OUKA
Osaka University Knowledge Archive

Title	Interaction of the RFTS domain of Dnmt1 with the SRA domain of Uhrf1 for the maintenance DNA methylation
Author(s)	Berkyurek, Ahmet Can
Citation	大阪大学, 2015, 博士論文
Version Type	VoR
URL	https://doi.org/10.18910/54037
rights	
Note	

Osaka University Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

Osaka University

Interaction of the RFTS domain of Dnmt1 with the SRA domain of Uhrf1 for the maintenance DNA methylation

PhD Thesis

2015

Ahmet Can BERKYUREK

Laboratory of Epigenetics

Institute for Protein Research, Osaka University

Supervisor: Prof. Shoji Tajima

Publication:

Berkyurek, A. C., Suetake, I., Arita, K., Takeshita, K., Nakagawa, A., Shirakawa, M., and Tajima, S. The DNA methyltransferase Dnmt1 directly interacts with the SET and RING associated (SRA) domain of the Multifunctional Protein Uhrf1 to facilitate the accession of the Catalytic Center to Hemi-methylated DNA. *J. Biol. Chem*. **289**, 379-386, 2014.

CONTENTS

Summary--4

1. INTRODUCTION--5

1.1. Epigenetics--5

1.2. 5-methylcytosine (5mC)--6

1.2.1. Discovery and physiological significance of 5mC ----------------------------6

1.2.2. Distribution of 5mC in the genome--6

1.2.3. Regulation of gene expression by 5mC--8

1.3. Mammalian DNA (C-5) methyltransferases---------------------------------------11

1.4. Maintenance DNA methylation--16

1.4.1. The Structure of Dnmt1---17

1.4.2. The Structure of the SRA domain of Uhrf1--------------------------------------18

1.5. Aims of this work---22

TABLE AND FIGURES

2. MATERIALS AND METHODS---24

3. RESULTS--28

3.1. Dnmt1 containing the RFTS cannot methylate short hemi-methylated DNA---28

3.2. Mutant Dnmt1(291-1620) impaired the hydrogen bonds significantly methylates 12-bp hemi-methylated DNA----------------------------31

3.3. The SRA domain of Uhrf1 promotes DNA methylation-------------------------34

3.4. SRA-dependent short DNA methylation activity is due to direct interaction between the SRA and RFTS -----------------------------------37

3.5. The interaction between the SRA and RFTS promotes accession of DNA to the catalytic center --39

FIGURES

4. DISCUSSION---42

4.1. DNA length-dependent DNA methylation activity of
Dnmt1 containing the RFTS--42
4.2. Significance of the interaction between the RFTS and the SRA--------------43
4.3 Proposed model and future aspects --44

5. REFERENCES--45
6. ACKNOWLEDGEMENTS ---55
7. SUPPLEMENTARY INFORMATION --56

Summary

DNA methylation plays an essential role in development through regulating gene expression. Genome-wide DNA methylation patterns are established during early stage of embryogenesis and germ cells, and are faithfully propagated to next generations during replication by Dnmt1, which preferentially methylates hemi-methylated DNA that transiently appears during replication and repair step. Dnmt1 is recruited to replicating region by its “replication foci targeting sequence” (RFTS) (Leonhardt et al., 1992). Interestingly, three-dimensional structure of Dnmt1 solved by our group exhibited that the RFTS forms independent domain and is plugging the catalytic pocket. The position is stabilized by four hydrogen bonds between the RFTS and catalytic domains (Takeshita et al., 2011). For this, DNA cannot access the catalytic center, and thus the RFTS should be removed from the catalytic pocket for the active DNA methylation.

I have found that Dnmt1 with the RFTS domain could not methylate DNA shorter than 12 bp. In addition, the SRA domain of Uhrf1, which is a prerequisite factor for maintenance DNA methylation and selectively binds to hemi-methylated DNA (Arita et al., 2008; Sharif et al., 2007), stimulated DNA methylation activity of Dnmt1 in a dose dependent manner. The SRA domain of Uhrf1 directly interacted with the RFTS domain to stimulate the DNA methylation activity of Dnmt1 since the mutation in the SRA domain that cannot bind to DNA stimulated DNA methylation activity of Dnmt1 to the same extent as to the wild-type SRA domain. The interaction between the SRA and RFTS domains facilitated DNA accession to the catalytic center forced the release of hemi-methylated DNA from the SRA domain. From these results I concluded that the SRA domain removes the RFTS domain from the catalytic pocket and handovers the DNA bound to the SRA domain to the catalytic center of Dnmt1. The interaction between the RFTS domain of Dnmt1 and the SRA domain of Uhrf1 plays a crucial role in the maintenance DNA methylation. Those results have been published (Berkyurek et al., 2014).

1. INTRODUCTION

1. 1. Epigenetics

Organisms inherit their characteristic genetic memory to next generations. During the course of evolution, eukaryotes developed the mechanisms to inherit genetic memories under the control of “epigenetics” in addition to the nucleotide sequence inheritance. “Epigenetics” is defined as a change of read out of genomic information without any alteration in the nucleotide sequence. In addition, importantly, the information must be inherited to next generation. Methylation at the 5th carbon of the cytosine in the CpG sequence is one of the major epigenetic mechanisms together with histone post-translational modifications and non-coding RNA (Reik, 2007).

Epigenetic mechanisms regulate gene expression via changing the conformation of chromatin, thereby control the accessibility of RNA polymerase to the target sequences. Genomic DNA in nucleus is packaged into basic structure called nucleosome, which comprises two molecules each of core histones H2A, H2B, H3, and H4, and 147 bp DNA. Beaded nucleosomes on DNA called “beads-on-a-string” form higher ordered structure of 30 nm fiber (Thomas and Kornberg, 1975). On a wider scope within the nucleus, chromatin forms coils and loops, thus builds a higher organization of the chromatin conformation (Huang et al., 2015). Further hierarchical chromatin packaging gives two distinct conformations, heterochromatin and euchromatin. Heterochromatin is tightly packed, contains silenced region of the genome, and positioned and anchored at the periphery of nuclei. Whereas, euchromatin is more relaxed and contains expressing genes. Increasing evidence suggests that spatial and temporal adaptation of three dimensional architecture of the chromatin contributes to the epigenetic regulation (Bell et al., 2011). On the chromatin, transcriptionally active regions carry post-translational modifications (PTMs) of histone H3K4 tri-methylation and H3K9 acetylation, whereas inactive genes are mostly associated with histone H3K9 di- or tri-methylation. Not only the histone modifications, but also ATP-dependent nucleosome remodeling complexes contribute to the distribution of histones on the chromatin (Bártová et al., 2008). DNA methylation and histone modifications are regulating each other via a crosstalk mechanism (Cedar and Bergman, 2009). Epigenetic mechanisms at the transcriptional level are not completely elucidated, and often work in concert with other regulatory

pathways such as non-coding RNA. Transcriptional regulatory mechanisms such as DNA methylation in case of imprinting and histone PTMs are mostly copied during cell division.

1.2. 5-methylcytosine (5mC)

1.2.1. Discovery and physiological significance of 5mC

5mC was first reported in the calf thymus DNA (Hotchkiss, 1948) and later in wheat DNA (Wyatt, 1951). Following the discovery, its presence was documented in several other animals and genomes. However, 5mC cannot be found in yeast and nematodes (Steele and Rae, 1980). Though this base modification is widely used as a defense mechanism in some prokaryotes called "restriction modification system", vertebrates adopted 5mC in regulating gene expression. Holliday and Pugh (1975), proposed that methylation modification of cytosine at specific sites might have a role in developmental regulation by affecting the binding ability of specific (transcription) factors, and thereby regulate events such as X-chromosome inactivation initiation and maintenance. DNA methylation patterns are established during early stage of embryogenesis and then inherited to daughter cells in a cell lineage-specific manner.

1.2.2. Distribution of 5mC in the mammalian genome

In vertebrates, cytosine methylation mostly is on the CpG sequence, and 80% of them in mammals are methylated (Antequera and Bird, 1993). In plants, not only the CpG sequence but CpHpG and CpHpH sequences are also methylated. The CpG sequence has been deleted possibly due to the methylation modification during evolution, is a rare sequence in mammalian genome. However, some part of the mammalian genome exists in relatively high CpG density, which are mostly found in the promoter of housekeeping genes and kept under methylated. These regions are named "CpG islands" (Law and Jacobsen, 2010). The promoters comprising CpG island are suggested to be a region of transcription start sites and can start transcription towards both directions (Sandelin et al., 2007). The CpG in CpG island promoters are methylated only 3%, while non-CpG island promoters, which regulate mostly tissue specific genes, are frequently methylated unless the genes are actively transcribed (Weber et al., 2005). It is also reported that some classes of CpG islands are

differentially methylated in different tissues and thus play an important role in cellular differentiation (Illingworth and Bird, 2015).

In addition to CpG methylation, non-CpG methylation is often reported especially in embryonic stem cells (Lister et al., 2009), brain (Kinde et al., 2015), and growing oocyte (Shirane et al., 2013). Although the function of non-CpG methylation is elusive, it is not surprising that those cells possess high level of non-CpG methylation, as those cells express high levels of *de novo*-type DNA methyltransferases Dnmt3a and/or Dnmt3b (Inano et al., 2000; Sakai et al., 2004; Watanabe et al., 2002), and they methylate not only CpG but CpA and/or CpT (Aoki et al., 2001; Suetake et al., 2003). Non-CpG methylation is not observed when embryonic stem cells are induced to become fibroblast cells. This is because that differentiation of embryonic stem cells quickly down regulates the expression of Dnmt3a and Dnmt3b (Sato et al., 2006), and the maintenance DNA methyltransferase Dnmt1 wipes off the non-CpG methylation.

Highly expressed genes exhibit low methylation levels in their promoters, but possess high gene body methylation (Ball et al., 2009). However, weakly expressed genes have higher methylation levels in both regions (Suzuki and Bird, 2008). These reports argue that gene body methylation is a common feature in a mammalian genome and they reflect not only the ancestral DNA methylation, but also a function to reduce the transcriptional noise (Huh et al., 2013). Others argue that gene body methylation might have a function in regulating different chromatin conformations (Jjingo et al., 2012). However, the experimental evidence is not very clear yet (Hahn et al., 2011).

