

Cooperative DNA binding and protein/DNA fiber formation increases the activity of the  
Dnmt3a DNA methyltransferase\*

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\*Running title: *Multimerization of Dnmt3a on DNA increases its DNA methylation rate*

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**Key words:** DNA methylation, enzyme mechanism, processivity, multimerization, protein/DNA  
interaction

**Background:** Dnmt3a has been reported to multimerize on DNA and methylate DNA processively, which is contradicting.

**Results:** We show that multimerization of Dnmt3a on DNA increases its activity, but processive DNA methylation is not detectable.

**Conclusion:** Dnmt3a forms enzyme/DNA fibers.

**Significance:** Our results help to understand the mechanism of DNA methylation and the effects of Dnmt3a somatic cancer mutations.

## ABSTRACT

The Dnmt3a DNA methyltransferase has been shown to bind cooperatively to DNA and to form large multimeric protein/DNA fibers. However, it has also been reported to methylate DNA in a processive manner, a property that is incompatible with protein/DNA fiber formation. We show here that the DNA methylation rate of Dnmt3a increases more than linearly with

increasing enzyme concentration on a long DNA substrate, but not on a short 30mer oligonucleotide substrate. We also show that addition of a catalytically inactive Dnmt3a mutant, which carries an amino acid exchange in the catalytic center, increases the DNA methylation rate by wild type Dnmt3a on the long substrate, but not on the short one. In agreement with this finding, pre-incubation experiments indicate that stable protein/DNA fibers are formed on the long, but not on the short substrate. In addition, methylation experiments with substrates containing one or two CpG sites did not provide evidence for a processive mechanism over a wide range of enzyme concentrations. These data clearly indicate that Dnmt3a binds to DNA in a

**cooperative reaction and that the formation of stable protein/DNA fibers increases the DNA methylation rate. Fiber formation occurs at low  $\mu$ M concentrations of Dnmt3a, which is in the range of Dnmt3a concentrations in the nucleus of ES cells. Understanding the mechanism of Dnmt3a is of vital importance, because Dnmt3a is a hotspot of somatic cancer mutations and one of the most abundant cancer mutation had been implicated in changing Dnmt3a's processivity.**

DNA methylation is a key epigenetic process involved in the control of gene expression, regulation of parental imprinting, stabilization of X-chromosome inactivation, as well as maintenance of the genome integrity through protection against endogenous retroviruses and transposons (1-4). Aberrant DNA methylation patterns are associated with several human diseases and contribute to both the initiation and the progression of various cancers. In mammals, DNA methylation occurs at the C5 position of the cytosine residues, primarily in the CpG dinucleotides. However, only certain CpG sites are methylated, resulting in the generation of a tissue and cell type specific pattern of methylation. The methyl group is introduced into DNA by DNA methyltransferases (Dnmts), which use *S*-adenosyl-*L*-methionine (AdoMet) as a methyl group donor. Dnmt3a belongs to the Dnmt3 family of DNA methyltransferases (1), members of which are involved in the initial generation of DNA methylation patterns during early development (5). The enzyme consist of a C-terminal catalytic domain that is active in an isolated form (6), and an N-terminal part involved in enzyme targeting and regulation (7-9). The structure of the C-terminal domain of Dnmt3a (Dnmt3a-C) in complex with the

analogous domain of its regulatory factor Dnmt3L showed that the complex forms a linear heterotetramer, consisting of two Dnm3L (at the edges of the tetramer) and two central Dnmt3a molecules, which together form the DNA binding site (10). In the absence of Dnmt3L, Dnmt3a self-associates to form a homodimer via the Dnmt3a/3L (FF) interface, which further dimerises through the RD interface, forming tetramers. Since DNA binding occurs at the RD interface, a Dnmt3a tetramer contains one DNA binding site (10-12). In addition, Dnmt3a and Dnmt3b were also shown to form heterodimers *in vitro* and in cells (13).

The fact that DNA is a linear polymer containing specific target sites for enzymatic turnover embedded in a non-specific sequence has important consequences for the mechanism of DNA enzymes and Dnmts in particular. First, the polymeric nature of DNA implies that several proteins could bind next to each other in a cooperative manner. This is particularly relevant for proteins, which bind non-specifically or with low specificity to DNA, like Dnmt3a. Cooperative DNA binding implies that an unbound enzyme will preferentially associate to a DNA molecule next to an already bound protein. Depending on the degree of cooperativity, this may lead to the complete coverage of some DNA molecules with protein and formation of a protein-DNA fiber, while other DNA molecules remain unbound. For Dnmt3a complexes, cooperative DNA binding was observed in equilibrium DNA binding experiments and the formation of large protein-DNA fibers was observed in gel retardation experiments and by direct scanning force microscopy imaging (10-12,14). Furthermore, the protein-protein interface

between adjacent Dnmt3a complexes bound to the DNA that is needed for cooperative DNA binding was identified and mutations in this region significantly reduced the cooperativity (14). However, the functional relevance of Dnmt3a multimerization on DNA has not been addressed so far.

A second consequence of the polymeric nature of DNA as a substrate for an enzymatic turnover is that binding of enzymes to the target site is usually a two-step process. At first, the enzyme binds to the DNA at a non-specific site and then it moves along the DNA in a process called linear (or facilitated) diffusion to reach its specific site. Similarly, dissociation from the DNA after an enzymatic turnover often is a two-step process, in which the enzyme first moves from the target site to a neighboring non-specific site. Then, it may stay on the DNA and move along the DNA by linear diffusion or dissociate from the DNA. Since typically several target sites are present on one DNA molecule, this process can lead to a processive reaction mechanism. For a DNA methyltransferase this means that the enzyme could subsequently methylate several cytosines on one DNA molecule without ever leaving the DNA, because it can move along the DNA after each turnover to reach the next target site. Processivity has been shown for many DNA enzymes, like DNA polymerases (15), RNA polymerase (16), nucleases (17,18), restriction enzymes (19) and also DnmTs, like M.SssI (20); EcoDam (21) or Dnmt1 (22-24). Processive DNA methylation has also been reported for Dnmt3a by the Reich group (25).

We were puzzled by the observation that Dnmt3a has been reported to multimerize on DNA

and to work in a processive mode. These properties appear mutually exclusive, because the concept of a processive turnover is based on individual enzyme molecules moving along a DNA substrate. In this study we investigated the functional role of Dnmt3a multimerization on DNA. In addition, we revisited the published evidence in favor of a processive mechanism for Dnmt3a and conducted an additional test for processivity. This mechanistic feature of Dnmt3a is of particular relevance, since in follow-up studies the Reich group has also reported that a frequently observed somatic cancer mutation in Dnmt3a leads to changes in enzyme processivity (26,27). We show here that multimerization of Dnmt3a on DNA stimulates the activity of the enzyme *in vitro* and processive DNA methylation is not detectable.

## EXPERIMENTAL PROCEDURES

*Protein purification* – The C-terminal domain (amino acid 608 - 908) of mouse Dnmt3a (Dnmt3a-C) and its catalytically inactive variant E752A were cloned into pET 28a+ vector (Novagen) as an N-terminal His<sub>6</sub>-tag fusion (28). The mutated glutamate is residue number 752 in wild type murine Dnmt3a, hence it is designated here E752A, even when used in the catalytic domain of Dnmt3a. Human and mouse Dnmt3a-C are identical in sequence. The wildtype and mutant Dnmt3a-C were overexpressed in BL21 (DE3) Codon+ RIL *E.coli* cells (Stratagene). The cells were grown in TB medium until an OD<sub>600nm</sub> of 0.8 was reached and Dnmt3a-C expression was induced for 12 hours at 20 °C by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The proteins were purified at high micromolar concentrations using Ni-NTA agarose and stored in 20 mM HEPES pH

7.5, 200 mM KCl, 0.2 mM DTT, 1 mM EDTA and 10% glycerol at -80 °C. The concentration and purity of the protein preparations was determined by UV absorption at 280 nm and by densitometric analysis of Coomassie stained SDS-polyacrylamide gels (Fig. 1A). M.SssI was purified as described (29).

*Radioactive DNA methylation kinetics* – Radioactive methylation kinetics were conducted with a biotinylated double stranded 30mer oligonucleotide substrate (GAG AAG CTG GGA CTT **CCG** GGA GGA GAG TGC) and a biotinylated 509mer DNA fragment using the avidin-biotin methylation assay, basically as described (30). The 509mer was amplified from the CpG island upstream of the human SUHW1 gene using the following primers (Bt-AGA TTA GGG AAG GGG GTG TG and AAG ATC CTT TCA AGG CCT CAG). Its sequence is given below.

