager (Amersham Imager 600 RGB) using 312 nm UV excitation and a 605BP40 emission filter.

1.3 Purification of mcherry-Tet1CD and MBD-YFP

1.3.1 Mcherry-Tet1CD purification

• Prepare PARP buffer with protease inhibitors. Add 200 µL AEBSF, 200 µL E64, 20 µL PepA in 20 mL PARP buffer (0.5 M NaCl) and store on ice.

• Thaw four 1 mL Sf9 cell pellets on ice by adding 2 mL PARP buffer (0.5 M NaCl) with inhibitors and incubate for 30 minutes on ice.

• Lyse cells by syringe treatment (5 strokes 21G, 5 strokes 23G).

• Combine all cell lysates in 50 mL tube and incubate 10 minutes on ice.

• Centrifuge for 30 minutes at 4°C, 9552 rpm (14000x g).

• Meanwhile prepare RBP-beads
  o Transfer 1 mL of slurry beads to a 15 mL tube.
  o Wash beads 3 times with 5 mL PARP buffer supplemented with protease inhibitors by centrifugation at 4°C, 800 rpm, 3 min.

• After clearing the cell lysate by centrifugation, transfer supernatant to the RBP-beads and incubate for 3 h on a rotator at 4°C.

(Note: Overnight incubation would increase protein binding.)

• Centrifugation at 4°C, 800 rpm, 3 min.

• Discard supernatant.

• Wash pellets 4 times with 6 mL PARP buffer (0.5 M NaCl) with inhibitors and two times with 6 mL PBS by centrifugation at 4°C, 800 rpm (129x g), 3 min.

• Elute the mcherry-Tet1CD protein by adding 2 mL MgCl₂ (pH 4.4) and incubate 10 minutes on ice.
• Centrifuge for 3 minutes at 4°C, 800 rpm (129x g), then transfer supernatant to a new 15 mL tube and add 10 mL PBS to dilute MgCl₂.

(Note: Do not transfer any of the beads.)

• Repeat elution step and combine ~2 mL supernatant with the previous supernatant.

• Centrifuge for 3 minutes at 4°C, 800 rpm (129x g) and transfer 13.5 mL of supernatant to Amicon Ultra falcon (50 mL).

(Note: This step is critical to avoid beads transfer.)

• Buffer exchange.
  o Prepare 50 mL ice-cold 1x PBS.
  o Transfer supernatant to an Amicon Ultra centrifugal filter unit.
  o Centrifuge for 20 minutes at 4°C, 3000 rpm (1811x g).
  o Discard flowthrough and fill up with 1x PBS and repeat until PBS is used up.
  o Centrifuge until final volume reaches around 200 µL.
  o Determine protein concentration by SDS-PAGE and protein-measurement using a commercial assay (Pierce™ 660nm Protein Assay Reagent).
  o Aliquot and store at -20°C.

(Note: All solutions should be kept on ice.)

1.3.2 MBD-YFP purification

MBD-YFP is purified the same way as mcherry-Tet1CD, except that PARP buffer with 1 M NaCl and GBP-beads were used.

(Note: Overnight incubation of cell lysates with GBP-bead is not suitable for GFP/YFP tagged proteins.)

1.4 Preparation of native polyacrylamide gels for EMSA

• Assemble the Dual Gel Caster for Mini Vertical Units (see Equipment Setup). Use notched and unnotched glass plates, as well as T-spacers with a height of 12 cm.

• Per gel, mix 3 mL of 30% acrylamide/bis-acrylamide with 2 mL of 5x TBE, 14 mL ddH₂O, 1 mL of 20% Glycerol, 150 µL of 10% APS and 15 µL TEMED.

• Insert the comb, pour the gel and let solidify at RT.

• Assemble the Tall Mighty Small Mini Vertical Electrophoresis Unit (SE280) (see Equipment Setup) and fill the upper and lower buffer chamber with 0.5% TBE.

• Connect the Tall Mighty Small (II) Mini Vertical Electrophoresis Unit (SE280) to the Power Pack 25 and pre-run the gel for 2 h at 10 mA, 4°C.

1.5 Preparation of the binding reaction

1.5.1 Methylated DNA versus poly(dI:dC)

• Protein dilution:
  MBD-YFP: dilute protein to 20 pmol/µL in PBS
  mcherry-Tet1CD: dilute protein to 20 pmol/µL in PBS

• Set up following reactions:
• Incubate samples for 1.5 h at 37°C.

### 1.5.2 Methylated versus unmethylated DNA

- **Protein dilution:**
  - **p1:** dilute protein to 20 pmol/µL in PBS
  - **p2:** dilute protein to 2.5 pmol/µL in PBS
- **Set up following reactions:**

|   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  |
|---|----|----|----|----|----|----|----|----|----|
| ddH₂O | 3.0 µL | 3.0 µL | 2.0 µL | 2.0 µL |
| MBD-YFP (20 pmol/µL) | / | 1.0 µL | / | 1.0 µL |
| mCherry-Tet1CD (20 pmol/µL) | 1.0 µL | / | 1.0 µL | / |
| mCpG DNA (ATTO647N) (10 pmol/µL) | 1.0 µL | 1.0 µL | 1.0 µL | 1.0 µL |
| poly(dI:dC) (200 ng/µL) | / | / | 1.0 µL | 1.0 µL |

|   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  |
|---|----|----|----|----|----|----|----|----|----|
| protein | 2.0 µL | 1.5 µL | 1.0 µL | 0.5 µL | 2.0 µL | 1.0 µL | 0.5 µL | 0.25 µL | / |
| mCpG DNA (ATTO647N) (10 pmol/µL) | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL |
| Cpg DNA (ATTO550) (10 pmol/µL) | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL |
| 2x Binding buffer | 5.0 µL | 5.0 µL | 5.0 µL | 5.0 µL | 5.0 µL | 5.0 µL | 5.0 µL | 5.0 µL |

• Incubate samples for 1.5 h at 37°C.