In addition to the spatial distribution of 5-methylcytosine, rewriting of methylation patterns takes place during two developmental stages; during early stage of embryogenesis and gametogenesis especially in primordial germ cells. During these stages global DNA demethylation event, which erases most of the methylation patterns, occurs (Monk et al., 1987). Nevertheless, some regions in the genome remain resistant to the global demethylation. Erasure of DNA methylation after fertilization skips the differentially methylated region (DMR) of imprinting genes, and some repetitive sequences in primordial germ cells. In primordial germ cells, some retrotransposons such as *IAP* partially retain their methylation (Morgan et al., 1999). DNA methylation patterns in male and female germ cells are reestablished in a sex-specific manner.

Given that the methylation levels are drastically decreased soon after fertilization, new patterns are written by DNA methyltransferases after implantation stage. During this stage, promoters which are required for the expression of proteins controlling the pluripotent state have to be kept un-methylated, while those for cellular differentiation are highly methylated. In the same way during differentiation, promoter of a gene required for a specific cell type differentiation has to be reactivated (Hajkova et al., 2002; Reik, 2007; Rougier et al., 1998).

1.2.3. Regulation of gene expression by DNA methylation

Regulation of gene expression by DNA methylation is performed in two ways; CpG methylation directly interferes the binding of transcriptional regulator to the target sequence, or the proteins specifically bind methylated CpG (**Table 1**) recruit repressor complexes that modify histone modifications (Clouaire and Stancheva, 2008; Hendrich and Tweedie, 2015). Former example is CTCF, which is a component of insulator that cannot bind to the target sequence when the site is methylated (Bell and Felsenfeld, 2000; Hark et al., 2000). As a second category, methyl CpG binding proteins (MBP) especially the MBD family proteins recruit co-repressor complex, many of which are ATP-dependent chromatin remodeling factors and/or DNA methyltransferases (Rottach et al., 2009a). MBP can be categorized into three classes; MBD family, Uhrf family, and Kaiso family (**Table 1**). These proteins recognize methylated DNA via distinct domains.

Among the MBD family, except MBD3, MBD1, 2, and 4 specifically bind to 5mC via their methylated DNA binding domain (MBD). Actually, mammalian MBD3, which shows no methylated DNA-binding property, is exceptional. However, MBD3 isolated from *Xenopus* can selectively bind to methylated CpG, and plays crucial role in development (Iwano et al., 2004). MBD family member proteins recruit histone deacetylases (HDAC) containing complex to form or maintain silent chromatin state (Hendrich and Tweedie, 2015). Different from other MBDs, MBD4 protein has a catalytic domain, which has T:G mismatch DNA glycosylase activity, in addition to its MBD. It has been argued that MBD4 might have a direct role in active DNA demethylation by removing 5mC (Zhu et al., 2000) and 5-hydroxymethylcytosine (5hmC), which is produced from 5mC by oxygenase TET and thought to be an intermediate form for demethylation (Hashimoto et al., 2012; Moréra et al., 2012), as

Table-1: Methyl-CpG binding proteins and their function.

Enzyme	Domains involved	Specificity
1. MBD family		
MBD1	MBD, CXXC	5mC
MBD2	MBD, TRD	5mC
MBD3	MBD	5mC
MBD4	MBD, Glycosylase	5mC, 5hmC
MeCP2	MBD, TRD	5mC
2. Uhrf family		
Uhrf1	Tudor, PHD, SRA, UBL, RING	hemi-methylated DNA, histone H3K9me3 H3R2me0, ubiquitin E3 ligase
Uhrf2	PHD, SRA, UBL, RING	5hmC
3. Kaiso family		
Kaiso	BTB/POZ, ZnF	5mC
ZBTB4	BTB/POZ, ZnF, CXXC	5mC
ZBTB38	BTB/POZ, ZnF, CXXC	5mC

MBD4 can bind not only 5mC but 5hmC (Otani et al., 2013a). Another member of the MBD family, MeCP2, binds also to histone H3K9 methyltransferases and recruit them to the genome (Fujita et al., 2003; Sarraf and Stancheva, 2015). MeCP2 mutations is the major cause of RETT Syndrome, of which gene is on X-chromosome and thus affecting females (Hite et al., 2009). Mutations on MeCP2 causing RETT Syndrome are affected the methylated DNA binding or impaired its interaction with other chromatin binding proteins such as heterochromatin protein 1 (HP1) (Clouaire and Stancheva, 2008).

Another well characterized MBP is the Uhrf family, Uhrf1 and Uhrf2. Uhrf1 was reported to flip out the hemi-methylated CpG with the SET and RING associated (SRA) domain (Arita et al., 2008; Avvakumov et al., 2008; Hashimoto et al., 2008). Uhrf1, but not Uhrf2 was shown to be crucial for maintenance DNA methylation (Pichler et al., 2011). As for knockout of *Uhrf1*, the embryonic stem cells cannot fully transmit the DNA methylation patterns through consecutive cell divisions and the embryos show serious development defects in the early stage, which results in embryonic lethality. These reports do not only show the co-localization of Uhrf1 with Dnmt1 in a cell-cycle dependent manner, but also reflect a possible direct interaction between these proteins (Sharif et al., 2007). However, experimental evidence for a direct interaction between Dnmt1 and Uhrf1 is lacking. Uhrf1 is reported to interact with other DNA methyltransferases, Dnmt3a and Dnmt3b (Meilinger et al., 2009). Uhrf1 acts as a crosstalk protein between DNA methylation and histone modifications, as the tandem-tudor and PHD domains of Uhrf1 can bind histone H3R2 un-methylated and H3K9 tri-methylated state (Hashimoto et al., 2009; Rottach et al., 2009b; Rajakumara et al., 2011; Arita et al., 2012). Interestingly, Uhrf2 can recognize 5hmC with its SRA domain (Zhou et al., 2015). As for the SRA domain of Uhrf1, there are controversial reports describing that the SRA domain of Uhrf1 also can recognize 5hmC (Frauer et al., 2011), or cannot bind hemi-hydroxymethylated DNA (Otani et al., 2013b).

In addition to the regulatory function of Uhrf1 in chromatin and DNA methylation, it was reported as a marker for carcinogenesis (Unoki et al., 2009). Uhrf1 is highly expressed in non-small lung cell carcinoma and colon cancer cells with a global hypomethylation (Daskalos et al., 2011). Since the transient expression of the Uhrf1 that cannot bind hemi-methylated DNA in cancer cells retrieve the expression of

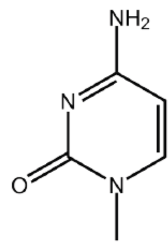
tumor suppressor genes (Achour et al., 2013), it is reasonable to speculate that hemi-methylated DNA-binding activity of Uhrf1 is necessary for progression of carcinogenesis.

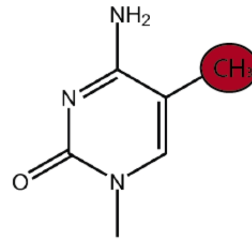
In contrast to MBD and Uhrf families, Kaiso protein of the Kaiso family recognizes two consecutive 5mC on a DNA strand by its Kruppel-like zinc finger domain. It was further reported that Kaiso can bind to nuclear corepressor (N-CoR) (Yoon et al., 2003). Two other members of Kaiso family are ZBTB4 and ZBTB38, which bind to single methylated CpG. Unlike Kaiso, ZBTB4 and ZBTB38 recruit Sin3/HDAC complex to Igf2 loci (Filion et al., 2006). Furthermore, Kaiso family proteins are reported to recognize sequence specific localization (Kim et al., 2004).

1.3. Mammalian DNA methyltransferases

In mammals, the 5th carbon of cytosine in the CpG sequence is often methylated (**Figure 1**), which is catalyzed by three distinct DNA methyltransferases, Dnmt1, Dnmt3a, and Dnmt3b. During early stages of development, DNA methylation patterns are established by Dnmt3a and Dnmt3b. Once the methylation patterns are established, Dnmt1 maintains these patterns to the daughter cells during replication (Goll and Bestor, 2005) (**Figure 2**). Dnmt2, which was firstly expected to be a *de novo*-type DNA methyltransferase, was turned out to be the tRNA methylase (Goll et al., 2006). Dnmt3L is a homologue of Dnmt3-type DNA methyltransferase, but has no DNA methylation activity. Instead, it binds to Dnmt3a and Dnmt3b through its C-terminal half to stimulate their DNA methylation activity (Suetake et al., 2004). All mammalian DNA methyltransferases possess a highly conserved ten motifs for the catalytic activity from bacterial DNA methyltransferase (Kumar et al., 1994) (**Figure 3**).

Dnmt1 is the first isolated mammalian DNA methyltransferase (Bestor et al., 1988). The N-terminal region comprising about 250 amino acid sequences forms independently folded domain (Suetake et al., 2006), which binds a variety of factors such as transcription repressor DMAP1 (Rountree et al., 2000), Dnmt3a and Dnmt3b (Kim et al., 2002), Rb2 (Pradhan and Kim, 2002), proliferating cell nuclear antigen (PCNA) (Chuang et al., 1997), CDKL5 kinase (Kameshita et al., 2008), casein kinase 1δ/ε (Sugiyama et al., 2010). For these, it is assumed that the N-terminal independently folded domain acts as a platform for the factors regulating Dnmt1 through tethering to





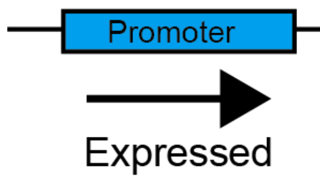


Figure 1. DNA methylation at promoter is associated with gene silencing.