To measure methylation at different concentrations of Dnmt3a-C, methylation reactions for each substrate (30mer / 509mer) were carried out using 0.25, 0.5, 0.75, 1, 1.5 and 2  $\mu$ M of Dnmt3a-C. To ensure the same buffer composition in all reactions, enzyme storage buffer was added to compensate for the different enzyme volumes. The methylation was carried out in methylation buffer composed of 20 mM HEPES pH 7.5, 50 mM KCl and 1 mM EDTA, supplemented with 0.25 mg/ml BSA using 250 nM of the 30mer or 100 nM of the 509mer. The methylation reaction was started by addition of an AdoMet mixture, yielding a final concentration of 2  $\mu$ M unlabeled AdoMet (Sigma) and 0.76  $\mu$ M radioactively labeled [methyl-<sup>3</sup>H]-AdoMet (PerkinElmer Life Sciences). To avoid protein binding to the wall of the reaction tubes in

the serial dilutions Protein LoBind Tubes (Eppendorf) were used.

To study the stimulatory effect of the inactive E752A variant on wild type (wt) Dnmt3a-C, methylation reactions were performed using 1  $\mu$ M wt (1W), 1  $\mu$ M wt and 1  $\mu$ M E752A mutant (1M1W), or 2  $\mu$ M wt (2W). As described above, different enzyme volumes were compensated with the storage buffer. The reaction conditions were as above, except that 1  $\mu$ M of the 30mer was used.

Pre-incubation experiments were carried out in methylation buffer containing 0.05 mg/ml BSA using 1.5  $\mu$ M Dnmt3a-C. The enzyme was pre-incubated with 50 nM (final concentration) of the first DNA substrate at ambient temperature for 25 min to allow for complex formation. Afterwards, 50 nM of the second DNA substrate (competitor with an identical sequence) was added and the methylation reactions were started immediately by addition of labeled AdoMet to a final concentration of 0.76  $\mu$ M.

Calibration reactions were carried out using the 30mer and the radioactively labelled AdoMet with the prokaryotic M.SssI C5 methyltransferase, which has a high activity and was used to obtain fully methylated DNA.

*Methylation of substrates with one or two CpG sites*

- To investigate if Dnmt3a-C could be potentially processive at low enzyme concentrations where fiber formation does not occur, we performed methylation kinetics using 0.25  $\mu$ M of the biotinylated 1-site and 2-sites substrates (1-site substrate: GAA GCT GGA CAG TAC **GTC** AAG AGA GTG CAA, 2-sites substrate: GGA CAG TAC **GTC** AAG CAG TAC **GTC** AAG AGA).

These 30mer substrates contain either one or two CpG sites in the same flanking sequence context. Dnmt3a-C was used at concentrations of 2, 1, 0.5, 0.25, and 0.0625  $\mu$ M. Each methylation reaction was started by addition of AdoMet to a final concentration of 1.5  $\mu$ M consisting of a 1:1 mixture of radioactively labeled and unlabeled AdoMet. Aliquots were taken after 60 minutes and the total methylation measured. Control experiments with M.SssI were conducted using 1  $\mu$ M of M.SssI and a final concentration of 5  $\mu$ M AdoMet consisting of a 1:4 mixture of radioactively labeled and unlabeled AdoMet.

*Bisulfite sequencing DNA methylation experiments*

– Bisulfite DNA methylation studies were carried out using the 509mer DNA fragment. Methylation reactions was carried out in methylation buffer containing 0.05 mg/ml BSA using 100 nM substrate (509mer) and Dnmt3a-C wt and/or mutant in concentrations as indicated in the main text. Control reactions using same amounts of the enzyme storage buffer but no enzyme were included to confirm the completeness of the bisulfite conversion. The samples were incubated for 25 min at room temperature and the methylation reaction was started by the addition of AdoMet to a final concentration of 0.32 mM. The reactions were stopped after 30, 60 or 90 min by flash-freezing in liquid nitrogen. Bisulfite conversion was performed as described (31) using primers specific for the converted DNA (GTG GGA TTT GGT TTT GTT TTG TAT TTT and GAA TAT TAC TAC TAC CCT CCT CCT TCT CAA TTT AAC). After PCR amplification, the converted DNA molecules were subcloned using StrataClone PCR cloning Kit (Stratagene) and individual clones were sequenced.

The sequencing results were analyzed using BISMA (32).

The sequence of the 509mer used for the radioactive and bisulfite DNA methylation analysis is given in the next paragraph. The primer binding sites used to amplify the fragment and to retrieve DNA after bisulfite conversion are colored black and dark grey, respectively. The region enclosed by the bisulfite primers contains 56 CpG sites, the methylation of which was analyzed (highlighted in light grey).

AGATTAGGGAAGGGGGTGTGCGTGAGGGGATCGTG  
 GGACCTGGTTCTGCCCTGCACTCCCCACAGAAAGC  
 GGCTGTATTGCAAAGGCCGCTCCGGCAGCGCACGGC  
 CAAGCGCGGTGTGCGGACCCGACAGGGCTGCGGCTC  
 GCCATGAAATCCCTCAAGTCTCCCCTCAGGGAACGCT  
 GAAGCCGCGCCACGCCCCCGTCTTACCAGTCCGGA  
 TCAGCTGCTGTTGCGGAGCTGCCGGCCACGCACCAG  
 CCCCAGAGGCGCTCCCGGGGCACAGCCGGCGGCGA  
 CTACGCCTCTCAGGCCCGCGCCGCGCCGACGCGC  
 ACGCTCCACACGCGCGCGTCCAGTGGAGACCTGCG  
 ATTGGCTGCCAGGTGCCGGCGGAGATCGGCGCGG  
 CTCCGAGCTAGGAGCATGCGCGGCTCTGACGCCCC  
 TGGTGGCGACGGCTGGACGCGGGGTTAAATTGAGA  
 AGGAGGAGGGCAGCAGCAATACCCCTGAGGCCTTG  
 AAAGGATCTT

*DNA binding experiments* - Equilibrium binding of Dnmt3a to Cy5-labeled oligonucleotide substrates was studied by fluorescence anisotropy, basically as described (14). Two Cy5 labelled 29mer DNA substrates were used for the DNA binding studies, one containing two CpG sites (5'-ACT TGC AAC GGT CCT AAC CGT CAC CTC TT-3') and one without CpG sites (5'-ACT TGC AAC AGT CCT AAC ATT CAC CTC TT-3'). The binding reactions were carried out in binding buffer containing 20 mM HEPES pH 7.5, 100 mM KCl, 1 mM EDTA, 2 nM of Cy5-labeled DNA and increasing concentration of protein after incubation at room

temperature for 5 min. Each data point was measured in triplicate and the average anisotropy values were taken for the analysis. Data were least squares fitted using a cooperative binding model to determine the dissociation constant ( $K_D$ ) and Hill coefficient ( $n$ ) of DNA binding.

*Western blot of Dnmt3a in mouse ES cells-* Mouse E14 embryonic stem cells (a kind gift from Wolf Reik's lab, Babraham Institute, UK) were grown feeder-free at 37 °C and 5% CO<sub>2</sub> in complete ES medium (DMEM 4.5 g/l glucose, 4 mM L-glutamine and 110 mg/l sodium pyruvate, 15% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, 0.1 mM non-essential amino acids, 50 µM β-mercaptoethanol, 10<sup>3</sup> U LIF ESGRO). 2.8x10<sup>6</sup> cells were collected and the cell pellet was lysed in buffer (50 mM Tris pH 8.0, 300 mM NaCl, 1 mM EDTA, 1% TritonX and 0.1% SDS), sonicated on ice for 4 min (with 30 s on/off duty cycles) and centrifuged at 16,000 x g for 10 min, yielding a soluble protein extract and the pellet. The pellet was resuspended in the SDS PAGE loading buffer and heated up at 95°C for 10 min. The amounts of protein extract corresponding to 0.8x10<sup>6</sup> or 1.5x10<sup>6</sup> of cells, as well as the pellet fraction corresponding to 0.8x10<sup>6</sup> of cells were separated on an 8% SDS-PAGE. 100 ng and 50 ng of recombinant Dnmt3a2-His purified from *E. coli* were loaded in parallel and served as a reference for calculation. The proteins were transferred to a nitrocellulose membrane, blocked with 5% milk in TBST and incubated with the monoclonal anti-Dnmt3a antibody from IMG-268A clone 64B1446 (Novus Biologicals) in a dilution of 1:250 ON at 4°C, followed by washing, 1h incubation with the anti-mouse secondary antibody coupled to

horseradish peroxidase (GE Healthcare) (dilution: 1:5000) at RT and washing in TBST. The Western blot was developed using Western Lightening Ultra ECL reagent from Perkin Elmer and the image captured with Fusion Advance detection system (Pepqab). Western blots with recombinant Dnmt3a and Dnmt3b catalytic domains showed that the primary antibody detects both proteins. The intensities of the bands were extracted using Image J and the antibody signals for the Dnmt3a and Dnmt3b from ES cells were compared with the signals obtained for purified Dnmt3a2-His. The pellet fraction yielded a signal that was twice as strong as the signal obtained in the corresponding amount of soluble protein. The volume of the ES cell nucleus was calculated assuming a radius of 10 µm.