### 1.6 Electrophoretic mobility shift

- Wash pockets with running buffer using a 100 µL pipette.
- Load samples (see step 1.5) into the pre-run native polyacrylamide gel (see step 1.4).
  (Note: Load the samples without any loading buffer carefully.)
- Load 1 µL of 6x DNA loading dye (containing the tracking dyes bromophenol blue and xylene cyanol FF) to the last lane.
- Connect Mighty Small (II) Mini Vertical Electrophoresis Unit (SE250) to the Power Pack 25 and let the gel run at 200 V at 4°C.
• Once the tracking dye bromophenol blue has reached 1/5 of the gel bottom, turn off the power supply, disconnect from the power pack and remove the gel.

(Note: Do not remove glass plates from the gel.)

1.7 Fluorescence signal detection and analysis

• Gels containing GFP- and mcherry-tagged proteins, as well as ATTO-550 and ATTO-647N labelled DNA oligonucleotides are imaged on a fluorescence Imager (Amersham Imager 600 RGB) using 460 nm, 520 nm and 630 nm excitation light sources, as well as 525BP20, 605BP40 and 705BP40 emission filters.

• Gels containing YFP-tagged proteins are imaged on a fluorescence microplate reader (TECAN Infinite® M200).
  - On a transparent plate with dimensions of 10 cm x 12 cm (width x height), mark gel-pockets with red tape and spaces between lanes with green tape, respectively.
  - Fix the labeled transparent plate onto the lid of a flat 96-well plate using transparent, adhesive tape.
  - Place the gel from step 1.6 onto gel template according to the red (gel-pockets) and green (spaces between lanes) labels and fix with transparent, adhesive tape.
  - Place the template-fixed gel (with pockets facing to the right side) into the TECAN Infinite® M200 microplate reader.
  - Measure fluorescence signals using 475 +/- 9 nm excitation and 520 +/- 20 nm emission wavelengths for YFP, 585 +/- 9 nm excitation and 630 +/- 20 nm emission wavelengths for mcherry, as well as 645 +/- 9 nm excitation and 675 +/- 20 nm emission wavelengths for ATTO-647N.
  - Save data as .xls file.
  - Plot heatmaps using self-written R script (Supplementary file).

(Note: Check fluorescence intensity of red tape in the cherry channel, make sure it is homogeneously distributed for all lanes, otherwise fix the gel again and repeat the measurement.)

2. Base flipping detection assay

2.1 Preparation of DNA Oligonucleotides

2.1.1 2AP oligonucleotides preparation

• Mix 10 µL of 100 µM 2AP DNA oligonucleotide with 10 µL of 100 µM MG-up DNA oligonucleotide, 10 µL NEBuffer 2 and 20 µL ddH2O in 1.5 mL tube.
  - Incubate at 95°C for 2 minutes in a ThermoMixer and then slowly cool down to 37°C by switching off the ThermoMixer.
  - Add 50 µL of ddH2O and store at -20°C.

2.1.2 Hemi-methylated CpG oligonucleotide preparation

• Mix 1 µL of 100 mM dATPs, 100 mM dGTPs, 100 mM dTTPs and 0.1 µL of 100 mM dCTPs in 46.9 µL ddH2O in 1.5 mL tube (dCTP nucleotides mix).
  - Mix 10 µL of 100 µM MG-up with 10 µL of 100 µM Fill-In, 10 µL NEBuffer 2 and 20 µL H2O in 1.5 mL tube.
  - Incubate oligo mixture at 95°C for 2 minutes in a ThermoMixer and then slowly cool down to 37°C by switching off ThermoMixer.
Add 50 µL of dCTP nucleotide mix and 1 µL (5 U/µL) DNA polymerase I, large (Klenow) fragment in a total reaction volume of 100 µL. Incubate for 1 h at 37˚C. Store at -20°C.

2.1.3 T:G mismatch oligonucleotide preparation
- Mix 10 µL of 100 µM T:G mismatch-down DNA oligonucleotide, 10 µL of 100 µM MG-up DNA oligonucleotide, 10 µL NEBuffer 2 and 70 µL ddH₂O in 1.5 mL tube.
- Incubate at 95°C for 2 minutes on a ThermoMixer and allow to slowly cool down to 37°C by switching off the ThermoMixer.