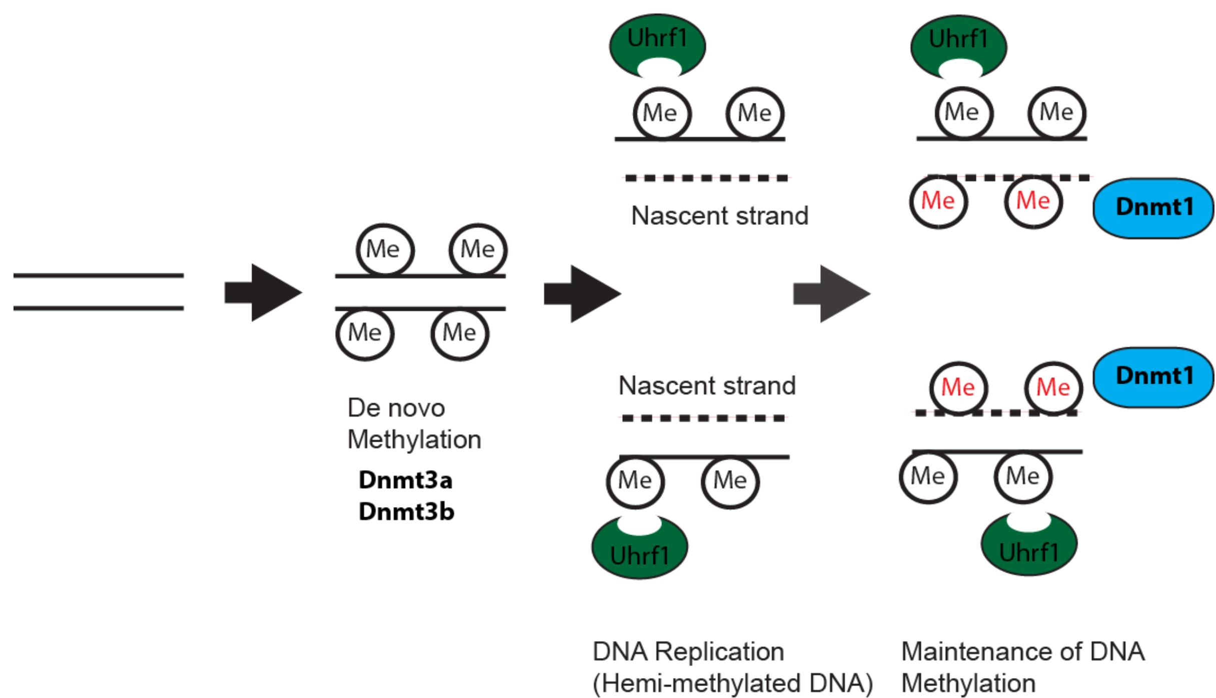


Figure 2. DNA methylation patterns are faithfully transmitted to next generation during replication. DNA methyltransferase-1 (Dnmt1) specifically recognizes hemi-methylated sites merges just after replication, and maintains DNA methylation patterns.

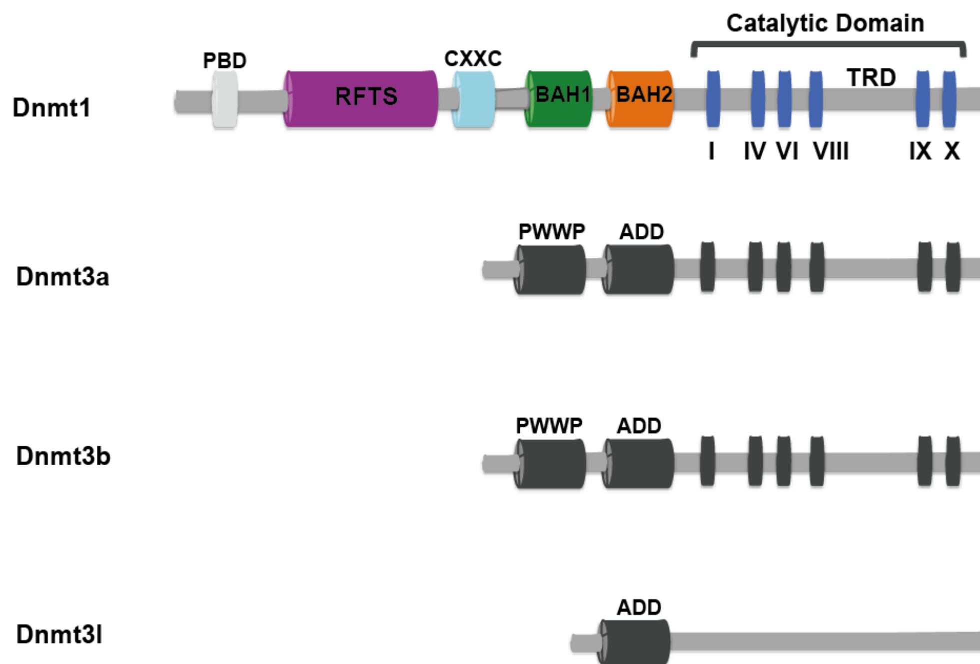


Figure 3. Schematic illustration of DNA methyltransferase family.
Dnmt1 consist of N-terminal independently folded domain containing many motifs including PCNA binding domain (PBD), replication foci-targeting sequence (RFTS) domain, two bromo-adjacent homology domains (BAH1 and BAH2), and the catalytic domain.

specific regions. Among these factors, PCNA, which binds DNA polymerases and the factors necessary for replication, and thus is a prerequisite factor for replication, is thought to contribute to maintenance methylation during replication (Chuang et al., 1997). Dnmt1 deleted the N-terminal independently folded domain has been solved the crystal structure (Takeshita et al., 2011) (**Figure 4**). According to the three-dimensional structure, the replication foci targeting sequence (RFTS) forms a domain that follows the N-terminal independently folded domain. This RFTS domain is dispensable for the selective DNA methylation activity towards hemi-methylated DNA *in vitro* (Takeshita et al., 2011; Song et al., 2011). The CXXC motif comprising two Zn-finger motifs follows the RFTS domain. The motif binds un-methylated DNA and is assumed to inhibit *de novo* methylation (Song et al., 2011). It was recently reported that the crystal structure of Dnmt1 lacking the CXXC motif allows selective methylation activity towards hemi-methylated DNA (Song et al., 2012). Two bromo-adjacent homology (BAH) domains, of which the function is not known, and the catalytic domain follow the CXXC motif. The catalytic domain is highly homologous to bacterial DNA methyltransferases containing 10 conserved motifs (Kumar et al., 1994) (**Figure 3**).

Dnmt1 can bind to long non-coding RNA with a stem loop conformation through its catalytic pocket. It was further discussed that the affinity of Dnmt1 to RNA was stronger than that of Dnmt1 to DNA (Di Ruscio et al., 2013). In the scope of current scientific evidence, the biological relevance of Dnmt1 and non-coding RNA interaction remains to be elucidated. One recent study reported that a long non-coding RNA *Dum* could interact with Dnmt1 and regulate myogenic differentiation and muscle regeneration (Wang et al., 2015). Despite of these exciting findings, it remains unclear whether or not there is a specific class of non-coding RNAs interacting with Dnmt1 or different developmental stages require different non-coding RNA and Dnmt1 complexes.

Dnmt1 is highly expressed during the S phase of the cell cycle (Szyf et al., 1991), with some exception that it is highly expressed in non-proliferating growing oocytes (Kimura et al., 1999; Mertineit et al., 1998), and in post-mitotic neurons (Inano et al., 2000), but are localized in cytoplasm. During the replication, the RFTS domain brings Dnmt1 to the replication foci (Leonhardt et al., 1992).

Other members of mammalian methyltransferases are Dnmt3a and Dnmt3b, which are called "*de novo*" methyltransferases. Different from differentiated cells, *de novo* methyltransferases are highly expressed in embryonic stem cells and primordial germ cells (Watanabe et al., 2002; Sakai et al., 2004). Unlike Dnmt1, they have similar activity toward hemi- and un-methylated DNA (Aoki et al., 2001; Suetake et al., 2003). The N-terminal half of Dnnmt3a and 3b contains the PWWP and ATRX-DNMT3-DNMT3L (ADD) domains (**Figure 3**). The PWWP domain of Dnmt3b is reported to be the motif to bind to DNA (Qiu et al., 2002) and, at the same time, the domain tethers the molecules to heterochromatin (Chen et al., 2004; Ge et al., 2004). The ADD domain is reported to bind histone H3K4 with no modification (Otani et al., 2009), and the interaction contributes to the activation of Dnmt3a methylation activity with histone H3 tail (Zhang et al., 2010). Recent structural study revealed that histone H3 tail-binding to the ADD domain releases an auto-inhibitory state of Dnmt3a (Guo et al., 2015).

1.4. Maintenance of DNA methylation

Dnmt1 is the responsible enzyme for the maintenance of DNA methylation as it favors to methylate hemi-methylated DNA produced during replication and repair, and is tethered to replication and repair regions (**Figure 2**). As the N-terminal independently folded and RFTS domains of Dnmt1 is responsible for the recruitment to the replication foci, there are substantial evidence for the interaction of Dnmt1 with other proteins for the maintenance DNA methylation (Tajima and Suetake, 1998). In this regard, PCNA and Uhrf1 are the key players (Chuang et al., 1997; Hervouet et al., 2010). Several independent reports discuss that Uhrf1 recruits Dnmt1 specifically to hemi-methylated sites on the nascent strand (Arita et al., 2008; Sharif et al., 2007). Likewise, PCNA interacts with the N-terminal independently folded domain of Dnmt1, and allows the interaction with the replication machinery (Chuang et al., 1997). Dnmt1 sequentially methylates hemi-methylated DNA merges during replication. It was thought that this may be due to the binding of Dnmt1 to PCNA, however, it was shown that the PCNA binding domain is dispensable in maintenance DNA methylation (Garvilles et al., unpublished, 2015). Actually, our laboratory has shown that Dnmt1 that lacks the PCNA binding domain can methylate hemi-methylated DNA in a processive manner, though skips one out of 20 hemi-methylated CpG (Vilkaitis et al., 2005). However, molecular mechanisms during replication process to maintain DNA

methylation remains elusive. Its interaction with other factors may enable faithful propagation of DNA methylation patterns to next generation.

1.4.1. Structure of Dnmt1

Recently, our group solved the three dimensional structure of mouse Dnmt1, which comprises 1,620 amino acid residues, lacking 1-290 N-terminal independently folded domain by X-ray crystallography analysis (PDB accession number, 3AV4) (Takeshita et al., 2011). A striking feature of the structure is that the RFTS domain, which is responsible for recruiting Dnmt1to the replication foci, is plugging the catalytic pocket. This structure is not an artifact of the crystal, as the position is fixed by four hydrogen bonds between the RFTS and catalytic domains (**Figure 4**). Because of the steric hindrance, the RFTS domain must be kicked out from the catalytic pocket to make hemi-methylated DNA access to the catalytic center. Recently, the mutations increasing the hydrophobic index in the RFTS domain at the interacting surface with the catalytic domain cause neurological disorders of cerebellar ataxia, deafness and narcolepsy (ADCA-DN), which is late onset, having no developmental defects (Winkelmann et al., 2012).

The bromo-adjacent homology domains, BAH1 and BAH2, of which functions in the maintenance methylation remain to be elucidated, are in close contact with the catalytic domain. The BAH1 domain has a hydrophobic cage resembles to that of Orc1, which specifically recognizes histone H4K20 di-methylation state (Yang and Xu, 2012). For this, the BAH1 domain may related to chromatin or histone methylation to connect Dnmt1 with chromatin. The BAH2 domain is connected to the target recognition domain (TRD) via a linker region. Considering the flexibility of the linker region between the TRD and the BAH2, the DNA binding ability of Dnmt1 might be regulated by conformational rearrangements.

