Supplementary Information

Characterisation of the purified proteins

In order to characterize the nucleosomal binding mode of the de novo methyltransferases, and to study de novo DNA methylation of nucleosomes, the human Dnmt3a and Dnmt3b2 proteins and the chromatin remodeling enzyme Snf2H (WT and KR211 mutant) were tested for the presence of contaminating genomic DNA (Figure 1B, S1B). Flag-tag Snf2H purified from baculovirus infected Sf21 insect cells exhibited no detectable levels of associated DNA (Figure S1B lanes 1-2). In contrast, Dnmt3a and Dnmt3b2 purified via their 6x-His-tags from Sf21 and E. coli cells respectively, showed detectable levels of contaminating DNA (Figure 1B, lanes 1-2). Importantly, we included a second purification step, involving a cation exchange matrix (SP FF, GE Healthcare), to remove the associated DNA (Figure 1B, lanes 3-4). This is a critical purification step, avoiding unspecific methylation of the co-purified DNA, a potential problem in the interpretation of the results of previous studies (1,2).

Figure S1 Protein purification and analysis of DNA content. (A) Snf2H WT (lane 1) and KR211-mutant (lane 2) were purified via affinity chromatography from infected Sf21 insect cells, using the N-terminal Flag-tag. Proteins were subjected to SDS-PAGE and Coomassie Blue staining. Sizes of the molecular weight marker are indicated. (B) recombinant Snf2H wildtype and KR211 (lanes 1 and 2) were analyzed for co-purifying DNA contaminations. 5 µg of each protein preparation was incubated with RNaseA and proteinaseK. The remaining DNA was purified and analyzed by agarose gel electrophoresis and staining with ethidium bromide. Molecular weight marker (M) and sizes are indicated.

Characterisation of the nucleosome Dnmt interactions

We used the highly purified recombinant proteins to study the characteristics of the Dnmt-nucleosome interaction and the role of the DNA linkers (Figure 1A, Figure S2). In agreement with previous findings (1,3), electromobility shift assays revealed that Dnmt3a bound with similar affinity to free DNA and nucleosomes with short DNA linkers (Figure S2A, B, lanes 1-4). However, we clearly demonstrate preferential binding of Dnmt3a to the mononucleosome
77-NPS1-77, showing that Dnmt3a is able to recognize specific nucleosomal structures in addition. In contrast, Dnmt3b2 showed similar affinities towards DNA and mononucleosomes with long DNA linkers (i.e. 77-NPS1-77) (Figure S2A, lanes 5-8). DNA is the preferred substrate for Dnmt3b2, as it binds with decreasing affinity to DNA, nucleosomes with symmetric and long DNA linkers over asymmetric DNA linkers and nucleosomes without DNA overhangs (Figure S2A, B, lanes 5-8). Recently, the ADD-domains of Dnmt3a and Dnmt3b were shown to bind similar well to the non-modified histone H3 tail (4). Albeit the fact that in non-competitive EMSA assays both enzymes bind to nucleosomes (Figure S2C), we qualitatively show that the DNA linkers presented a much better substrate than the H3 tail. Accordingly, the binding characteristics of both proteins are strongly affected by the different DNA binding affinities of the PWWP domains (5,6) and a nucleosomal interface for Dnmt3a.

**Figure S2** Dnmt3a and Dnmt3b2 bind to nucleosomes in vitro. DNA fragments containing the NPS1 sequence located either in the center or close to the DNA border were partially assembled into mononucleosomes with the salt dialysis method. The length of the DNA linkers are indicated. (A) A stochiometric mixture of nucleosomal templates with symmetric DNA linkers (20 nM) was incubated with increasing amounts of Dnmt3a (300-1200 nM, lanes 2 to 4) and Dnmt3b2 (250-1000 nM, lanes 6 to 8). Nucleosome binding reactions were analyzed by native PAA gel electrophoresis (5% PAA and 0.4xTBE). (B) Same experiment as described in (A) but using nucleosomes with asymmetric DNA linkers. (C) Same experiment as described in (A) using 30 nM of a fully reconstituted nucleosome without DNA linkers, representing just the NPS1 sequence. Nucleosomes were incubated with 30-240 nM Dnmt3a (lanes 2 to 4) and 45-320 nM Dnmt3b2 (lanes 6 to 9). (D) Disruption of Dnmt-nucleosome interactions with an excess of competitor DNA. 20 nM of fully reconstituted 40-NPS1 DNA was incubated with 480 nM and 640 nM Dnmt3a and Dnmt3b2 respectively (lanes 2 and 6). Increasing amounts of competitor DNA were added to the Dnmt-nucleosome complexes shortly prior to gel electrophoresis.
loading (lanes 3 to 6 and lanes 7 to 9). The released nucleosomal particles were analyzed by native \( \text{PAA} \) gel electrophoresis as described. The molecular weight marker (M) is indicated.