2.2 Purification of proteins
2.2.1 His-Tet1CD purification
- Thaw four 1 mL Sf9 cell pellets on ice by adding 2 mL TLB (supplemented with protease inhibitors) each and incubate for 30 minutes on ice.
- Lyse cell pellets by syringe treatment (5 strokes 21G, 5 strokes 23G).
- Unify all lysates in a 50 mL tube and incubate on ice for 10 minutes.
- Centrifuge for 30 minutes at 9552 rpm (14000 x g), 4°C.
- Meanwhile prepare TALON® resin.
  - Transfer 600 µL TALON® resin to a new 15 mL tube.
  - Wash resin 3 times with 5 mL TWB by spinning down for 2 minutes at 800 rpm (129 x g), 4°C.
- Add supernatant to the TALON® resin.
- Incubate on a rotator for 2 hours at 4°C.
- Centrifuge for 3 minutes at 800 rpm (129 x g) and 4°C and collect supernatant in a new tube.
  - Wash resin 3 times with 4 mL TWB by centrifugation for 3 minutes at 800 rpm (129 x g) and 4°C.
  - Wash resin 3 times with 4 mL 1x PBS by centrifugation for 3 minutes at 800 rpm (129 x g), 4°C.
- Elute His-tagged proteins by adding 2.5 mL TEB.
  - Incubate on ice for 10 minutes.
  - Centrifuge for 3 minutes at 800 rpm (129 x g) and 4°C.
  - Collect the supernatant in a new tube.
  - Repeat elution one more time and combine supernatants.
  - Centrifuge for 3 minutes at 800 rpm (129 x g), 4°C.
- Buffer exchange using a 15 mL Amicon Ultra (30 KDa) centrifugal filter unit.
  - Prepare 50 mL ice-cold 1x PBS.
  - Transfer supernatant to an Amicon Ultra centrifugal filter unit.
  - Centrifuge supernatant for 20 minutes at 3000 rpm (1811 x g), 4°C.
  - Discard flowthrough and fill up with 1x PBS
  - Repeat until PBS is used up.
  - Centrifuge until final volume reaches around 200 µL.
- Determine protein concentration by SDS-PAGE and protein-measurement using a commercial assay (Pierce™ 660nm Protein Assay Reagent).
- Aliquot and store at -20°C.
2.2.2 Strep-tagged Mbd4 purification

- Prepare PARP buffer with protease inhibitors. Add 200 µL AEBSF, 200 µL E64, 20 µL PepA in 20 mL PARP buffer (0.5 M NaCl) and store on ice.
- Thaw eight 1 mL Sf9 cell pellets on ice by adding 2 mL PARP buffer (0.5 M NaCl) with inhibitors and incubate for 30 minutes on ice.
- Lyse cells by syringe treatment (5 strokes 21G, 5 strokes 23G).
- Combine all cell lysates in 50 mL tube and incubate 10 minutes on ice.
- Centrifuge for 30 minutes at 4°C, 9552 rpm (14000x g).
- Meanwhile prepare Strep-beads
  - Transfer 1 mL of slurry beads to a 15 mL tube.
  - Wash beads 3 times with 5 mL PARP buffer supplemented with protease inhibitors by centrifugation at 4°C, 800 rpm, 3 min.
- After clearing the cell lysate by centrifugation, transfer supernatant to the Strep-beads and incubate for 3 h on a rotator at 4°C.
- Centrifugation at 4°C, 800 rpm, 3 min.
- Discard supernatant.
- Wash pellets 4 times with 6 mL PARP buffer (0.5 M NaCl) with inhibitors and 2 times with 6 mL PBS by centrifugation at 4°C, 800 rpm (129x g), 3 min.
- Elute the Strep-Mbd4 protein by adding 4 mL elution buffer and incubate 10 minutes on ice.
- Centrifuge for 3 minutes at 4°C, 800 rpm (129x g), then transfer supernatant to a new 15 mL tube and add 6 mL PBS.
- Buffer exchange.
  - Prepare 50 mL ice-cold 1x PBS.
  - Transfer supernatant to an Amicon Ultra centrifugal filter unit.
  - Centrifuge for 20 minutes at 4°C, 3000 rpm (1811x g).
  - Discard flowthrough and fill up with 1x PBS and repeat until PBS is used up.
  - Centrifuge until final volume reaches around 200 µL.
  - Determine protein concentration by SDS-PAGE and protein-measurement using a commercial assay (Pierce™ 660 nm Protein Assay Reagent).
  - Aliquot and store at -20°C.

(Note: All solutions should be kept on ice.)

2.3 CAA treatment and base flipping assay (Tet1)
2.3.1 DNA base flipping reaction preparation
• Prepare reactions in MicroAmp® Optical 8-Tube Strip tubes as listed below

|                | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL |
|----------------|--------|--------|--------|--------|
| DNA (10 pmol/µL) | (M:C)  | (M:C)  | (X:X)  | (X:X)  |
| M.HpaII buffer  | 1 µL   | 1 µL   | 1 µL   | 1 µL   |
| CAA (100 mM)    | 3 µL   | 3 µL   | 3 µL   | 3 µL   |
| His-Tet1CD (20 pmol) | 1 µL / | 1 µL / | 1 µL / |
| PBS             | /      | 1 µL   | /      | 1 µL   |
| ddH2O           | 4.5 µL | 4.5 µL | 4.5 µL | 4.5 µL |

(Note: All reactions should be prepared on ice. All reactions should be prepared as triplicates. Prepare mastermixes where possible, as the DNA amount per reaction is crucial for the outcome.)