## RESULTS

As described in the introduction, we wanted to investigate whether multimerization of the catalytic domain of Dnmt3a (Dnmt3a-C) on DNA affects its catalytic properties. Therefore, we aimed to determine the DNA methylation rates on a long substrate, which allows cooperative DNA binding and protein/DNA fiber formation and to compare it with the methylation rate on a short substrate, which does not support fiber formation. Previous biochemical and structural data indicated that one Dnmt3a-C complex (consisting of 4 or more Dnmt3a-C molecules) contacts the DNA with its inner two subunits and methylates cytosine residues in the upper and lower DNA strand located at a distance of 8-10 bps (10,11,14). DNA binding of these Dnmt3a complexes occurs in a tilted fashion, such that adjacent complexes can bind the upper and lower cytosine of one CpG site. Based on these

observations, we used 29mers and 30mers as short substrates, because they provide enough space for unconstrained binding of one or few Dnmt3a complexes, but do not allow for fiber formation. As shown in Fig. 2A and 2B, the binding curves of Dnmt3a-C to 29mer substrates were hyperbolic. Their fitting resulted in Hill coefficients of  $1.3 \pm 0.3$  (SD) for the substrate containing CpG sites and  $1.3 \pm 0.1$  (SD) for the substrate without CpG sites. However, both curves could also be convincingly fitted with a Hill coefficient of 1.0 (as shown in Fig. 2 A and B). Binding was slightly stronger to the oligonucleotide lacking CpG sites, clearly demonstrating that DNA binding by Dnmt3a-C is non-specific. In contrast, as shown previously, binding to a 60mer substrate was cooperative, as indicated by the fact that the binding curve was sigmoidal and could only be fitted with a Hill-coefficient of 2.2 (Fig. 2C) (14). Based on these results, we conclude that binding of Dnmt3a-C to the 29mer DNA shows only a very weak cooperativity, indicating that an oligonucleotide of this size can be used as a reference substrate to study Dnmt3a-C methylation activity with little influence of cooperative DNA binding and multimerization of Dnmt3a-C complexes on the DNA.

*The in vitro catalytic turnover rate of Dnmt3a-C is increased on longer DNA substrates* - Having shown that DNA binding to the short oligonucleotides shows very low cooperativity, we next determined the enzymatic rate of DNA methylation using two biotinylated substrates of different length, a 30-bp oligonucleotide containing one CpG site (at 250 nM) (which has been used as “standard” substrate in many of our studies before) and a 509 bp long substrate, which corresponds to a

part of the CpG island upstream of the human SUHW1 gene and contains 58 CpG sites (at 100 nM). The reaction progress was followed by incorporation of radioactivity from radioactively labelled AdoMet into the DNA. Prior to the start of the methylation reactions, the samples were pre-incubated for 25 min to allow for complex formation between the enzyme and the DNA. As shown in Fig. 1B, the rate of DNA methylation by Dnmt3a-C was significantly faster on the 509mer substrate than on the 30mer under these conditions.

*Higher Dnmt3a-C concentrations lead to an exponential increase in DNA methylation activity* -

We speculated that the difference in the methylation rates between the long and the short substrate might be due to the multimerization of Dnmt3a-C on the long DNA. To further investigate this hypothesis, we measured the methylation rates of both substrates using increasing concentrations of Dnmt3a-C. As shown in Fig. 1C, the methylation rate of the oligonucleotide increased linearly with the enzyme concentration, which was an expected result for a reaction where DNA is not saturated with the enzyme. This condition is fulfilled under our experimental settings, since the DNA provides binding sites for 2-3 Dnmt3a-C complexes (see above), each of which is at least a tetramer (10,12). In contrast, the methylation rate of the long substrate showed a higher order dependence on the enzyme concentration (Fig. 1C and 1D). The more than linear increase in reaction rates for the long substrate is not due to technical problems, like loss of protein in the dilution steps, because with the 30mer in parallel experiments a linear increase of the DNA methylation rate with enzyme concentration was observed. It can be explained by

the cooperative DNA binding of Dnmt3a-C, leading to a sigmoidal DNA binding curve, the first part of which is sampled in our DNA methylation experiments. Therefore, we conclude that multimerization of Dnmt3a-C on the long DNA substrate stimulates the rate of DNA methylation.

*Addition of a catalytically inactive Dnmt3a-C variant increases the rate of DNA methylation of the wild type enzyme* - Based on the finding that addition of more Dnmt3a-C stimulates its rate of DNA methylation due to cooperative DNA binding, we next investigated if addition of a catalytically inactive Dnmt3a-C variant could also have the same effect. To this end, methylation reactions were conducted using either 1  $\mu$ M wt Dnmt3a-C or 1  $\mu$ M wt together with 1  $\mu$ M of an inactive variant of Dnmt3a-C (E752A). This variant carries a Glu to Ala exchange in the ENV motif of Dnmt3a, which is essential for catalysis. Therefore, it has only a very low residual catalytic activity, but its folding and DNA binding are not changed (33,34). As shown in Fig. 3, addition of the mutated E752A Dnmt3a-C protein strongly stimulated the activity of wt Dnmt3a-C on the 509mer, but not on the 30mer. As expected, the E752A mutant itself was almost inactive (Fig. 4A). This strong, almost 4-fold stimulation of the methylation rate of wt Dnmt3a-C on the long substrate is a very striking result, because, intuitively, the inactive mutant would be expected to compete with wt Dnmt3a-C for DNA and AdoMet binding and, consequently, addition of E752A should have reduced the activity of the wild type enzyme. The stimulation of Dnmt3a-C observed here can be easily rationalized in the context of cooperative DNA binding and multimerization of Dnmt3a-C on DNA, because this

reaction is supported by the higher combined concentration of Dnmt3a-C and E752A.

*Multimerization of Dnmt3a-C on DNA reduces its dissociation rate from DNA* - The results described in the last paragraph provided clear evidence that Dnmt3a-C multimerizes on the 509mer substrate after pre-incubation. Formation of stable protein/DNA fibers would be expected to reduce the dissociation rate of Dnmt3a-C from the DNA. To investigate this hypothesis, we conducted pre-incubation/chase experiments with the 30mer oligonucleotide and the 509 bp DNA substrate. Both substrates were used in a biotinylated (substrate) version, which was recovered afterwards on the avidin plate, such that its methylation was detected, and an unbiotinylated (competitor) version of an identical sequence, which was not recovered, such that its methylation was invisible. The experiments were conducted in three parallel setups. In the first setting, the enzyme was pre-incubated with the biotinylated substrate, then the unbiotinylated competitor substrate was added and the methylation reaction was started by addition of AdoMet. In the second setting, pre-incubation was done in the opposite order. In the third setting, the enzyme was pre-incubated without any substrate and both substrates were added together and the reaction started immediately by adding AdoMet. Importantly, at the start of the reaction, the composition of the reaction mixtures was identical in all three settings. The initial reaction rates were determined over 16 min (Fig. 5). With the oligonucleotide substrate, the methylation rates of the biotinylated substrates were almost indistinguishable in all pre-incubation setups. This indicates a rapid equilibrium of binding of the



enzyme to both substrates. The methylation rate was slightly lower if the enzyme was pre-incubated without DNA, which may indicate that the enzyme is stabilized by DNA during the pre-incubation phase. However, the methylation rate of the biotinylated long substrate was about 3 fold higher after its pre-incubation with the enzyme, indicating that the binding of enzyme molecules on the DNA accelerated the methylation of the biotinylated DNA. In contrast, pre-incubation with the unbiotinylated substrate reduced the methylation rate of the biotinylated DNA, indicating that some enzyme molecules were trapped on the competitor substrate. This suggests that the pre-formed enzyme-DNA fibers are stable on long DNA substrates. In contrast, enzyme-DNA complexes formed on the 30mer substrate, which does not provide enough space for fiber formation, are not stable.

*Processivity is not detected at low enzyme concentrations* - All results presented so far indicate that multimerization of Dnmt3a-C on DNA occurs at low  $\mu\text{M}$  protein concentrations. Since the multimerisation would obviously prevent a processive reaction, in which one enzyme complex moves along the DNA and conducts several successive turnovers, we wanted to study, if processivity of Dnmt3a-C could be detected at very low enzyme concentrations, where not more than one enzyme complex is bound to each DNA molecule. To this end, we used two different substrates, one with one CpG site and the other with two CpG sites (all in an identical flank context) and measured their methylation at different Dnmt3a-C concentrations. In case of a processive reaction, the methylation of the second target site in the two site

substrate should be faster than the first, resulting in an increased rate of methyl group transfer to the two sites substrate. The experiment was carried out using 0.25  $\mu\text{M}$  substrate (0.5  $\mu\text{M}$  target cytosines in case of one site and 1  $\mu\text{M}$  target cytosine with the two sites substrate). Dnmt3a-C was used at 2  $\mu\text{M}$ , 1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 0.25  $\mu\text{M}$  and 0.0625  $\mu\text{M}$ , which ensured that at the lower concentrations not more than one enzyme complex was bound to each DNA duplex. After one hour reaction, the methylation was measured and compared between both substrates. As shown in Fig. 6, both substrates were always methylated with the same relative efficiency even at the lowest concentration of Dnmt3a-C, indicating that processivity is undetectable. This result is in full agreement with previous results obtained at high concentrations of Dnmt3a-C using substrates with different flanking sequences (35).