In addition to the crystal structure published from our group, crystal structures of a human DNMT1 fragment deleted the RFTS domain in complex with un-methylated DNA has been reported (Song et al., 2011) **(Figure 5A**). The structure of human DNMT1(651-1616) in complex with un-methylated DNA (PDB accession number, 3PT6) shows that DNA is bound to the CXXC motif, and thus the CXXC is pushed into the catalytic pocket. For this CXXC position with un-methylated DNA, un-methylated DNA cannot access to the catalytic center. Song et al. proposed that this

is the auto-inhibition mechanism to inhibit *de novo* DNA methylation. Human DNMT1(600-1600) fragment contains CXXC motif in complex with sinefungin (PDB accession number, 3SWR), which is a *S*-adenosyl-L-methionine mimicked inhibitor, shows the CXXC motif in a similar position as to that of the DNMT1-un-methylated DNA complex. This may raise a question whether or not the CXXC position in that particular complex is an artifact due to the removal of the RFTS domain.

When the conformations of all available Dnmt1 structures are aligned, minor but significant changes are observed (**Figure 5B**). One remarkable difference between the structures of Dnmt1 with or without DNA is the position of the CXXC domain. According to the conformational alignment, the CXXC must be out of the catalytic pocket as a result of hemi-methylated DNA accession to the catalytic center. Compared to the DNMT1-un-methylated DNA complex, DNMT1(732-1616) in complex with hemi-methylated DNA (PDB accession number, 4DA4) shows different DNA binding patterns. Interestingly, DNMT1 flips out the cytosine to be methylated out of the double stranded DNA.

Another conformational change was the position of the TRD domain. The TRD in the structures of Dnmt1-DNA complexes is likely to bend over the catalytic pocket compared to that without a DNA. Importantly, the structure harboring the hemi-methylated DNA has the TRD closer to the catalytic pocket than that with an un-methylated DNA. This observation might well explain the preferential binding of Dnmt1 to the hemi-methylated DNA. Notably, a loop connecting the CXXC and the RFTS to the catalytic pocket exhibits 9Å shift upon hemi-methylated DNA binding (**Figure 5C**). As well, the structure of the RFTS in free form shows noticeable conformational deviations than the one interacting with the catalytic pocket (**Figure 6**). Conformational differences resulted by hemi-methylated DNA binding may result in the removal of the RFTS and enable the catalytic pocket to access to DNA.

1.4.2. Structure of the SRA domain of Uhrf1

Up to the present, no crystal structure of a full-length Uhrf1 is reported. The structures of the SRA domain in complex with a hemi-methylated DNA are available (PDB accession numbers, 2ZKE, 3DWH, and 2ZO0) (Arita et al., 2008; Avvakumov et al., 2008; Hashimoto et al., 2008). According to the structures, the SRA binds to DNA by two loops in both major and minor groove. 5mC completely flipped out is stabilized

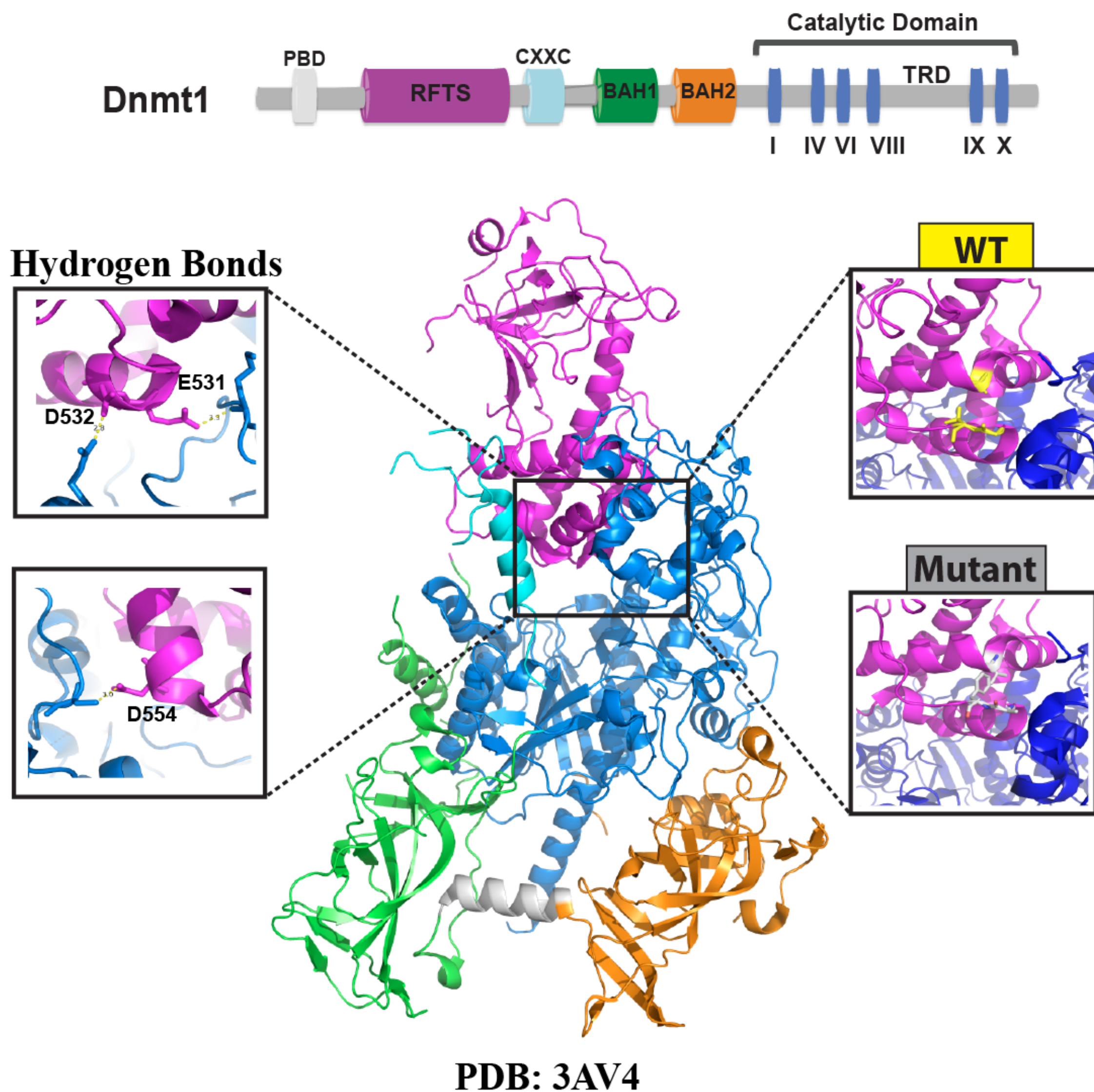


Figure 4. Crystal Structure of the mouse Dnmt1 coding 291-1620.
The RFTS domain plugs the catalytic pocket, which is stabilized by four hydrogen bonds between the RFTS and catalytic domains (Takeshita et al., 2011). Reported disease mutations (Winkelmann et al. 2011) on the RFTS domain increases local hydrophobicity at the interface of the RFTS and catalytic domain.

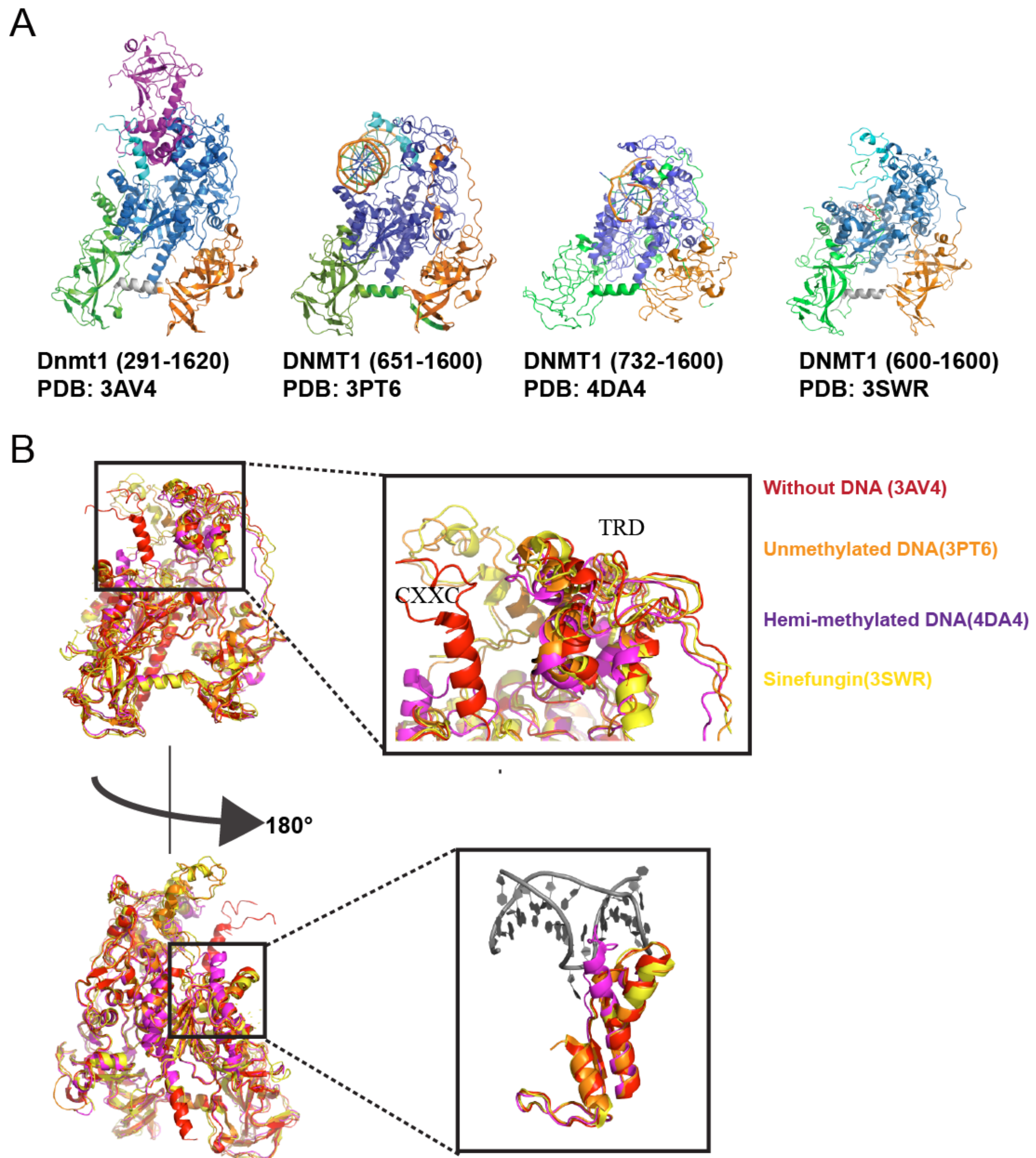


Figure 5. Crystal structures of Dnmt1 reported from different groups.
A. Illustration of published crystal structures of mouse Dnmt1 and human DNMT1.
B. Conformational changes as a result of DNA binding.