(Note: The purified Tet1CD proteins were diluted in 1x PBS, therefore in all reactions without protein the same amount of 1x PBS was used, as the salt concentration can affect the outcome.)

• Close the tubes with MicroAmp® Optical 8-Cap Strip lids.
• Incubate reactions in a ThermoMixer for 2 hours at 37°C and put on ice afterwards.

2.3.2 High resolution melting temperature analysis

• Keep reactions on ice.
• Prepare a detection mastermix containing 5 µL Platinum® SYBR® Green qPCR SuperMix-UDG and 5 µL ddH2O per reaction.
• Inactivate UDG for 2 minutes at 50°C on a ThermoMixer
• Discard MicroAmp® Optical 8-Cap Strip lids.
• Add 10 µL detection mastermix to every reaction.
• Close tubes with new MicroAmp® Optical 8-Cap Strip lids to ensure unimpeded readout.
• Place the tubes in the StepOnePlus™ Real-Time PCR Systems and run the following protocol:
  o 95°C for 30 seconds.
  o Decreasing ramp to 50°C with a decreasing rate of 2%.
  o 1 minute at 50°C.
  o Increasing ramp to 90°C with 0.1°C steps.
  o Keep at 90°C for 15 seconds.

2.3.3 Data presentation

• Export experiment data from StepOne™ software as “.txt” files.
• Fluorescence intensity plotting: normalized intensities and the derivative of fluorescence intensities were plotted against temperature using self-written R script as line plots (Supplementary file).

(Note: Fluorescence intensity normalization: The original fluorescence of SYBR Green was normalized to the fluorescence of the ROX reference dye. Then for each reaction, the intensities were further normalized to the maximal and minimal values.)

2.4 CAA treatment and base flipping assay (M.HpaII)

2.4.1 DNA base flipping reaction preparation

• Prepare reactions in MicroAmp® Optical 8-Tube Strip tubes as listed below
| Components          | 0.5 µL | 0.5 µL | 0.5 µL | 0.5 µL |
|---------------------|-------|-------|-------|-------|
| DNA (10 pmol/µL)    | (M:C) | (M:C) | (X:X) | (X:X) |
| M.HpaII buffer      | 1 µL  | 1 µL  | 1 µL  | 1 µL  |
| CAA (100 mM)        | 3 µL  | 3 µL  | 3 µL  | 3 µL  |
| M.HpaII (4 U)       | 1 µL  | /     | 1 µL  | /     |
| M.HpaII storage buffer | /     | 1 µL  | /     | 1 µL  |
| ddH₂O              | 4.5 µL| 4.5 µL| 4.5 µL| 4.5 µL|

(Note: All reactions should be prepared on ice. All reactions should be prepared as triplicates. Prepare mastermixes where possible, as the DNA amount per reaction is crucial for the outcome.)

2.4.2 High resolution melting temperature analysis
See 2.3.2

2.4.3 Data presentation
See 2.3.3

2.5 Mbd4 induced base excision detection assay

2.5.1 Reactions preparation

| Components          | 1 µL | 1 µL | 1 µL | 1 µL |
|---------------------|------|------|------|------|
| DNA (10 pmol/µL)    | (M:2AP) | (M:TG) | (M:C) | (M:2AP) |
| 2x reaction buffer  | 5 µL | 5 µL | 5 µL | 5 µL |
| Mbd4 (20 pmol/µL)   | 1 µL | 1 µL | 1 µL | / |
| PBS                 | /    | /    | /    | 1 µL |
| ddH₂O              | 3 µL | 3 µL | 3 µL | 3 µL |

Note: If the reactions are used for fluorescence intensity measurement, prepare the reactions in 50 µL. For each component, use 5 times the amounts as listed above.

• Incubate at 37°C for 2 hours.
• Add 1 µL 20 mg/ml proteinase K and incubate at 50°C for 1 hour.

2.5.2 2AP fluorescence intensity measurement
• Spin down the reactions.
• Transfer all reactions to 96-well plate.
• Measure the fluorescence intensity using 320 nm and 370 nm excitation and emission wavelengths, respectively, using the TECAN M200 fluorescence microplate reader.

2.5.3 Denaturing polyacrylamide gel electrophoresis
• Assemble the Dual Gel Caster for Mini Vertical Units according to the manufacturer’s instruction. Use notched and unnotched glass plates, as well as T-spacers with a height of 8 cm.
• Per gel, mix 4.024 g Urea with 2 mL of 5x TBE and 5 mL of 30% acrylamide/bis-acrylamide, heat the solution in the microwave for 10 seconds, then add 2.5 mL of formamide, 100 µL of 10% APS and 10 µL TEMED.
• Insert the comb, pour the gel and let solidify at RT.
• Assemble the Mighty Small (II) Mini Vertical Electrophoresis Unit (SE250) and fill the upper and lower buffer chamber with 1x TBE.

• Connect Mighty Small (II) Mini Vertical Electrophoresis Unit (SE250) to the Power Pack 25 and pre run the gel at 100 V and 50˚C for 1 hour.

• Meanwhile prepare the samples.
  • Add 1 µL of 1 M NaOH to each sample (10 µL) and incubate at 99˚C for 10 minutes.
  • Add 10 µL 2x loading buffer and incubate at 99˚C for 10 minutes. After incubation, quickly put the samples on ice.