*Bisulfite methylation analysis supports multimerization of Dnmt3a on DNA* - Next, we wanted to investigate the distribution of methylation events on the 509 bp DNA molecule at a single site resolution. To this end, we methylated the 509 substrate using 1  $\mu\text{M}$  and 2  $\mu\text{M}$  Dnmt3a-C (1W and 2W) and a mixture of 1  $\mu\text{M}$  wt Dnmt3a-C plus 1  $\mu\text{M}$  of the Dnmt3a-C E752A mutant (1W1M). The enzymes were pre-incubated with the DNA for 25 min and the methylation reactions started by addition of AdoMet. Samples were taken after 30 min, 1 h and 2 h, the DNA was converted by bisulfite treatment, PCR amplified, sub-cloned and individual clones were sequenced (Fig. 7 and 8A). The overall methylation rate calculated based on the methylation levels observed after 30 and 60 min was 0.64  $\text{h}^{-1}$  in the 1W sample. With 2  $\mu\text{M}$  Dnmt3a-C the methylation rate based on the methylation

observed after 30 min, was  $3.0 \text{ h}^{-1}$ . Hence, the enzyme showed an about fivefold higher activity when comparing the 1W and 2W samples (with twofold increase in enzyme concentration). This more than linear increase of activity with increasing enzyme concentrations is similar as observed before in the radioactive methylation reactions. The addition of  $1 \mu\text{M}$  E752A variant to  $1 \mu\text{M}$  of wt Dnmt3a-C resulted in an increase of the methylation rate to  $1.6 \text{ h}^{-1}$ , indicating that the catalytically inactive variant stimulated the activity of the wild type enzyme, again similarly as observed in the radioactive methylation kinetics. This effect cannot be due to the residual activity of E752A in this experiment, because we have confirmed that this variant is almost inactive in the bisulfite methylation analysis as well (Fig. 4B).

*DNA methylation patterns observed after methylation of the 509mer illustrate the mechanism of DNA methylation in Dnmt3a-DNA fibers* - In the bisulfite conversion assays coupled to sequencing of individual clones, each clone reflects the methylation pattern of one DNA molecule in the reaction mixture. The general methylation patterns observed with wt Dnmt3a-C here are similar to what has been reported previously by us and others for Dnmt3a-C and full length Dnmt3a (10,36). To follow the cooperative DNA binding of Dnmt3a-C, we analyzed the number of methylation events in each clone and fitted the resulting distributions to the sum of two binomial distribution curves. As shown in Fig. 8B, the distribution of methylation levels in the 1W sample was dominated by one component with an average methylation of 16%, which contributed about 74% to the overall distribution. These clones correspond to the DNA

molecules, which experienced a low stochastic methylation, indicative of a dynamic association and dissociation of individual enzyme complexes or formation of small unstable oligomers. A second component showed an average methylation of 37%, which we attribute to larger multimers formed on the DNA, which, however, did not completely cover the DNA (“partial fibers”). In the 2W sample (Fig. 8C), the low methylation fraction was lost, but two major populations of DNA molecules with medium and high average methylation levels were detected. One population showed a methylation of about 48%, corresponding to the partial fibers seen before. In addition, a novel fraction of DNAs with a high methylation average of 73% was found, which we attribute to DNA molecules highly occupied by Dnmt3a-C (“long fibers”). Due to the cooperativity in DNA binding, full fibers only appear if the concentration of free enzyme was high enough. Formation of a stable protein/DNA fiber increases the residence time of Dnmt3a-C on this DNA molecule, leading to the strong increase in methylation with increasing concentration of Dnmt3a-C.

In the 1W1M sample (Fig. 8D), the same two populations of full and partial fibers appeared. The full fiber population showed 54% average methylation and the partial fibers 24%. This result illustrates the mechanism of the stimulation of DNA methylation by the addition of the inactive variant. Due to the increased overall concentration of Dnmt3a-C (wt plus mutant, which both equally bind DNA and multimerize on DNA), protein/DNA fiber formation is stimulated when compared with the reaction with only  $1 \mu\text{M}$  of wild type Dnmt3a-C. Of note, the methylation levels of the full and partial

fibers were reduced in 1W1M, as compared to 2W, which can be explained by the statistical incorporation of the inactive variant into the protein/DNA fiber, which blocks some of the CpG target sites from methylation. In the case of partial fibers, the methylation dropped from 48% in 2W to 24% in 1W1M, which is exactly what would be expected on the basis of the 1:1 stoichiometry of active and inactive Dnmt3a-C molecules in the 1W1M sample. In the case of the full fibers, methylation dropped from 73% to 54%, which can be explained because the methylation of full fibers already reached saturation in 2W.

These data are in full agreement with the radioactive methylation data. Below a certain critical enzyme concentration (which is around 1  $\mu$ M under our conditions), individual enzyme complexes bind to the DNA and eventually form small fibers, which are not stable. Above the critical Dnmt3a concentration, stable protein/DNA fibers are formed. In the protein/DNA fiber, methylation reactions are catalyzed by individual active sites in a stochastic manner, based on the access of individual active centers to the target sites and the flanking sequence of the target site. Most of the fibers showed methylation levels of 60-70% after long incubation with enzyme. The lack of full substrate methylation could be due to the fact that the conformational dynamics of the fibers allow the active sites of the methyltransferase to reach most of the CpG target sites, but not all. Alternatively, it can be explained by the strong flanking sequence preferences of Dnmt3a-C (36-39). Because of this, CpG sites situated in an unfavorable flanking sequence context are almost refractory to methylation, which may also explain why some

sites on the long DNA substrate always remained unmethylated.

*Estimation of the nuclear content of Dnmt3 enzymes in ES cells* – In the light of the concentration dependent multimerization of Dnmt3a on DNA, we were interested to estimate the concentration of Dnmt3a and Dnmt3b in mouse embryonic stem (ES) cell nuclei, where these enzymes catalyze *de novo* methylation of DNA. To this end, we performed western blots using ES cell lysates and compared the intensity of the antibody signal to the signal obtained with purified recombinant Dnmt3a2 (Fig. 9). Assuming a nuclear radius of an ES cell of 10  $\mu$ m, this experiment provided an estimate of the nuclear concentration of Dnmt3 enzymes of about 4  $\mu$ M. Although this number must be considered a rough estimate, it indicates that the multimerization observed in this study occurs at enzyme concentrations that are within the relevant cellular range.

## DISCUSSION

The exact mechanisms, by which DNA methylation pattern are established during development and altered in cancer remain a fundamental unanswered question. For this, a detailed functional understanding of the mechanism of the Dnmt3a DNA methyltransferase, which is a key enzyme involved in this process, is needed. Up to date two very different views on the mode of action of Dnmt3a were described in the literature. On one hand, using a variety of techniques, including gel shift DNA binding experiments (10,11), fluorescence polarization equilibrium DNA binding experiments (14), AFM experiments (14)

and catalytic studies on a multimeric substrate (11,14) our group observed that Dnmt3a binds to DNA in a cooperative manner and forms stable protein/DNA fibers. On the other hand, the group of Reich and coworkers published that Dnmt3a methylates DNA in a processive reaction (25-27). Since these models are mutually exclusive, because a processive mechanism implies that individual enzyme complexes move along the DNA undergoing multiple turnovers, we wanted to further investigate the mechanism of Dnmt3a here. We conducted several dedicated experiments, which determined the activity of Dnmt3a at different concentrations on long and short DNA substrates and in the presence of a catalytically inactive Dnmt3a variant, which were designed to decide which of these two models describes experimental evidence better.

*Different methylation experiment did not provide evidence of a processive DNA methylation* - We have shown here that Dnmt3a-C is more active on a longer DNA substrate, where DNA binding can occur cooperatively, than on a short oligonucleotide substrate. In principle, such stimulation could also arise from a processive mechanism, because the turnover of many enzymes acting on DNA is limited by product release. In such situation, a processive enzyme (which does not have to release the bound DNA) can be much faster than a non-processive one. Therefore, it would be expected that the catalytic rates on substrates with multiple sites should be higher than on a substrate with a single site. However, early studies with Dnmt3a had already shown that this is not the case for this enzyme. Reactions with oligonucleotides, which contained one or two identical methylation sites

showed that both substrates were methylated with almost identical rates over 60 minutes (35). We have performed similar experiments here using a wide range of enzyme concentrations and shown that even at low concentrations of Dnmt3a, where only one enzyme complex is bound to a substrate DNA, methylation rates of these substrates were identical. This result does not rule out that the Dnmt3a-C complex stays bound to the DNA after one turnover, moves around and later methylates the second target site. However, it does show that the rate of the second methylation event cannot be massively higher than the methylation during the first turnover.