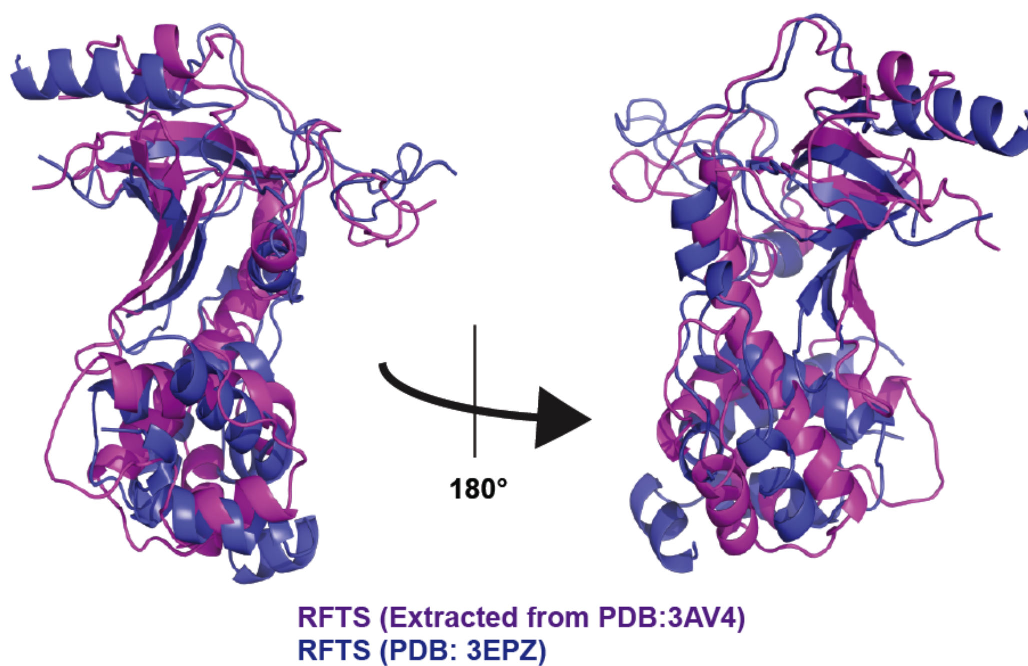


Figure 6. RFTS domain structures, independently expressed or in the Dnmt1.
The RFTS domain plugging the catalytic pocket of Dnmt1 and independently expressed form slightly different conformations.

by hydrogen bonds and van der Waals interactions. Furthermore, the orphanated guanine resulted by flipping out of the 5mC is captured by another loop to stabilize the position of the SRA on DNA. Mouse and human SRA in complex with hemi-methylated DNA display similar folding patterns (**Figure 7**).

1.5. Aims of This Work

Requirement of Dnmt1 in the maintenance DNA methylation is well established and there is substantial numbers of reports regarding the interacting partners of Dnmt1. Dnmt1 is necessary but not sufficient for faithful propagation of maintenance DNA methylation. According to the widely accepted model, Dnmt1 is recruited to the hemi-methylated sites by Uhrf1. However, detailed molecular mechanisms regarding how Dnmt1 is loaded onto the replication foci, and how Dnmt1 specifically finds the hemi-methylated sites are remain to be investigated. The aim of the present study is to reveal mechanistic aspects of the maintenance DNA methylation.

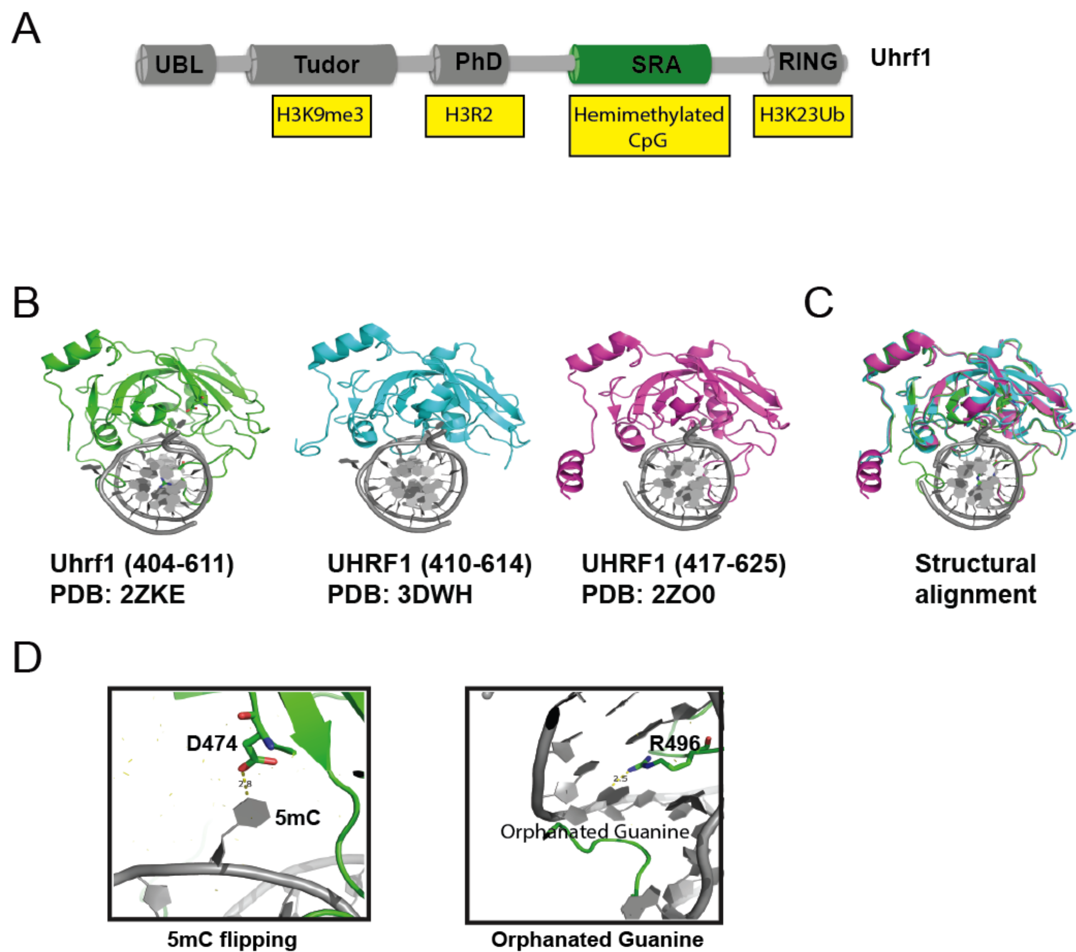


Figure 7. Schematic illustration of Uhrf1 and crystal structures of the SRA domain.
A. Uhrf1 is a multifunctional protein which mediates the crosstalk between epigenetic marks.
B. Crystal structures of the SRA and DNA complex.
C. Structural alignment of the SRA and DNA complexes.
D. Amino acid residues responsible for holding 5mC and guanine recognition in mouse SRA:DNA complex.

2. MATERIALS AND METHODS

2.1. Preparation of Recombinant Dnmt1:

Truncated and mutated mouse Dnmt1 cDNAs were subcloned into pFastBac-Htb (Invitrogen) with the GST cDNA inserted in frame at the 5' end. All the DNA sequences were confirmed by the dideoxy method (Sanger et al., 1977). The baculovirus harboring Dnmt1 cDNAs coding 291-1620 or 602-1620 was expressed in Sf9 cells and purified as described elsewhere (Takeshita et al., 2011; Berkyurek et al., 2014). The mutant Dnmt1 coding 291-1620 {Dnmt1(291)} with E531A and D532A, E531K and D532K, and D554K were purified as wild-type Dnmt1. The concentrations of purified Dnmt1 were determined from the absorbance at 280 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.2. Preparation of recombinant SRA of Uhrf1:

Mouse Uhrf1, the SRA domain coding 409-617, or with mutations was subcloned into the BamHI and XhoI sites of pGEX6P-1 or pET30a with a GST sequence added at the 5' end in frame. DNA sequences were confirmed by the dideoxy method (Sanger et al., 1977). All the proteins were induced the expression in *Escherichia coli* BL21(DE3) CondonPlus-RIL with 0.5 mM IPTG and then further cultured at 18°C for 16 h. The expressed proteins were purified with a HiTrap Chelating column (GE Healthcare) and/or GSH-Sepharose column. To remove the GST tag, PreScission protease was used. The RFTS protein concentrations were determined with a BCA Protein Assay Kit.

2.3. Synthesized DNA:

The following DNA sequences show the methylated strands of the hemi-methylated DNA used for methylation activity measurements (Gene Design, Mino, Osaka);

12-bp: 5'-GCAATCMGGTAG-3',

16-bp: 5'-CGGCAATCMGGTAGAC-3',

20-bp: 5'-GACGGCAATCMGGTAGACGA-3',

30-bp: 5'ACGACGACGGCAATCMGGTAGACGACGACG-3',

42-bp: 5’-GATCCGACGACGACGGCAATCMGGTAGACGACGACGACGATC-3’,

M in the sequences denotes 5mC. As for the 12-bp F-DNA, anti-sense strand of the hemi-methylated CpG’s un-methylated cytosine was replaced by 5-fluorocytosine. Equal amounts of the methylated and complementary strand oligomers (100 μM each) in 50 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0, were incubated at 97°C for 5 min, and then gradually targeted to 70°C after 2 h, 50°C after 4 h, and then to 15°C after 2 h. The annealed DNA concentrations were determined with a NanoDrop 2000 spectrophotometer. The DNA was stored at -80°C until use.

2.4. DNA methylation reaction:

DNA methylation activities were determined as described elsewhere (Vilkaitis et al., 2005). In brief, 6.4 nM Dnmt1, 66 nM DNA, and 2.2 μM [^{3}H]-*S*-adenosyl-L-methionine (AdoMet) (10,15 or 18 Ci/mmol) (Perkin Elmer) were incubated at indicated temperature in a total volume of 25 μl of reaction buffer comprising 5 mM EDTA, 50 mM NaCl, 2.7 M glycerol, 0.2 mM PMSF, and 20 mM Tris-HCl, pH 7.4 unless otherwise stated. After the incubation, radioactivity incorporated into DNA was determined in a liquid scintillation counter. The specific activities (mol CH_3 transferred to DNA/h/mol Dnmt1 enzyme) were determined within a linear time-course range.