Furthermore, we show that Dnmt binding to nucleosomes did not lead to histone eviction or nucleosome re-positioning, as the addition of an excess of DNA to Dnmt-nucleosome complexes resulted in binding competition and the release of intact nucleosomes (Figure S2D).

Mapping and quantification of nucleosomal DNA methylation in vitro

For the detailed analysis of DNA methylation, we prepared mononucleosomes of high quality, using the salt dialysis method (Figure 1A, S3A). In contrast to the nucleosomes used in the electromobility shift assays (Figure 1A, S2A, B), histone:DNA ratios were carefully titrated such that DNA was completely reconstituted into mononucleosomes, without the formation of unspecific aggregates (Figure S3A). Nucleosomes were almost exclusively positioned on the NPS2 sequence as visualized by the discrete mononucleosomal band on the native polyacrylamide gel and as verified by a restriction enzyme accessibility assay (Figure S3B).

**Figure S3** Reconstitution and mapping of the nucleosome binding site NPS2 (A) The DNA template C91-NPS2-C104 (342 bp, 27 CpG sites) includes the modified 601 nucleosome positioning sequence NPS2 in the center of the DNA fragment. DNA was reconstituted into mononucleosomes with increasing histone to DNA ratios as indicated. Reconstitution was performed with the salt dialysis method in the presence of the CpG less competitor DNA (pCpGL basic, 250 ng). Nucleosome assembly was monitored by native PAA gel electrophoresis and visualized by ethidium bromide staining. The molecular weight marker is indicated (M). An arrow marks the nucleosomal substrate used for non-radioactive in vitro DNA methylation assays. Asterisks (*) indicate minor nucleosome positions. (B) Although it is highly similar to the 601 sequence (7) we characterized the nucleosome positioning behavior of the NPS2 sequence (C91-NPS2-C104) in detail with an restriction enzyme
protection assay. Partially assembled nucleosomes were digested with the indicated restriction enzymes (RE). Native nucleo-protein complexes (lanes 1-5, I.), or the purified DNA (lanes 6-10, II.) were loaded on a 5% PAA gel. StyI (S.) cuts within the linker region and at a second site within the nucleosome positioning sequence. DNA cleavage gives rise to DNA fragments of 70 bp and 272 bp length if the NPS2 site is protected by a nucleosome. The free DNA is fully cleaved into fragments of 70 bp, 118 bp, 154 bp length. After StyI digestion (I., lane 2) the free DNA of the partially assembled nucleosome is fully hydrolyzed, whereas the nucleosomal StyI-site is protected (* marks the theoretic position of the protected DNA fragment). After proteinaseK treatment, the DNA fragment (*, lane 9) that had been protected from RE digest appears. Smaller fragments are not longer visible due to reduced efficiency in ethanol precipitation. The restriction enzymes NdeI (N.), XbaI (Xb.) and XhoI (Xh.) cut within the linker region and shift the nucleosome to smaller size.