Furthermore, methylation patterns generated by Dnmt3a provided additional evidence against a processive mechanism: A “classical” processive DNA methyltransferase would typically generate characteristic methylation patterns, consisting of a mixture of molecules with high methylation coexisting with completely unmethylated DNA molecules. This is due to the fact that a processive enzyme will stay on one DNA molecule and subsequently methylate several sites on it, while other DNA molecules which do not carry a bound methyltransferase will remain unmethylated. Several methylation studies on longer DNA substrates were conducted with Dnmt3a or Dnmt3a-C to detect such patterns, but they did not provide any evidence in favor of a processive mechanism for this enzyme (6,10,11,35). Another characteristic of processive DNA methyltransferases is that continuous stretches of methylated sites are formed (see for example 24). This has never been observed with Dnmt3a, despite several bisulfite analyses carried out in our lab,

which included the methylation of >1000 individual clones so far (see this work and (10-12,14)). However, due to the low activity of Dnmt3a and Dnmt3a-C, all these experiments were conducted at high enzyme concentrations, such that protein/DNA fiber formation could have prevented an enzyme complex from moving along the DNA and undergoing multiple turnovers, such that these negative results do not formally exclude a processive mechanism.

In summary, the increased methylation rate of long substrates observed here can be connected to the cooperative DNA binding of Dnmt3a, but not to the processive methylation of several sites on the substrate. In addition, several experiments aiming to detect processive DNA methylation by Dnmt3a failed to provide evidence of an individual enzyme complex moving along the DNA and undergoing several successive turnovers.

*Stimulation of DNA methylation by increasing concentration of Dnmt3a* - To understand the mechanism underlying the increased methylation of the long substrate, we studied the dependence of the methylation rate of the long and short substrates on the Dnmt3a-C concentration. In these experiments we observed a strong and more than linear increase in methylation rates on the long substrate with increasing concentration of Dnmt3a-C, both in the radioactive kinetics and in the bisulfite methylation studies, which we attribute to cooperative DNA binding and formation of stable protein/DNA fibers. A sigmoidal concentration dependence of Dnmt3a activity was also observed at lower enzyme concentrations (26), where it was interpreted as a reflection of the dimer/tetramer equilibrium of Dnmt3a. However, we observed a non-linear

increase in reaction rates with the 509mer DNA substrate distinctively, but not with the 30mer oligonucleotide, where DNA binding is not cooperative and a stable protein/DNA fiber is not formed. Such results cannot be interpreted by the interference with the dimer/tetramer formation, because this would equally affect the methylation of the long and short substrate. Our interpretation was further confirmed by pre-incubation/chase experiments. While pre-incubation of the 509mer DNA with Dnmt3a-C led to a strong increase in its methylation, such effect was not observed with the 30mer, indicating again that stable protein/DNA fibers are formed on the 509mer, but not on the 30mer.

*Chase of DNA methylation experiments* - The conclusion that Dnmt3a is a processive enzyme was based on a single type of experiments conducted by Holz-Schietinger & Reich (25), which were later reproduced by the same group (26,27). In the initial study (25), methylation reactions were conducted with different long DNA substrates for up to 2 hours. After 20 minutes of methylation, an excess of a plasmid not containing CpG target sites was added in one reaction (chase), while a parallel reaction was continued without adding the chase substrate. The authors observed that the chase did not cause a rapid stop of the methylation of the primary substrate, which they interpreted as an evidence of a processive methylation. As a control, reactions were conducted, in which the chase substrate was added at the beginning and strongly reduced methylation rates were observed. These observations clearly show that the dissociation of Dnmt3a from DNA must be very slow, which is in agreement with the results of our premixing methylation experiments.

Of note, in our experiments only a single turnover was observed, such that the effects can only be connected to a slow release of Dnmt3a-C, but not to a potential processivity in DNA methylation. Based on their calibration of the radioactive assay and measurement of the enzyme concentration, Holz-Schietinger & Reich (2010) concluded that several turnovers took place during the chase period, which would be indicative of a processive methylation. Our results are not in agreement with multiple processive turnovers of Dnmt3a-C on one DNA substrate with two CpG sites within the time scale of 1 hour.

Interestingly, the same paper Holz-Schietinger & Reich reported that the apparent processivity of Dnmt3a was only observed after pre-incubation of the enzyme with DNA (25), which cannot be easily interpreted in a conceptual framework of a processive turnover. In the context of our multimerization model, however, this result reflects the fact that pre-incubation allows the formation of stable Dnmt3a fibers on the DNA.

*Stimulation of DNA methylation by addition of the catalytically inactive Dnmt3a variant* - In order to set up an experiment, which can clearly discriminate between the cooperative formation of the protein/DNA fiber and individual Dnmt3a complexes moving along the DNA and undergoing several processive turnovers, we conducted DNA methylation experiments with Dnmt3a-C in the presence of the catalytically inactive Dnmt3a-C E752A variant. Our data show that addition of the inactive variant led to a significant stimulation of the activity of Dnmt3a-C on the long DNA substrate in both radioactive kinetics and methylation reactions followed by bisulfite-sequencing. In

contrast, methylation of the 30mer substrate was not stimulated by the addition of the E752A variant. This variant contains an exchange of a critical amino acid residue in the catalytic center of the enzyme. Consequently, it has a strongly reduced activity, but it is correctly folded and its binding to DNA is unaffected (33,34). Hence, it can be incorporated into Dnmt3a-C protein fibers on the DNA and contribute to the cooperative DNA binding. Thereby, it can increase the DNA methylation rate of Dnmt3a-C although its catalytic center is almost inactive. We could not find a plausible explanation for the stimulation of Dnmt3a by addition of the inactive variant in the conceptual framework of the processive methylation model.

*DNA methylation patterns observed after addition of a catalytically inactive Dnmt3a variant* - The analysis of methylation patterns in the bisulfite sequencing experiments obtained for Dnmt3a-C wt alone at 1 $\mu$ M (1W) and 2  $\mu$ M (2W) and for a 1:1 mixture of Dnmt3a-C wt and its inactive E752A mutant (1W1M) provides a final evidence in favor of the cooperative fiber model. If the individual Dnmt3a enzyme complexes moved freely on the DNA and methylated DNA processively, the results for the 1W and 1W1M samples should be similar, because additional inactive enzymes moving on the DNA should not influence the active enzyme molecules, as long as binding sites are in large excess, which is the case in our experiment. However, in case of the cooperative fiber formation, the general distributions of fibers should be similar in the 2W and 1W1M samples, because the overall protein concentration is identical. Still, since the 1W1M fiber will contain 50% inactive enzymes, the methylation levels of fibers in the 1W1M sample are

expected to be about two fold lower, as compared to the 2W sample. Clearly, our data are not compatible with the processive enzyme model, because the results obtained with 1W and 1W1M samples were completely different. In contrast, the results obtained with 1W1M and 2W were very similar, because in both cases incomplete and full fibers were observed and the methylation levels of the fibers were approximately half in the case of 1W1M. Hence, the methylation patterns observed in the presence of the E752A mutant strongly support the model of cooperative fiber formation and exclude a processive enzyme model.

*Mechanism of the R882H Dnmt3a somatic cancer mutant* – Understanding the catalytic mechanism of Dnmt3a is very relevant from a biomedical perspective. In some forms of leukemia, Dnmt3a is a hotspot of somatic cancer mutations, the most prominent of which is R882H in the DNA binding interface (40). Holz-Schietinger & Reich (2011 and 2012) have studied the kinetics of DNA methylation by the R882H variant in chase experiments and showed that the chase with a competitor substrate led to a stronger decline in catalytic activity when compared with the wild-type Dnmt3a (26,27). This observation was interpreted as an indication of a loss of processive DNA methylation. Based on our data, we propose as an alternative interpretation that the R882H mutation reduces the stability of DNA binding by Dnmt3a similarly as observed previously for the corresponding exchange to

alanine (34). Recent publications indicated that this mutation influences the tetramer formation of Dnmt3a (41,42).

## CONCLUSIONS

In summary, our data are fully consistent with a cooperative DNA binding and fiber formation model of Dnmt3a, but not with a processive DNA methylation model. The DNA methylation of Dnmt3a-C is initiated by a cooperative DNA binding reaction. Below a critical enzyme concentration, individual enzyme complexes will associate to the DNA and may form small oligomers, but they are not stably bound. At higher concentration of Dnmt3a, which are in the range of the nuclear concentration of Dnmt3 enzymes in ES cells, stable protein/DNA fibers are formed in a cooperative reaction. In the protein/DNA fiber, methylation of individual active sites occurs stochastically, depending on the specific flanking sequences of the target sites and their access to the individual active centers. It will be interesting to investigate, if fiber formation enhances catalysis mainly by increasing the residence time of the enzyme at its target site, or whether it induces a conformational change of the enzyme that increases the catalytic activity. The stimulation of Dnmt3a's catalytic activity by multimerisation on DNA suggests that this process could contribute to the efficient methylation of CpG rich DNA loci in cells.