To determine the effect of the SRA domain on the DNA methylation activity, the SRA was pre-incubated for 30 min on ice with DNA unless otherwise indicated in the figure legends.

2.5. Effect of the SRA domain on the DNA binding activity to Dnmt1:

The 5’ end of 12-bp F-DNA was labeled with T4 polynucleotide kinase (Toyobo) and [γ-^{32}P]-ATP (6,000 Ci/mmol, MP Biomedicals). Dnmt1(291) or Dnmt1(602) (6.4 pmol) was incubated with 10 pmol of labeled F-DNA and 60 pmol AdoMet, in the absence or presence of 5 or 20 pmol of the SRA in a 25 μl reaction mixture comprising 50 mM NaCl, 20% glycerol (w/v), 0.2 mM EDTA, 0.2 mM PMSF, 1mM DTT, and 20 mM Tris-HCl pH 7.4, followed by incubation at 37°C for 1 h. After the incubation, the mixtures were subjected to SDS-polyacrylamide gel electrophoresis in a 7.5% gel. The protein bands were visualized with Coomassie Brilliant Blue R250 (CBB) (Nacalai Tesque) staining and a BAS2000 Bioimage analyzer (Fuji Film), and

then exposed to X-ray film (Fuji Film). The amount of F-DNA bound to Dnmt1 was quantitated with Image-Gauge software (Fuji Film), and the protein band stained with CBB was quantitated with Quantity One (BioRad).

2.6. Pull-down assaying of Dnmt1 with Uhrf1:

SRA or SRA(D474A, R496A) (1.5 μg) was mixed with GST-Dnmt1(291) (1.2 μg), GST-Dnmt1(602) (1 μg), or GST-RFTS (2.3 μg) coupled to GSH-Sepharose, respectively, and then incubated at 25ºC for 30 min in a 40 μl of binding buffer comprising 50 mM NaCl, 10% glycerol, 0.1% (w/v) Nonidet P-40, and 50 mM Tris-HCl, pH 7.4. Dnmt1(291) (1 μg) or Dnmt1(602) (0.8 μg) was mixed with GST-SRA (3 μg) coupled to GSH-Sepharose, and then incubated under the same conditions to as above. Then, the beads were washed three times with binding buffer. The bound, unbound, and washed fractions were precipitated with 10% (w/v) trichloroacetic acid. The protein bands were separated by SDS-polyacrylamide gel electrophoresis in a 12% gel. The protein bands were visualized with CBB.

2.7. Gel shift assaying:

Indicated amounts of the RFTS, SRA, or SRA and RFTS domains were incubated with 12-bp or 42-bp DNA with one hemi-methylated CpG (0.2 μM) in a solution comprising 50 mM NaCl, 20% glycerol (w/v), 0.2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, and 20 mM Tris-HCl, pH 7.4, at 4ºC for 30 min. After the incubation, the mixtures were subjected to 0.8% agarose gel electrophoresis in 0.2 x TBE and 2% glycerol at 4ºC with 150 V for 10 min. DNA was stained with GelGreen (Biotium), and visualized in a fluoro-imager, Typhoon FLA 9500 (GE Healthcare).

2.8. Competition of the SRA domain with Dnmt1 on the DNA binding:

The SRA (0.6 μM) and 0.2 μM 12-bp hemi-methylated DNA were mixed in a total volume of 25 μl comprising 50 mM NaCl, 20 % glycerol (w/v), 0.2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, and 20 mM Tris-HCl, pH 7.4, without or with 1 to 50 folds higher concentrations of the RFTS to that of SRA domains, and then incubated at 4°C for 30 min. After the incubation, the mixtures were subjected to 0.8% agarose gel electrophoresis in 0.25x TBE and 2.5% glycerol at 4°C with 150 V for 10 min. DNA

was stained with GelGreen (Biotium), and visualized with a fluoro-imager, Tyhoon FLA9500 (GE Healthcare).

3. RESULTS

3.1. Dnmt1 containing the RFTS domain cannot methylate short hemi-methylated DNA

According to the X-ray crystal structure of mouse Dnmt1(291), the RFTS domain is plugging the catalytic pocket (Takeshita et al., 2011). This indicates that the RFTS has to be removed from its position to perform the DNA methylation. Interestingly, however, even in the presence of the RFTS, Dnmt1 can methylate hemi-methylated DNA (Takeshita et al., 2011; Vilkaitis et al., 2005). On the contrary, Syeda et al. reported that the recombinant Dnmt1 containing the RFTS has no DNA methylation activity when a short hairpin hemi-methylated DNA was used as the methyl acceptor (Syeda et al., 2011). I assumed that the difference between our and Syeda's results may due to the length of the methyl-group acceptor DNA. Short DNA may not be able to access catalytic center when the RFTS is plugging the catalytic pocket.

To determine whether or not the DNA length is the determinant for the accession of DNA to the catalytic pocket for DNA methylation activity, I prepared 12, 16, 20, 30, and 42-bp double-stranded hemi-methylated DNA, each of which contained one hemi-methylated CpG site. Recombinant Dnmt1(291) and Dnmt1(602) (**Figure 8A**), containing and lacking the RFTS, respectively, were purified and determined the DNA methylation activity towards these substrates. As shown in **Figure 8B**, Dnmt1(291) could not methylate 12-bp hemi-methylated DNA, but showed significant activity toward the DNA longer than 16-bp hemi-methylated DNA, and then increased the activity as the DNA length increased. Different from Dnmt1(291), Dnmt1(602) lacking the RFTS domain showed significant DNA methylation activity even when 12-bp hemi-methylated DNA was used as methyl group acceptor. Increase in the DNA length only mildly increased the DNA methylation activity of Dnmt1(602). These results indicate that 12-bp short hemi-methylated DNA used in the present study cannot access the catalytic center by replacing the RFTS from the catalytic pocket to be methylated. To access the catalytic center, DNA length must be longer than 12-bp.

The RFTS was separately purified and added to Dnmt1(602), and determined the DNA methylation activity towards 12-bp and 42-bp hemi-methylated DNA. The

RFTS domain added to the reaction mixture did not affect the DNA methylation activity when 42-bp hemi-methylated DNA was used as methyl-group acceptor. On the contrary, the RFTS domain inhibited the DNA methylation activity towards 12-bp hemi-methylated DNA in a dose-dependent manner (**Figure 8C**). The result suggests that short DNA such as 12-bp cannot efficiently access the catalytic center when the RFTS domain occupies the catalytic pocket, while long DNA does.

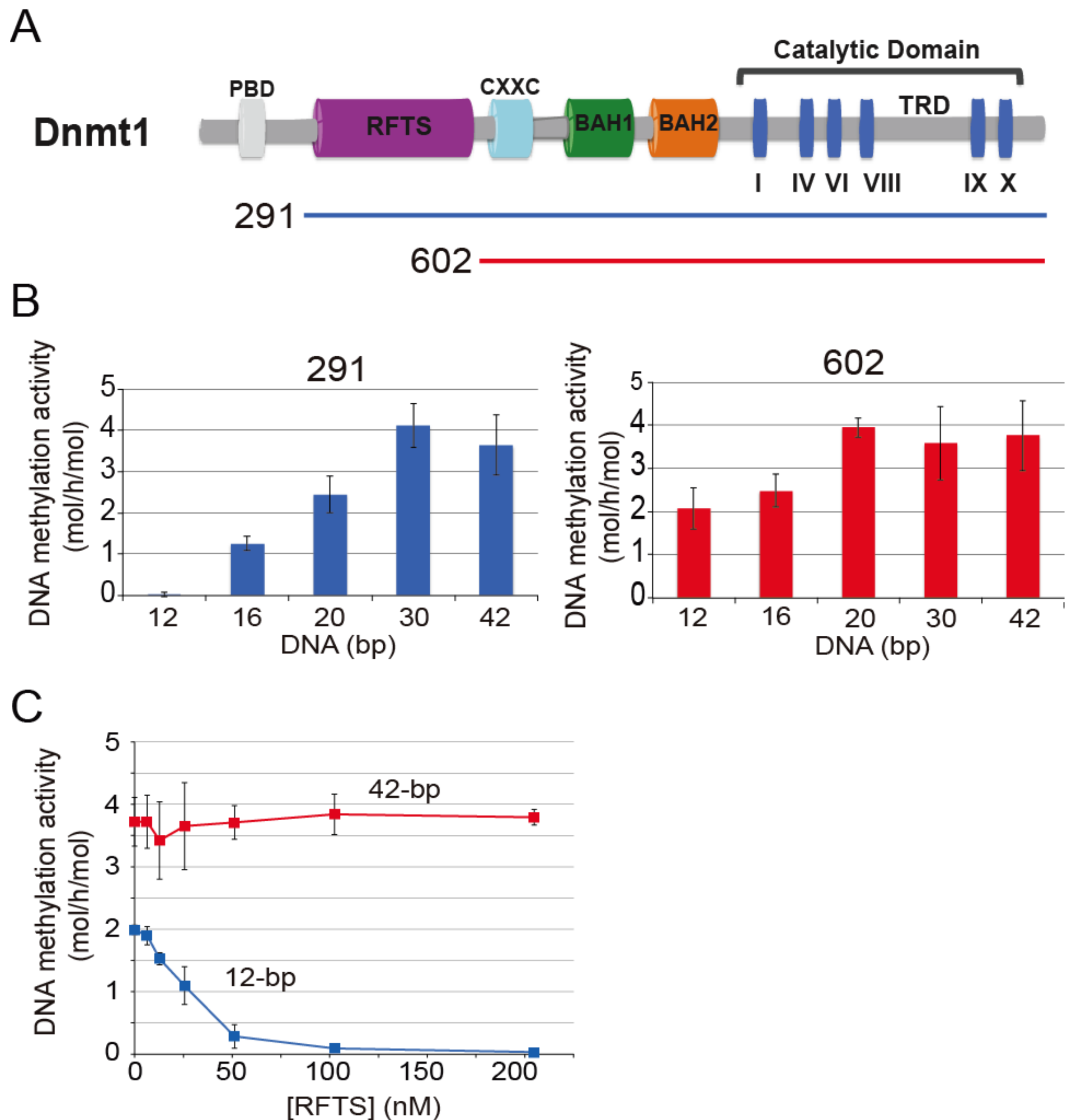


Figure 8. DNA length-dependent DNA methylation activities of Dnmt1(291) and Dnmt1(602). A. Schematic illustration of Dnmt1: Dnmt1 has a multi-domain structure. Two Dnmt1 constructs (291) and (602) are indicated. **B.** DNA length-dependent activity of Dnmt1 (291) and (602) towards 12, 16, 20, 30, and 42-bp hemi-methylated DNA with one CpG. **C.** Dnmt1(602) was incubated with the RFTS, and then the DNA methylation activity towards 12-bp (blue) and 42-bp hemi-methylated DNA (red) was determined. Specific activities of DNA methylation (mol CH3 transferred/h/mol Dnmt1) were determined. The average activities ± SD (n=3) are shown. The inhibition of Dnmt1(602) was statistically significant at 50, 100 and 200 µM RFTS ($P<0.001$).