Characterisation of the DNA methylation reactions
In order to characterize the kinetics of the DNA methylation reactions and to choose the optimal conditions, we performed time-course experiments with different templates either as free DNA or reconstituted into mononucleosomes (Figure S4). It is apparent that under the conditions used, the DNA methylation reactions, presented here and in the main text of this manuscript, were not end-point reactions. By comparing the DNA methylation activity on DNA and nucleosomal DNA a DNA:NUC ratio was calculated (Figure S4). This DNA:NUC ratio shows different methylation efficiencies towards the distinct nucleosomal substrates. Strongest repressions of the DNA methylation activity are observed with the CpG containing core nucleosome and the template without CpG sites in the linker DNA.
Figure S4 *In vitro* DNA methylation of nucleosomal templates by Dnmt3a, Dnmt3b2 and M.SssI. Time course of DNA methylation reactions using Dnmt3a, Dnmt3b2 and M.SssI with DNA or mononucleosomes. The following substrates were used: C91-NPS2-C104 (27 CpG sites, 342 bp), N78-NPS2-N79 (15 CpG sites, 304 bp), and NPS2 (15 CpG sites, 150 bp). 100 nM Dnmt3a and Dnmt3b2 were used in a reaction mix containing 200 ng BSA/µl, 480 nM 3H-SAM and DNA substrate with 500 nM CpG sites in 50 µl of Dnmt buffer (20 mM Tris, pH 7.6, 1.0 mM EDTA, 1.0 mM DTT). Reactions containing the bacterial DNA methyltransferase M.SssI (NEB, 4 U) were carried out in 50 mM Tris, pH 8.0, 10 mM MgCl2, 1.0 mM DTT. Reactions were stopped after 20, 40 and 60 min as indicated, by the addition of 10 mM SAM. The incorporated 3H-methyl groups for Dnmt3a, Dnmt3b2 and M.SssI (expressed as cpm) are plotted against the respective DNA templates. In addition, the ratios of DNA methylation activity of free DNA to nucleosomal DNA are given (indicated as DNA/NUC). (A) Dnmt3a was tested in experimental setup described above (B) The same setup as described in (A), using Dnmt3b2 (C) The same setup as described in (A), using M.SssI.
Analysis of chromatin dependent DNA methylation in HeLa cells
The relative level of CpG methylation within nucleosomal DNA and linker DNA was quantified using the 5-methyl-cytidine-sensitive ELISA (Sigma) (Figure 5B). To exclude possible effects of DNA length on the assay readout and to prove quantification, we generated a methylated and a non-methylated control DNA of 150 bp length (NPS2; Figure S5A). As expected, the ELISA assay was only capable to detect DNA methylation using the in vitro methylated DNA as substrate. The DNA concentration- and DNA methylation-dependent increase of relative methylation units with this assay confirms the reliable and robust quantification of nucleosomal DNA methylation by the enzyme-linked assay (Figure 5B).

In order to test whether decreased nucleosomal DNA methylation levels of the isolated mononucleosomal DNA are due to reduced CpG dinucleotide levels in the nucleosome cores, we compared the M.SssI activity on sonified genomic DNA of nucleosomal size and the purified mononucleosomal DNA (Figure S5B). The result clearly shows similar levels of methylatable sites, arguing against the absence of CpG dinucleotides within nucleosomes. Absolute levels of nucleosides and 5-methyl 2'-deoxycytidine (5-meC) of HeLa genomic DNA and mono-nucleosomes were quantified by micellar capillary electrophoresis (8) in reference to standard 2'-deoxy-nucleosides (Figure 5C, Figure S5C). We calculated the 5-meC content as percentage of the area 5-meC / area C + area 5-meC and found a 5-meC level of 3.06% (± 0.17 %) (Figure 5D) consistent with previous findings (8). Taking into account the average nucleosomal repeat length of 188 bp (9) and a nucleosome length of 147 bp, we determined the relative 5-meC contents of linker and nucleosomal DNA (Figure 4D). As a result of our calculations we reach a total of 62 % of methylated CpG sites throughout the genome. The latter is very well in agreement with early studies (10), revealing 70 % (± 10 %) total methylated CpG sites in HeLa cells. Accordingly we suggest that DNA linkers are fully methylated and that nucleosomal cores exhibit reduced CpG methylation levels.
Figure S5 Mononucleosomal DNA constitutes methylatable CpG sites. (A) The DNA fragment NPS2 (15 CpG sites, 150 bp in length) either non-methylated or fully methylated in vitro (mNPS2) was used for methylated CpG quantification with the enzyme-linked assay (Sigma). The relative DNA methylation signal is plotted against increasing DNA concentrations. The mNPS2 DNA (light grey bars) shows a concentration-dependent increase of the DNA methylation signal, whereas the NPS2 DNA (white bars) does not. (B) Mononucleosomal DNA (lane 1, light grey) and sonified genomic DNA (lane 2, dark grey) were methylated with the bacterial DNA methyltransferase M.SssI and the incorporation of ²H-methyl groups (cpm) is plotted against the DNA levels used in the reaction. (C) Micellar capillary electrophoresis of 0.5 mM 2′-deoxy-nucleosides (G, C, T, A) and 5-methyl 2′-deoxycytidine (5-meC) (light grey) and digested genomic HeLa DNA (dark grey, 4 µg/µl). The absorbance (AU) is plotted against the migration time (t; min). Impurities are marked by asterisks (*).