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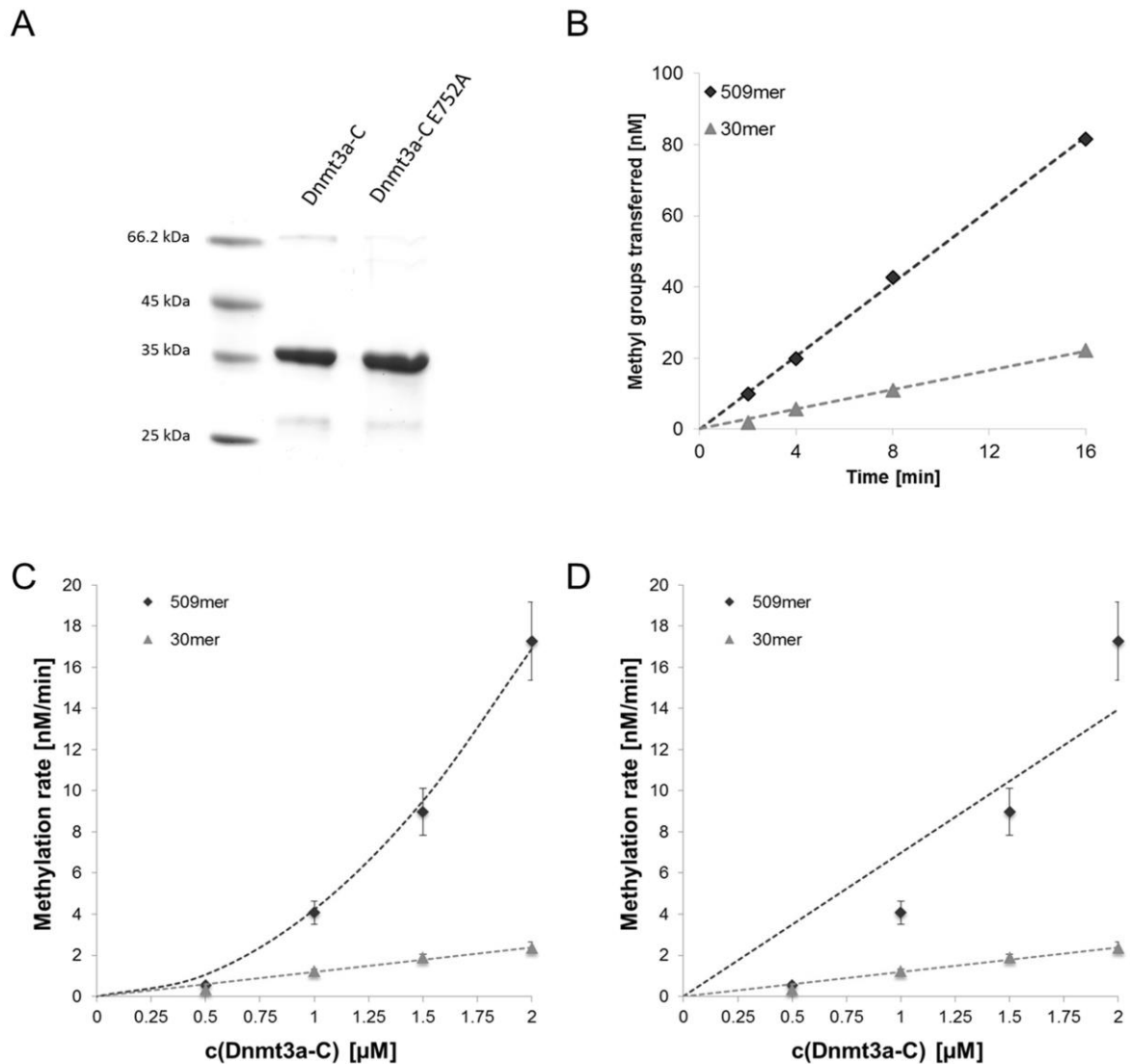
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*Acknowledgements* – We are grateful to Dr. Wolf Reik and Dr. Ferdinand von Meyenn for help with the ES cell work.

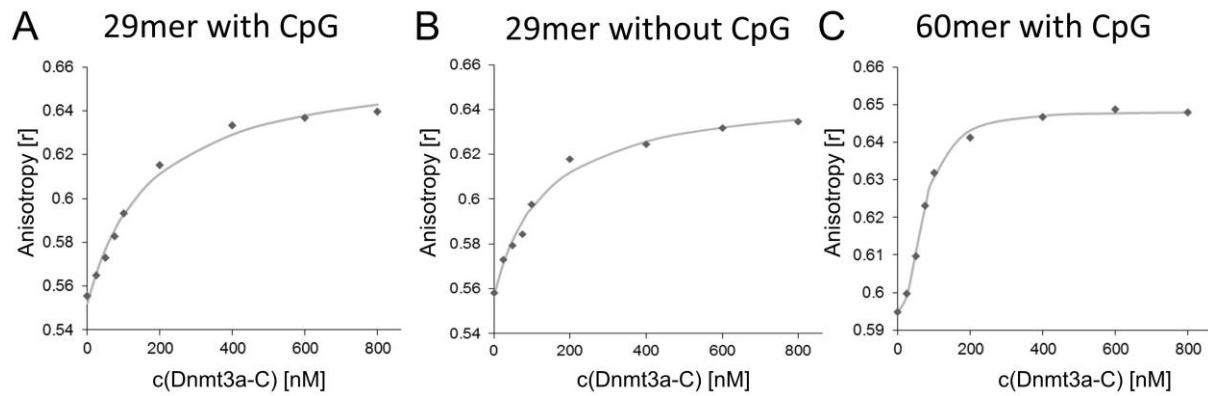
## FOOTNOTES

\*This work was supported by DFG grant JE 252/10 to A.J. and the BMBF to R.R. FKZ 01GS0805.