3.2. Mutant Dnmt1(291) impaired the hydrogen bonds between the RFTS and catalytic domains significantly methylates 12-bp hemi-methylated DNA

The RFTS is anchored to the catalytic pocked with four hydrogen bonds between E531, D532, D554, L593 in the RFTS domain and K1537, R1576, S1495, T1505 in the catalytic domain, respectively. For this, there exists an energy barrier in removing the RFTS domain from the catalytic pocket to make DNA accessible to the catalytic center. The difference in energy barrier between Dnmt1(291) and Dnmt1(602) for DNA methylation is about 80 kJ/mol (Takeshita et al., 2011). It is, therefore, expected that impairment of the hydrogen bonds formed between the RFTS and catalytic domains makes the RFTS rather easy to be removed from the catalytic pocket by lowering the activation barrier, which means that even the 12-bp DNA may access the catalytic center.

I replaced the amino acid residues, E531 and D532, or D554 in the RFTS domain, which form hydrogen bonds through their side chains (**Figure 9A**), purified the recombinants, and determined the DNA methylation activity of Dnmt1(291) containing the RFTS domain. As shown in **Figure 9B**, the Dnmt1(291) mutations harboring E531A and D532A, and D554K in the RFTS significantly lowered the activation barrier for the DNA methylation activity, which were calculated to be 97 and 90 kJ/mol, respectively. Under identical conditions, the activation energy of Dnmt1(291) and Dnmt1(602) were 133 and 34 kJ/mol, respectively. Expectedly, the replacement of E531 and D532 with alanine or lysine, or D554 with lysine in the RFTS domain of Dnmt1(291) partially but significantly exhibited the DNA methylation activity towards 12-bp hemi-methylated DNA (**Figure 9C**). The result further supports that the 12-bp short hemi-methylated DNA cannot remove the RFTS from the catalytic pocket of wild-type Dnmt1(291), and thus cannot access the catalytic center of Dnmt1.

As shown in **Figure 8C**, the addition of separately prepared RFTS to the reaction mixture inhibited the DNA methylation activity of Dnmt1(602) toward 12-bp hemi-methylated DNA. This is consistent with the report that the DNA methylation activity of Dnmt1 lacking the RFTS domain is inhibited by the addition of the RFTS when a short hemi-methylated hairpin DNA was used as a substrate (Syeda et al., 2011). The inhibition by the RFTS domain carrying the mutations of E531A and D532A, or D554K was less effective compared to that of the wild-type RFTS domain

(**Figure 9D**). Considering that the RFTS cannot stably bind to substrate DNA by itself (**Figure 9E**), this result can be the reflection that the mutants RFTS were less stably anchored into the catalytic pocket due to their impairment in the hydrogen bond formations with the catalytic domain.

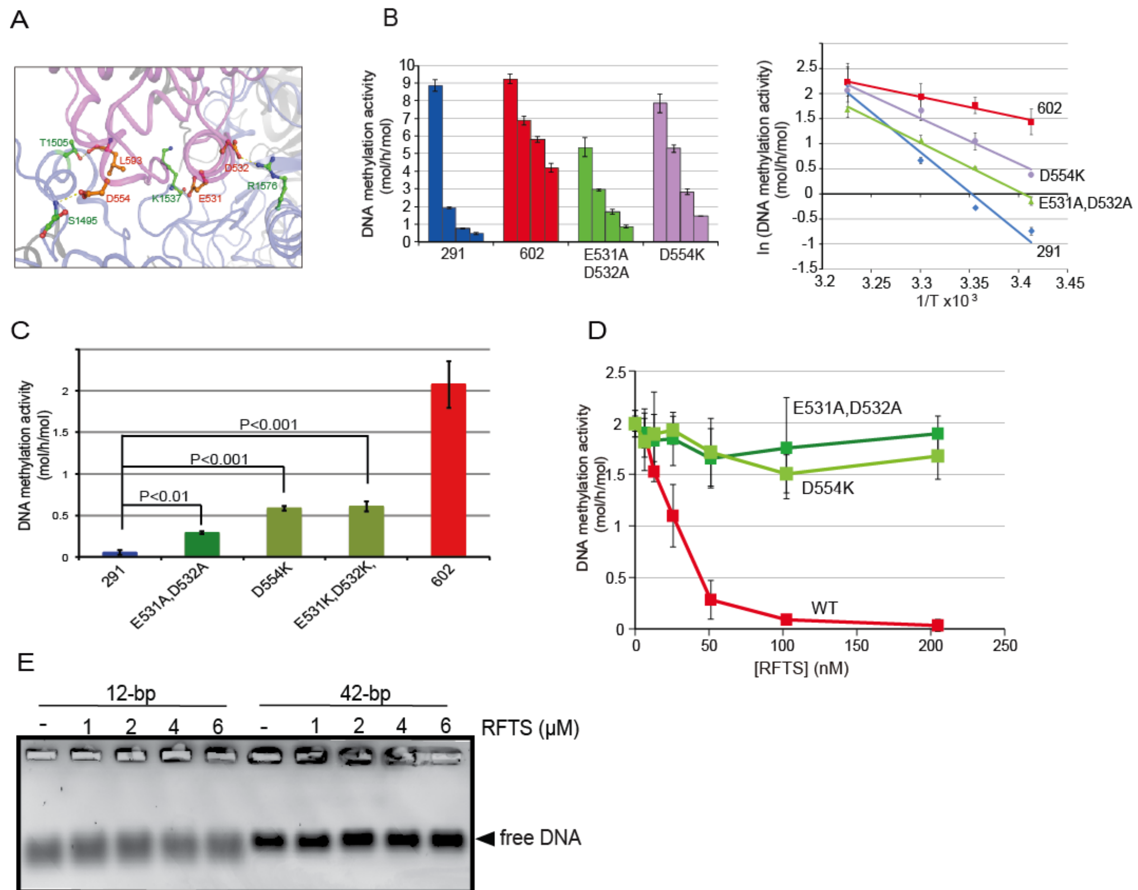


Figure 9. Disruption of the hydrogen bonds facilitates the removal of the RFTS domain from the catalytic pocket of Dnmt1(291). A. Four hydrogen bonds between the RFTS (light violet) and catalytic domains (light blue) of Dnmt1 (PDB accession number, 3AV6). The hydrogen bonds between the side chains of E531, D532, and D554, and the main chain of L593 in the RFTS, and K1537, R1576, S1495, and T1505 in the catalytic domain, respectively, are shown. **B.** DNA methylation activities of Dnmt1(291) (291), Dnmt1(602) (602), and Dnmt1(291) with E531A and D532A, and D554K were determined at 37, 30, 25, and 20ºC (bars from left to right), respectively, towards 42-bp DNA with 12 hemi-methylated CpG. Average activities ± SD (n=3) are shown (left panel). The logarithms of DNA methylation activities obtained {ln(DNA methylation activity), ordinate axis} against inverse temperatures (1/T, abscissa) are plotted (Arrhenius plot) (right panel). **C.** DNA methylation activities of Dnmt1(291) (291), Dnmt1(602) (602), and Dnmt1(291) with the mutations E531A and D532A (E531A, D532A), D554K (D554K), and E531K and D532A (E531K, D532K) were determined towards 12-bp hemi-methylated DNA as in Figure 1. **D.** Dnmt1(602) was incubated with the RFTS domain without any mutation (WT, red), or with mutation E531A, D532A (dark green) or D544K (light green), and then the DNA methylation activity toward 12-bp hemi-methylated DNA was determined. The average values ± SD (n=3) are plotted. E. The indicated amounts of the RFTS were incubated with 12-bp or 42-bp DNA with one hemi-methylated CpG (0.2 μM). After the incubation, the mixtures were subjected to gel electrophoresis, and then DNA was visualized.

3.3. The SRA domain of Uhrf1 promotes DNA methylation

Uhrf1 is a prerequisite factor for the maintenance DNA methylation *in vivo* (Sharif et al., 2007). The SRA domain of Uhrf1 specifically binds hemi-methylated CpG containing DNA and flips the methylated cytosine out of the double-stranded DNA (Arita et al., 2008; Avvakumov et al., 2008; Hashimoto et al., 2008). Since the RFTS domain is responsible in bringing Dnmt1 to the replicating region (Leonhardt et al., 1992) and Uhrf1 is co-localized with Dnmt1 at replication region (Sharif et al., 2007), it is reasonable to speculate that direct or indirect interaction of the RFTS and SRA domains contributes to the removal of the RFTS domain from the catalytic pocket, and thus making DNA access to the catalytic center. According to the *in vitro* binding study, the SRA domain bound to Dnmt1(291) containing the RFTS and the RFTS domain itself. On the other hand, the SRA domain did not bind to the Dnmt1 lacking the RFTS domain (**Figure 10A** and **B**). The results indicate that the RFTS is directly interacting with the SRA domain.

As described above, Dnmt1(291) could not methylate 12-bp hemi-methylated DNA but Dnmt1(602) could. If the SRA domain contributes to the removal of the RFTS domain from the catalytic pocket, Dnmt1(291) containing the RFTS domain may exhibit DNA methylation activity toward the 12-bp DNA by the addition of the SRA domain. As shown in **Figure 10C**, the DNA methylation activity of Dnmt1(291) exhibited significant level of DNA methylation activity depending on the addition of the SRA domain in a dose-dependent manner. Under the conditions, the DNA methylation activity of Dnmt1(602) was not affected by the addition of the SRA. The result indicates that the SRA domain contributes in making 12-bp hemi-methylated DNA access to the catalytic center possibly by removing the RFTS domain from the catalytic pocket. Dnmt1(291) activity was determined under three different concentrations of Dnmt1 titrating with the SRA domain with a fixed amount of 12-bp hemi-methylated DNA (**Figure 10D**). The SRA concentrations necessary for the maximum activation were high when the Dnmt1(291) concentrations used for the DNA methylation activity measurement were high. Interestingly, when the SRA concentrations were normalized with that of Dnmt1(291) and re-plotted, the titration curves using different concentrations of Dnmt1(291) fitted almost into an identical curve (**Figure 10E**). The results strongly suggest that direct interaction between the

SRA and RFTS domains is crucial for the 12-bp hemi-methylated DNA methylation activity.