Chromatin remodeling-dependent DNA methylation

In order to study the effect of ATP dependent nucleosome remodeling on nucleosomal DNA methylation we combined the recombinant remodeling enzyme Snf2H with the de novo methyltransferases and a defined nucleosomal substrate. The DNA template N78-NPS2-N79 (304 bp) was fully reconstituted into mononucleosomes by salt dialysis with an increasing histone to DNA ratio (Figure S6A). As DNA methylation reactions are performed at 37°C, the stability of mononucleosomes and remodeled mononucleosomes under these conditions was tested. In comparison with standard reaction temperature of 26°C, temperature elevation to 37°C did not have an effect on the stability or position of the nucleosome (Figure S6B). The addition of free DNA (competitor DNA) to chromatin remodeling reactions, which competes for Snf2H binding to nucleosomes, is required to stop remodeling reactions without disruption of the nucleosomal substrate. DNA methylation reactions were performed after the addition of the competitor DNA, in order to quantify nucleosomal accessibility and to avoid inhibition of DNA methylation by Snf2H-nucleosome complexes. We titrated the minimal amount of competitor DNA required to efficiently compete the remodeling enzyme (Figure S6C). Furthermore, we determined the minimal Snf2H concentration necessary to fully move the nucleosomes from the middle to the border positions of the DNA fragment (Figure S6D).
Dnmt3b was shown to interact with Snf2H in vivo (11) and the binding affinity of the maintenance DNA methyltransferase Dnmt1 towards mono-nucleosomes was enhanced in the presence of Snf2H (1). However, in our experiments we can exclude such a stimulatory effect as competitor DNA disrupted nucleosome-remodeler interactions (Figure S6C) and Snf2H was used in a 4-fold lower molar ratio compared to Dnmt3a and Dnmt3b2. Therefore the stimulatory effect of Dnmt3a on the remodeled nucleosome corresponds to the recognition of structural changes in the remodeled nucleosome.

In order to reveal whether Dnmt3a binds with higher affinity to the remodeled nucleosome, we mixed non-remodeled and remodeled nucleosomes, as well as free DNA, non-remodeled and remodeled nucleosomes at equimolar ratios. Substrate mixture were incubated in the presence or absence of ATP and increasing levels of Dnmt3a. In contrast to the bandshifts shown in Figure 1 (Dnmt3a binds with higher affinity to the symmetric nucleosome compared to the asymmetric nucleosome), Dnmt3a bound with similar affinity to the remodeled nucleosome and to the symmetric substrate (the non-remodeled nucleosome), suggesting an increased affinity for the remodeled substrate (Figure S6E and F).

**Figure S6** *In vitro* chromatin remodeling on N78-NPS2-N79. (A) The DNA template N78-NPS2-N79 (304 bp) carrying the nucleosome positioning NPS2 sequence was reconstituted into mononucleosomes by salt dialysis with an increasing histone to DNA ratio. Free DNA was progressively reconstituted into nucleosomes as indicated. Competitor DNA lacking CpG sites (pCpGL-basic (12)) was included at a 1:1 mass ratio in this assembly reaction. (B) DNA methylation reactions are performed at 37°C. To test the stability of the mononucleosomes, chromatin remodeling
reactions in RB90 buffer using 1.0 picomol of reconstituted N78-NPS2-N79 nucleosomes in the presence or absence of 1.0 picomol Snf2H, and 1.0 mM ATP were incubated at 26°C (lanes 1 and 3) or 37°C (*, lanes 2 and 4) for 90 min. (C) Addition of free DNA to nucleosomes competes for Snf2H binding. The minimum amount of competitor DNA to stop remodeling reactions was determined. Snf2H-dependent sliding reactions (10 µl, 1.0 picomol Snf2H) in RB90 buffer (20 mM Tris pH 7.6, 1.5 mM MgCl$_2$, 1.0 mM EDTA, 10 % glycerol, 90 mM KCl, 1.0 mM DTT) supplemented with 1.0 mM ATP were carried out for 90 min at 26°C and subsequently stopped with the addition of decreasing amounts competitor DNA (100 ng - 6.25 ng; 0.5 – 0.0375 femtomol). 50 ng of competitor DNA were used for the standard remodeling reactions described in the manuscript. (D) Same as for (C) but constant competitor DNA levels (0.25 femtomol) and increasing amounts of Snf2H (0.125 -1.0 picomol) were used. All reactions were analyzed on 5 % native PAA gels stained with ethidium bromide. Molecular weight marker (M) is indicated. (E-F) Competitive nucleosome-Dnmt3a electromobility shift assays. Either an equimolar mixture of remodeled and non-remodeled nucleosomes (E) or an equimolar mixture of free DNA, remodeled and non-remodeled nucleosomes (F), were incubated with a 3.5 to 15 fold molar excess of Dnmt3a, in the presence or absence of ATP, as indicated. Reactions were analyzed by native polyacrylamide gel electrophoresis and the positions of the nucleosomal substrates and the free DNA are indicated.
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