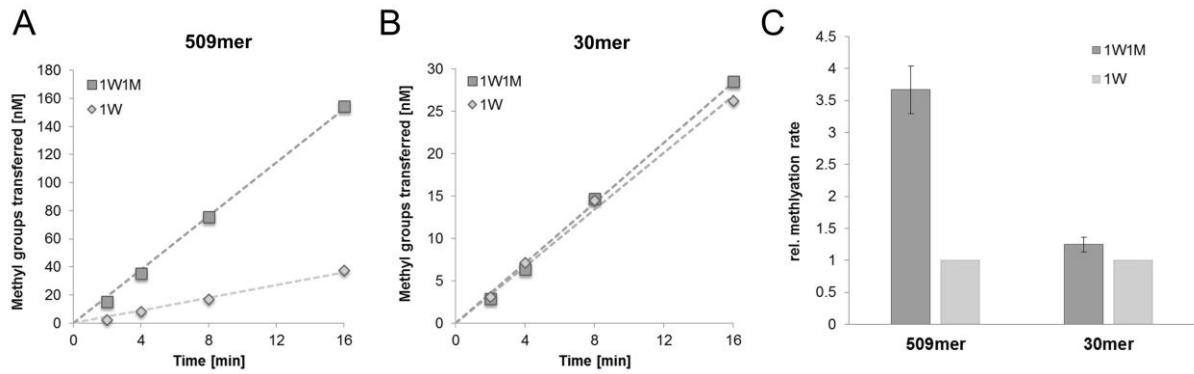
FIGURES AND FIGURE LEGENDS



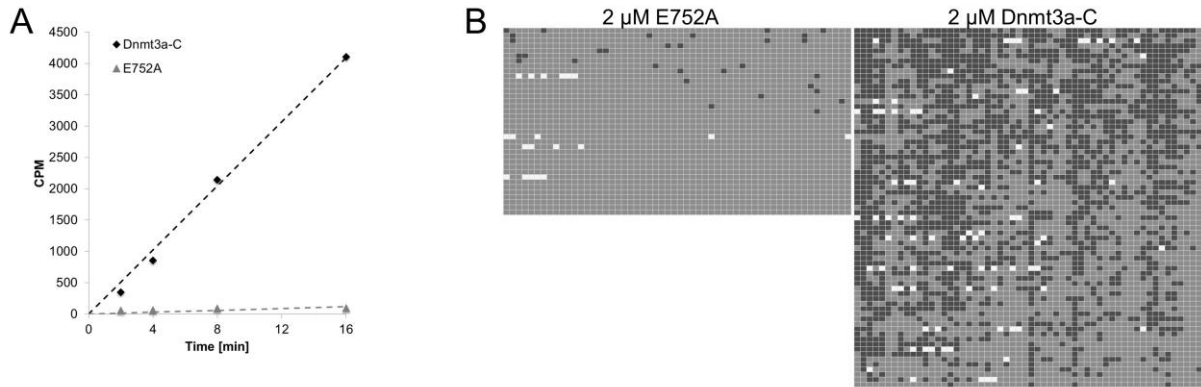
**Figure 1.** Catalytic activity of Dnmt3a-C on the 509mer long DNA substrate and on the 30mer oligonucleotide. A) Representative protein preparations used in this study. The image shows a Coomassie blue stained SDS gel loaded with purified Dnmt3a-C and E752A mutant. B) Example kinetics showing the methylation of 509mer (0.1 μM) (dark grey diamonds) and 30mer (0.25 μM) (light grey triangles) with 1 μM Dnmt3a-C. C) Methylation of the 509mer (0.1 μM) (dark grey diamonds) and 30mer (0.25 μM) (light grey triangles) using different concentrations of Dnmt3a-C. All reactions were pre-incubated for 25 min at room temperature prior to the start of the methylation rates of the 30mer. Average data of three independent experiments are shown, the error bars indicate the SEM (standard error of the mean). The light grey line shows a linear regression of the 30mer methylation rates. The dark grey line, included for illustration, shows a second order polynomial fit of the 509mer methylation rates. D) The 509mer methylation rates could not be fitted by linear regression (dark grey line).



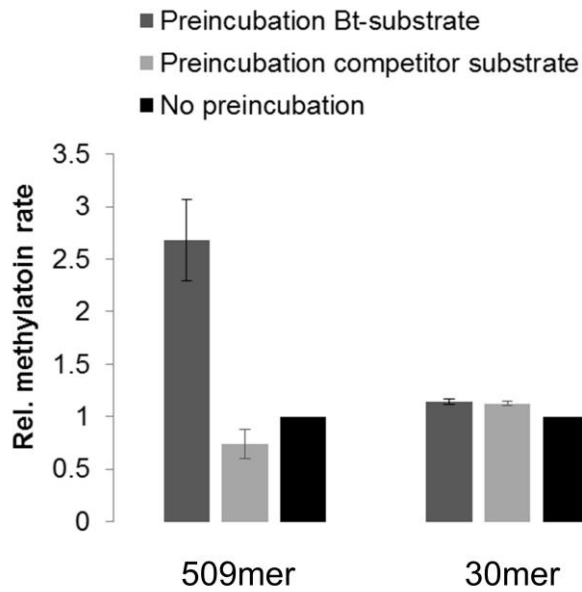
**Figure 2.** DNA binding of Dnmt3a-C studied by fluorescence depolarization. Binding was studied using 29mer oligonucleotides containing 2 CpG sites (A) or no CpG site (B) and a 60mer (C data were taken from (14)). Data were fitted to a binary binding equilibrium (A and B) and to the Hill equation (C). The derived binding constants based on  $n=1$  were  $183 (\pm 19 \text{ SD})$  nM for A,  $137 (\pm 5 \text{ SD})$  nM for B and  $86 (\pm 15 \text{ SD})$  nM for C, respectively. The Hill coefficient for binding of the 60mer was  $2.2 (\pm 0.1 \text{ SD})$ . The figure shows the average data of two repeats of the experiment, each individual measurement was done in triplicate. SD: standard deviation.



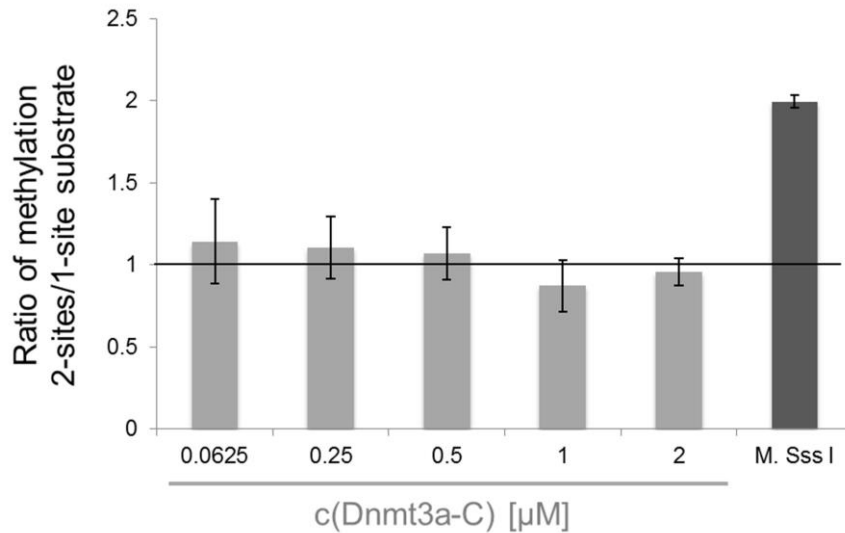
**Figure 3.** Stimulation of DNA methylation of Dnmt3a-C wt by the addition of the catalytically inactive Dnmt3a-C E752A variant. Panels A and B show examples of methylation reactions conducted using 1  $\mu$ M Dnmt3a-C alone (light grey diamonds) or with the addition of 1  $\mu$ M of E752 (dark grey squares) using 100 nM 509mer (A) or 1  $\mu$ M 30mer (B). The lines indicate linear regression fits. C) Summary of three independent experiments. Data were normalized to the respective rates measured in the absence of E752A and averaged. The error bars indicate the SEM.



**Figure 4.** The Dnmt3a-C E752A variant is catalytically almost inactive. A) Results of methylation kinetics with the 509mer using radioactive AdoMet and 1  $\mu$ M of Dnmt3a-C or 1  $\mu$ M E752A. B) Results of a bisulfite methylation analysis conducted with 2  $\mu$ M Dnmt3a-C or 2  $\mu$ M E752A. Each column represents a CpG site, each line represents one clone. Dark grey squares indicate methylation, light grey squares absence of methylation and white squares the sites where the methylation could not be resolved due to sequencing problems. These experiments were carried out side by side, the Dnmt3a-C data are included in the overall analysis presented in Fig. 7. In both cases the residual activity of the E752A variant is very low, although clearly above the background of the corresponding methods.