• Load the sample and run the gel at 130V and 50˚C until the tracking dye (bromophenol blue) has reached the bottom of the gel, turn off the power supply, disconnect the leads and remove the gel.

• Wash the gel with 1x TBE for 20 minutes at RT.

• Stain gel for 30 minutes in ethidium bromide staining solution (see Reagent Setup) or SYBR Green II (1:10000 dilution in 1x TBE).

• Rinse gel for 5 minutes in ddH₂O.

• Detect the signal on a fluorescent Imager (Amersham Imager 600 RGB) using 312 nm UV excitation and a 605BP40 emission filter for ethidium bromide or 460 nm excitation and a 525BP20 emission filter for SYBR Green II.

2.5.4 High resolution melting temperature analysis.
See 2.3.2.

2.5.5 Data presentation.
See 2.3.3.

3 Base modification activity detection assay

3.1 Preparation of oligonucleotides

• Mix 1 µL of 100 µM sense and 1 µL of 100 µM antisense containing 5mC single strand oligo with 8 µL of ddH₂O in 1.5 mL tube. Repeat this for 5hmC, 5fC and 5caC containing oligos.

• Mix 10 µL of 100 µM 20 nt CG-up/MG-up, 10 µL of 100 µM 20 nt CG-down/MG-down single strand oligo, 10 µL NEbuffer 2 with 70 µL of ddH₂O in 1.5 mL tube.

• Denature the oligo mixtures at 95˚C for 2 minutes in a ThermoMixer, then slowly cool down to room temperature by switching off the ThermoMixer.

(Note: The cooling-down process takes approximately 50 minutes and should not be accelerated to ensure proper annealing of the oligos.)

3.2 Protein purification
His-Tet1CD was purified as described in 2.2.1.

3.3 Double strand DNA stability measurement with DNA base modifications

3.3.1 Reaction preparation

• Mix 130 µL of SYBR Green mixture with 130 µL of ddH₂O.

• Transfer 19 µL of the mixture to a single well of MicroAmp® Optical 8-Cap Strips.

• Add 1 µL of double stranded DNA (3.1) to each well. For each modification, perform three technical repeats.
• Close the 8-Cap Strips with MicroAmp® Optical 8-Cap Strip lids.

3.3.2 HRM analysis

Perform the HRM analysis as described in 2.3.2.

3.3.3 Data presentation

Analyze the data by RStudio as described in 2.3.3.

3.4 Tet1 oxidation activity detection (oligonucleotides)

3.4.1 Preparation of reactions

Prepare the reactions as listed below:

|            | Tet1CD  | Oxidation buffer 1 | Oxidation buffer 2.1 | DNA (10 pmol/µL) | ddH₂O |
|------------|---------|--------------------|-----------------------|------------------|-------|
| 1          | 1 µL (Tet1CD) | 0.7 µL            | 3 µL                  | 0.5 µL           | 4.8 µL |
| 2          | 1 µL (PBS)    | 0.7 µL            | 3 µL                  | 0.5 µL           | 4.8 µL |

• Mix 33.6 µL of ddH₂O, 3.5 µL of fully methylated CpG DNA (1.1.1), 21 µL of oxidation buffer 2 and 4.2 µL of oxidation buffer 1. Mix well and transfer 9 µL of mastermix to MicroAmp® Optical 8-Cap Strip.
• Add 1 µL of Tet1CD proteins or PBS to each well.
• Close the MicroAmp® Optical 8-Cap Strips with MicroAmp® Optical 8-Cap lids.
• Incubate the reaction at 37°C for 2 h.

3.4.2 HRM analysis

Perform the HRM analysis as described in 2.3.2 except adding 4 µL of unmethylated 20 bp oligo (3.1.2) and 6 µL of SYBR Green Mix.

3.4.3 Data presentation

Analyze the data with RStudio as described in 2.3.3.

3.5 Tet1 oxidation activity detection (PCR fragment)

3.5.1 Preparation of reactions

Prepare the reactions as listed below:

|            | Tet1CD  | Oxidation buffer 1 | Oxidation buffer 2.1 | DNA (100 ng/µL) | ddH₂O |
|------------|---------|--------------------|-----------------------|------------------|-------|
| 1          | 1 µL (Tet1CD) | 0.7 µL            | 3 µL                  | 0.5 µL           | 4.8 µL |
| 2          | 1 µL (PBS)    | 0.7 µL            | 3 µL                  | 0.5 µL           | 4.8 µL |

• Mix 33.6 µL of ddH₂O, 3.5 µL of PCR fragment, which was amplified from a plasmid containing MINX sequence (7), 21 µL of oxidation buffer 2 and 4.2 µL of oxidation buffer 1. Mix well and transfer 9 µL of mastermix to MicroAmp® Optical 8-Cap Strip.
• Add 1 µL of Tet1CD proteins or PBS to each well.
• Close the MicroAmp® Optical 8-Cap Strips with MicroAmp® Optical 8-Cap lids.
• Incubate the reaction at 37°C for 2 h.