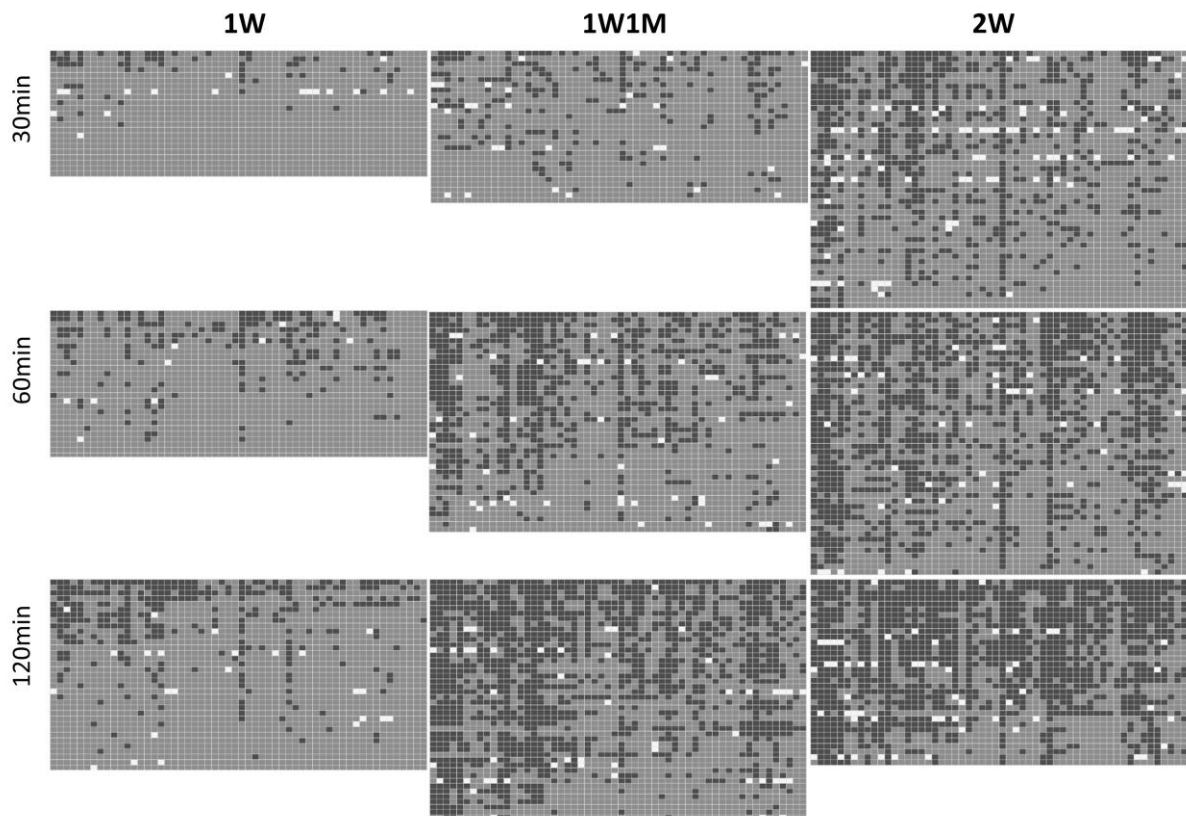


**Figure 5.** Activity of Dnmt3a-C on the 509mer (100 nM) and 30mer (2  $\mu$ M) substrates after pre-incubation and chase with competitor substrate. All reaction mixtures contained half of the substrate in a biotinylated form and the other half unbiotinylated. They were carried out in three settings: i) the enzyme was pre-incubated with the biotinylated substrate and afterwards the unbiotinylated competitor substrate was added and the reaction was started by addition of radioactively labelled AdoMet (dark grey), ii) the pre-incubation was done with the unbiotinylated competitor substrate and the biotinylated substrate was added later and the reaction was started by addition of  $^3\text{H}$ -labelled AdoMet (light grey), or iii) the enzyme was incubated alone for 25min and both substrate were added together and the reaction was started by addition of  $^3\text{H}$ -labelled AdoMet (black). The figure shows averages of 4 independent experiments with the 509mer and 2 with the 30mer. The error bars indicate the SEM.

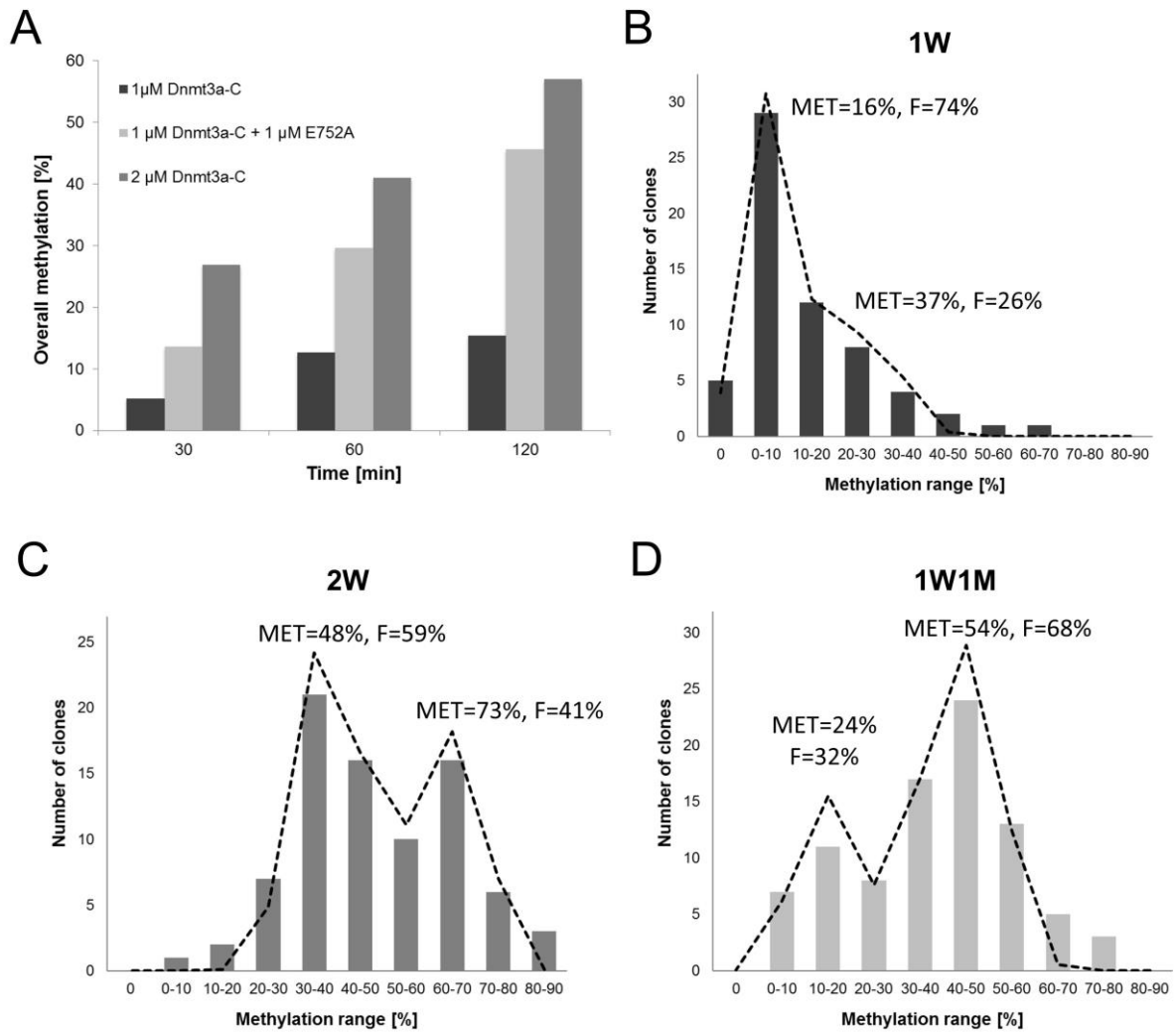


**Figure 6.** Methylation of the 1-site and 2-sites substrates at different concentrations of Dnmt3a-C. All reactions contained 0.25 µM oligonucleotide substrate, 1.5 µM AdoMet (0.75 µM radioactively labelled AdoMet and 0.75 µM unlabeled AdoMet), and Dnmt3a-C as indicated. Samples were incubated for 60 min and the final incorporation of radioactivity determined. The figure shows averages of 5 independent experiments. The error bars indicate the SEM. A control experiment with M.SssI confirmed a twofold higher incorporation of radioactivity into the 2-sites substrate when compared to the 1-site substrate (dark grey bar).

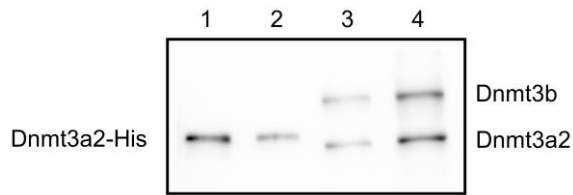




**Figure 7.** DNA methylation of the 509mer substrate analyzed after bisulfite conversion and sequencing of individual clones. Each column represents a CpG site, each line represents one clone. Dark grey squares indicate methylation, light grey squares absence of methylation and white squares the sites where the methylation could not be resolved due to sequencing problems. Methylation reactions were conducted using 1  $\mu$ M and 2  $\mu$ M wild type Dnmt3a-C (1W and 2W, respectively) and a mixture of 1  $\mu$ M wild type Dnmt3a-C and 1  $\mu$ M of the inactive Dnmt3a-C E752A mutant (1W1M). The time points were 30, 60 and 120 min.



**Figure 8.** Analysis of the Bisulfite DNA methylation experiments using the 509mer substrate as shown in Fig. 7. A) Overall methylation observed after methylation with Dnmt3a-C (1  $\mu$ M), Dnmt3a-C plus E752A (1  $\mu$ M each) or Dnmt3a-C (2  $\mu$ M). B-D) Distribution of the methylation levels observed in the individual clones after methylation with 1  $\mu$ M wild type Dnmt3a-C (1W), 2  $\mu$ M Dnmt3a-C (2W) or 1  $\mu$ M Dnmt3a-C plus 1  $\mu$ M E752A (1W1M). The dashed lines display a fit of the distributions to the sum of two binomial distributions. The average methylation of the individual binomial distributions (MET) in %, and the contribution of this component to the overall distribution (F in %) are indicated.



**Figure 9.** Estimation of the nuclear content of Dnmt3 enzymes in ES cells. Picture of the Western blot experiment with an anti-Dnmt3a antibody raised against the C-terminus of Dnmt3a. The antibody recognizes both Dnmt3a and Dnmt3b. For calibration, 100 ng and 50 ng of the recombinant His-tagged Dnmt3a2 were loaded in lanes 1 and 2, respectively. The amount of lysate corresponding to  $0.8 \times 10^6$  or  $1.5 \times 10^6$  of cells was loaded in lanes 3 and 4, respectively. The concentration of Dnmt3a2 (the isoform of Dnmt3a in ES cells) and Dnmt3b in the cell nucleus was calculated assuming the radius of an ES cell nucleus of 10  $\mu\text{m}$ .

**Cooperative DNA binding and protein/DNA fiber formation increases the activity of the Dnmt3a DNA methyltransferase**

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*J. Biol. Chem.* published online August 21, 2014

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Access the most updated version of this article at doi: [10.1074/jbc.M114.572032](https://doi.org/10.1074/jbc.M114.572032)

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