3.5.2 HRM analysis
Perform the HRM analysis as described in 2.3.2 except increasing temperature from 60°C to 99°C.

3.5.3 Data presentation
Analyze the data with RStudio as described in 2.3.3.

3.6 M.HpaII methyltransferase activity detection

3.6.1 Preparations of reactions

- Mix 10 µL of 10 pmol of 20 bp DNA (3.1) with 90 µL ddH2O.
- Mix 0.1 µL of 32 mM SAM with 50 µL M.HpaII buffer.
- Enzyme dilution:
  - p1: undiluted protein (4 U/µL)
  - p2: dilute protein to 0.4 U/µL in storage buffer
  - p3: dilute protein to 0.08 U/µL in storage buffer
  - p4: dilute protein to 0.016 U/µL in storage buffer
- Prepare the reactions as listed below:

|   | 1   | 2   | 3   | 4   | 5   | 6   |
|---|-----|-----|-----|-----|-----|-----|
| M.HpaII | 0.5 µL | 1 µL | 1 µL | 1 µL | /   | /   |
| Storage buffer | 0.5 µL | /   | /   | /   | 1 µL | 1 µL |
| 20 bp unmethylated DNA (1 pmol/µL) | 1 µL | 1 µL | 1 µL | 1 µL | 1 µL | /   |
| 20 bp methylated DNA (1 pmol/µL) | /   | /   | /   | /   | /   | 1 µL |
| M.HpaII buffer + SAM | 1 µL | 1 µL | 1 µL | 1 µL | 1 µL | 1 µL |
| ddH2O | 7 µL | 7 µL | 7 µL | 7 µL | 7 µL | 7 µL |

- Incubate the reaction at 37 °C for 2 h.

3.6.2 HRM analysis
Perform the HRM analysis as described in 2.3.2 except increasing temperature from 40°C to 80°C.

3.6.3 Data presentation
Analyze the data with RStudio as described in 2.3.3.
References

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Supplementary figures

Figure S1 Oligonucleotide preparation and protein purification.

(A) Oligonucleotides (42-mer) preparation. Either non-CpG (Non-CG up), unmethylated CpG (CG up) or methylated CpG (MG up) containing oligonucleotides were mixed with the respective fill-in oligonucleotides. After denaturing and renaturing, fill-in reactions were performed in the presence of Klenow DNA polymerase and a dNTP mixture as described in the methods. X: non-CpG, C: unmethylated CpG, M: methylated CpG. Grey shape represents base G. Yellow shape represents base C. Yellow shape with black border represents 5mC.
(B) Confirmation of the methylation status of all oligonucleotides. Double stranded oligonucleotides were incubated with or without the restriction enzymes MspI or HpaII. After two hours of incubation, DNA was separated on a native 20% polyacrylamide gel. Large and small fragments indicate undigested and digested DNA, respectively.

(C) Representative image of purified Tet1CD and MBD proteins separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue.
A

**Fluorescence imager**

| Signal Type | Ex. | Em. |
|-------------|-----|-----|
| GFP/YFP     | 470 +/- 30 | 532 +/- 28 |
| mCherry     | 530 +/- 28 | 607 +/- 50 |

**Fluorescence microplate reader**

| Signal Type | Ex. | Em. |
|-------------|-----|-----|
| mCherry     | 585 +/- 9 | 630 +/- 20 |

B

Transparent plate (10 cm x 12 cm)

C

Fix the transparent plate on top of a flat 96-well plate lid

D

Place gel on top of the template according to red tape and fix

E

Place the template-fixed gel into a fluorescence microplate reader (TECAN)

F

Plot intensity as a heatmap using RStudio

G

Check intensity of red tape

Step E

no

1 2 3 4 5 6 7 8 9 10

yes
Figure S2 Gel fluorescence detection optimization.

(A) Comparison of fluorescence protein signals separated on a polyacrylamide gel detected on a fluorescence imager (Amersham 600 RGB) and fluorescence microplate reader (TECAN M200), respectively. Spectral bleed through from YFP upon excitation with the 530 nm light source used to excite mcherry was observed using the fluorescence imager, but not with the fluorescence microplate reader (indicated with the yellow boxes and arrow).

(B-F) Optimization of the gel detection protocol using a fluorescence microplate reader. To control the position of the gel, a gel template was prepared. Gel pockets are marked with red tape and spaces between pockets are labeled with green tape (see methods). To measure the fluorescence intensity, we made a measurement protocol in i-control (1.10) software (TECAN) with following settings: number of rows: 10, number of columns: 43; length: 127800 µm; width: 85500 µm; left well position: 10 µm; right well position: 127800 µm; upper well position: 7500 µm; lower well position: 78500 µm; well size: 3000 µm x 3000 µm, well depth: 11700 µm.

To control the gel position, the fluorescence signals of the red and green tapes were used. If the fluorescence intensity (585/635 nm excitation and emission wavelengths) showed a homogeneous intensity distribution for all lanes, then intensities of the whole gel were plotted as a heatmap. Otherwise, the gel was repositioned properly and the measurement done again.
Figure S3 Binding specificity of Tet1CD to methylated and non-CpG containing DNA.

Purified His-Tet1CD or YFP-MBD was incubated with methylated CpG and non-CpG containing DNA. After 90 minutes of incubation at 37°C, free DNA and DNA-protein complexes were separated on a 4.5% native polyacrylamide gel. To quantify the binding, the amounts of unbound DNA (intensities of free DNA bands measured using Image J) were plotted below the gel. Independent experiments were repeated twice. Shown is one representative result. mC:mC, fully methylated CpG containing DNA; X:X, non-CpG containing DNA. Free DNA and protein-DNA complexes are indicated on the gel.
Figure S4 Detection of M.HpaII mediated DNA base flipping.

(A) CAA concentration test. 1 µM of hemimethylated oligonucleotides were incubated with varying concentrations of CAA and HRM analysis was performed. CAA: chloroacetaldehyde.

(B) Effect of different CAA concentrations on the detection of M.HpaII mediated base flipping. Red dashed lines indicate reactions with M.HpaII and blue dashed lines indicate reactions without M.HpaII,
respectively. Red and blue solid lines indicate $T_m$ difference. The green box indicates the best CAA concentration for base flipping reaction.

(C) Binding specificity of M.HpaII to CCGG containing or non-CpG containing DNA. M.HpaII was incubated with 0.1 $\mu$M CCGG containing DNA and 0.1 $\mu$M non-CpG containing DNA. After 90 minutes of incubation at 37°C, free DNA and DNA-protein complexes were separated on a 4.5% native polyacrylamide gel. To quantify the binding, the amounts of unbound DNA (intensities of free DNA bands measured using Image J) were plotted below the gel. mC:mC, fully methylated CpG containing DNA; C:C, unmethylated CpG containing DNA; Free DNA and protein-DNA complexes are indicated on the gel.

Figure S5 Detection of Tet1CD (catalytic domain of Tet1) mediated DNA base flipping.

(A) Binding of Tet1CD to hemimethylated DNA (method as described in Fig. S3) in the presence of varying CAA concentrations. 2.8 $\mu$M of Tet1CD protein was incubated with 0.5 $\mu$M of hemimethylated DNA in the presence of 30, 50 and 100 mM of CAA, respectively. mC: methylated CpG. C: unmethylated CpG.

(B) HRM analysis of DNA base flipping induced by Tet1 in the presence of CAA (method as described in Fig. 5). Derivative of fluorescent SYBR green intensities were plotted using RStudio. Independent experiments were repeated at least three times. Shown is one representative result of three averaged technical replicates. The $p$ values of student’s $t$ test are indicated in the plot. C: cytosine. mC: 5-methylcytosine.
Figure S6 2-aminopurine oligonucleotide preparation and Mbd4 protein purification.

(A) Testing 2AP fluorescence using single stranded DNA. Various amounts of single stranded 2AP containing DNA (42 nucleotides long) were diluted in ddH₂O and fluorescence intensities were measured on a fluorescence microplate reader using 320 nm and 370 nm excitation and emission wavelengths, respectively. As a control, single stranded oligonucleotides (42 nucleotides long) containing a methylated CpG site were used.

(B) Preparation and verification of double stranded DNA containing 2AP. mC: methylated CpG. C: unmethylated CpG. Grey shape represents base G. Yellow shape represents base C. Yellow shape with black border represents 5mC. The blue shape represents 2AP (2-aminopurine).
(C) Confirmation of the methylation status of all oligonucleotides. Double stranded oligonucleotides were incubated with or without the restriction enzymes MspI or HpaII. After two hours of incubation, DNA was separated on a native 20% polyacrylamide gel. Large and small fragments indicate undigested and digested DNA, respectively.

(D) Representative image of purified Mbd4-Strep proteins, separated by SDS-PAGE and stained with Coomassie Brilliant Blue.
Figure S7 Detection of base excision induced by Mbd4.

(A) Detection of Mbd4-mediated base flipping and excision activity. 2 μM Mbd4 was incubated with 1 μM double-stranded oligonucleotides containing 2AP in the context of methylated CpG dinucleotides at 37°C in a total volume of 50 μL for 2 hours. As a control, hemimethylated dinucleotides and T:G mismatched dinucleotides (in the context of methylated CpGs) were used. The 2AP fluorescence intensities were measured on a fluorescence microplate reader (TECAN M200) using 320 nm and 370 nm excitation and emission wavelengths, respectively at 25°C. Three independent experiments were performed. Shown are the mean value and standard deviation. (B) Detection of base excision using a denaturing gel. 2 μM Mbd4 was incubated with 1 μM double-stranded oligonucleotides containing 2AP in the context of methylated CpG dinucleotides at 37°C in 10 μL volume for 2 hours. As a control,
hemimethylated dinucleotides and T:G mismatched dinucleotides (in the context of methylated CpGs) were used. After incubation, the reactions were shifted to 99°C for 10 minutes in the presence of 0.1 M NaOH. After another 10 minutes at 99°C in 1x loading buffer and 5 minutes on ice, the samples were loaded on a denaturing gel. As a molecular weight marker, 100 pmol of single stranded 42 and 20 nucleotides (nt) DNA were used. The gel was stained with ethidium bromide for 30 minutes and destained in ddH$_2$O for 5 minutes. The DNA signal was detected using a fluorescence imager. M:C, hemimethylated DNA. M:TG, T:G mismatch in the context of methylated CpG.
Figure S8 Binding specificity of Mbd4 to methylated, unmethylated or T:G mismatch containing DNA.

(A) Purified Strep-tagged Mbd4 was incubated with methylated CpG (ATTO 647N labeled) and unmethylated CpG (ATTO 550 labeled) containing DNA. After 90 minutes of incubation at 37°C, free DNA and DNA-protein complexes were separated on a 4.5% native polyacrylamide gel. The methylated and unmethylated DNA signals were detected using a fluorescence imager. To quantify the binding, the amounts of unbound DNA (intensities of free DNA bands measured using Image J) were
plotted below the gel. The results show that Mbd4 has a binding preference for methylated DNA according to the amount of free DNA.

(B) Purified Strep-tagged Mbd4 was incubated with T:G mismatch (ATTO 647N labeled) and hemimethylated CpG (ATTO 550 labeled) containing DNA. After 90 minutes of incubation at 37°C, free DNA and DNA-protein complexes were separated on a 4.5% native polyacrylamide gel. The methylated DNA signals were detected using a fluorescence imager. To quantify the binding, the amounts of unbound DNA (intensities of free DNA bands measured using Image J) were plotted below the gel. The results show that Mbd4 has a binding preference for T:G mismatched DNA according to the amount of free DNA.
Figure S9 Detection of Tet1CD mediated 5mC oxidation.

(A) Effect of Tet oxidation products on DNA double strand thermostability. 1 μM of 42 bp oligonucleotides containing a single 5mC, 5hmC, 5fC and 5caC nucleotide in the middle of each strand were analyzed by HRM analysis. Independent experiments were repeated twice. Shown is one representative result.
(B) HRM analysis test with 5 pmol of 10 bp, 20 bp and 42 bp unmethylated DNA. The results showed that only 20 bp and 42 bp DNA give melting temperature above 50°C.

(C) Detection of Tet1CD mediated 5-methylcytosine (5mC) oxidation using 42 bp oligonucleotides containing a symmetrically methylated CpG. 0.5 µM of fully methylated DNA was incubated with 2 µM of Tet1CD for 120 minutes at 37°C. After incubation, HRM analysis was performed. Solid and dashed lines indicate incubation of DNA with or without Tet1CD, respectively. Independent experiments were repeated at least two times. Shown is one representative result with three technical replicates. The $p$ values of student’s $t$-test are indicated in the plot.

(D) Detection of Tet1CD mediated 5-methylcytosine (5mC) oxidation using 42 bp oligonucleotides as in (C). After incubation, 40 pmol of unmethylated 20 bp oligonucleotides were added in the reactions and then HRM analysis was performed. Solid and dashed lines indicate incubation of DNA with or without Tet1CD, respectively. Independent experiments were repeated at least three times. Shown is one representative result with three technical replicates. The $p$ values of student’s $t$-test are indicated in the plot.

(E) The behavior of DNA mixtures with different $Tm$ values in HRM analysis. DNA with high $Tm$ (mCmC, 42 bp oligonucleotides containing a methylated symmetric CpG) and low $Tm$ values (CC, 42 bp oligonucleotides containing an unmethylated symmetric CpG) were mixed at different ratios as indicated on the plot and studied by HRM analysis.

(F) Detection of Tet1CD mediated 5mC oxidation using 42 bp oligonucleotides containing a mismatched base. 0.5 µM of oligonucleotides containing a T:G mismatch in the context of methylated CpGs were incubated with 2 µM of Tet1CD for 120 minutes at 37°C. After incubation 40 pmol of unmethylated 20 bp oligonucleotides were added in the reactions, then HRM analysis was performed. Independent experiments were repeated twice. Shown is one representative result with three technical replicates. The $p$ values of student’s $t$-test are indicated in the plot. Solid and dashed lines indicate incubation of DNA with or without Tet1CD, respectively. mC: 5-methylcytosine. hmC: 5-hydroxymethylcytosine. fC: 5-Formylcytosine. caC: 5-Carboxylcytosine. T: thymine.
Figure S10 Effect of cofactors on Tet1 mediated oxidation using melting temperature analysis.
(A, B) Effect of ATP and DTT on Tet1 mediated 5mC oxidation. 0.5 µM of fully methylated DNA was incubated with 2 µM of Tet1CD in the presence (B) or absence of (A) ATP and DTT (oxidation buffer 2.1 versus oxidation buffer 2.4). After 120 minutes of incubation, DNA was used for HRM analysis. Independent experiments were repeated twice. Shown is one representative result with three technical replicates. The p values of student’s t.test are indicated in the plot.
Figure S11 Detection of methyltransferase activity.

(A) 0.1 µM of unmethylated 42 bp DNA was incubated with 0.125 µM (4 units) of M.HpaII for two hours at 37°C. After incubation, 40 µL of NEBuffer 1, 5 µl of 50 mM MgCl₂ and 20 units of HpaII were added to the reactions and the reactions were incubated at 37°C for two hours. Then, the DNA was separated on 20% PAA gel. As size markers, 20 bp DNA and 42 bp DNA without treatment were used. (B) 0.1 µM of unmethylated DNA was incubated with 0.125 µM (4 units) of M.HpaII for 120 minutes at 37°C. After incubation, HRM analysis was performed. Solid and dashed lines indicate incubation of DNA with or without M.HpaII, respectively. Independent experiments were repeated at least three times. Shown is one representative result with three technical replicates. The p values of student’s t.test are indicated in the plot. C: cytosine. mC: 5-methylcytosine.