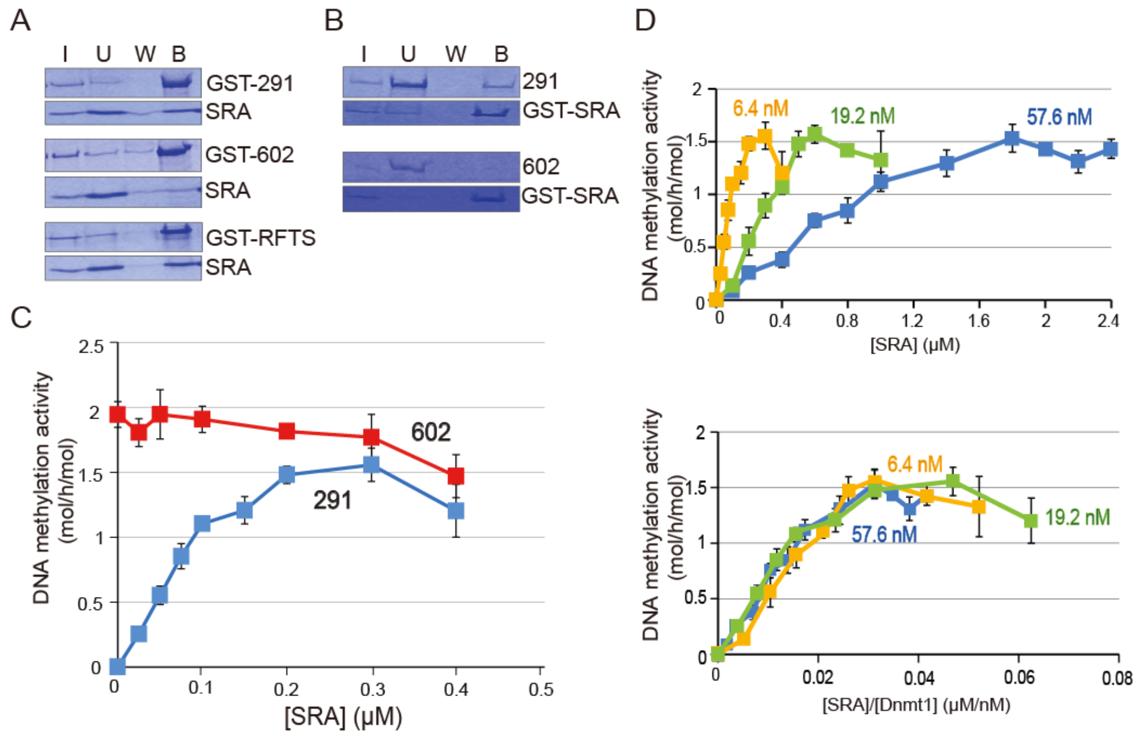


Figure 10. The SRA domain of Uhrf1 binds to the RFTS domain of Dnmt1 and induces DNA methylation activity toward 12-bp hemi-methylated DNA. A. The SRA was incubated with GST-tagged Dnmt1(291) (GST-291), Dnmt1(602) (GST-602), or RFTS (GST-RFTS) bound to GSH-Sepharose. After the incubation, input (I), unbound (U), wash (W), and bound (B) fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The protein bands were visualized by CBB staining. Equivalent amounts of samples were loaded, other than for the input (I) fractions, the amounts loaded being 1/4 of those of the other fractions. **B.** Dnmt1(291) (291) or Dnmt1(602) (602) was incubated with GST-tagged SRA (GST-SRA) bound to GSH-Sepharose. Input (I), unbound (U), wash (W), and bound (B) fractions were analyzed as in panel A. **C.** DNA methylation activities of Dnmt1(291) (blue) and Dnmt1(602) (red) toward 12-bp hemi-methylated DNA were titrated with the SRA. The specific activities of DNA methylation (mol CH_3 transferred/h/mol Dnmt1) were determined, and the average activities ± SD (n=3) are shown. Stimulation of the DNA methylation activities of Dnmt1(291) was statistically significant at all the SRA concentrations examined ($P<0.001$), except for 0.05 μM SRA where $P<0.01$. **D.** DNA methylation activities toward 12-bp hemi-methylated DNA were determined by titrating with the SRA with a fixed amount of DNA and three different concentrations of Dnmt1(291); 6.4 (light brown), 19.2 (light green), and 57.6 nM (blue). Average activities ± SD (n=3) are shown (upper panel). The concentrations of SRA in the upper panel were normalized with Dnmt1 and the DNA methylation activities are re-plotted.

3.4. SRA-dependent short DNA methylation activity is due to direct interaction between the SRA and RFTS domains

Because it is known that the SRA domain specifically binds to hemi-methylated DNA by flipping out the 5mC out of the double stranded DNA (Arita et al., 2008; Avvakumov et al., 2008; Hashimoto et al., 2008), we next asked whether or not the SRA domain could still activate Dnmt1 without binding to DNA. Based on the crystal structure of the SRA in complex with a hemi-methylated DNA, we introduced site-directed mutagenesis on D474 and R496 residues of Uhrf1, which recognize 5mC and orphanated guanine, respectively (**Figure 11A**). The mutant SRA domain with D474A and 496A did not show significant 12-bp DNA binding activity (**Figure 11B**). The methylation activity of Dnmt1(291) was titrated against the mutant SRA domain. Despite the lack of DNA binding activity, the mutant SRA stimulated the DNA methylation activity of Dnmt1(291) (**Figure 11C**). As shown in **Figure 11D**, the mutant SRA domain significantly bound to Dnmt1(291) although its amount was apparently less compared to that of the wild-type SRA domain. The result clearly indicates that the interaction between the RFTS and DNA-free SRA domains solely acts on the stimulation of the DNA methylation activity.

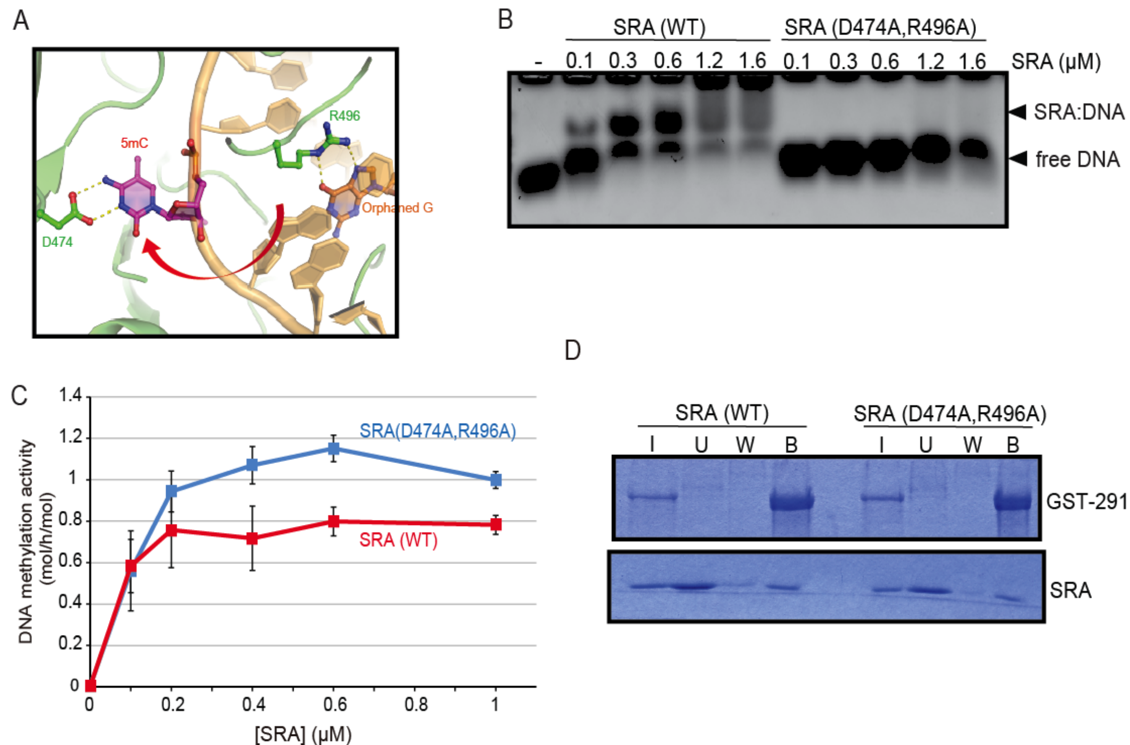


Figure 11. A mutant SRA which cannot bind to hemi-methylated DNA stimulates DNA methylation activity of Dnmt1. A. Image of the structure of the SRA bound to hemi-methylated DNA and flipping out of the 5mC of the double-stranded DNA is shown. D474 is holding 5mC and R496 is stabilizing the orphaned G. The red arrow indicates the flipping of 5mC. The structure data was taken from PDB accession number 2ZKD. **B.** Gel-shift assaying of the SRA and that with D474A, R496A was performed as in Figure 9E. 12-bp hemi-methylated DNA (0.2 μM) was incubated with the indicated amounts of the SRA or the mutant. Free DNA (arrows) and DNA-bound SRA (arrowheads) are shown. **C.** DNA methylation activities of Dnmt1(291) (12.8 μM) towards 12-bp hemi-methylated DNA were determined by titrated with SRA (WT, red) or SRA (D474A, R496A, blue). The average activities ± SD (n=4) are shown. Stimulation of the DNA methylation activities of Dnmt1(291) was statistically significant at all the SRA (D474A, R496A) concentrations examined ($P<0.001$), except for 0.1 μM SRA (D474A, R496A) where $P<0.05$. **D.** The SRA or mutant was incubated with GST-tagged Dnmt1(291) (GST-291) bound to GSH-Sepahrose. After the incubation, input (I), unbound (U), wash (W), and bound (B) fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The protein bands were visualized by CBB staining. Equivalent amounts of samples were loaded, other than for the input (I) fractions, the amounts loaded being 1/4 of those of the other fractions.

3.5. The interaction between the SRA and RFTS domains promotes accession of DNA to the catalytic center

Since the effect of the SRA domain was examined by DNA methylation activity, the assay system involves multi-steps; removal of the RFTS domain from the catalytic pocket, accession of hemi-methylated DNA to the catalytic center, and transfer of methyl group to cytosine. However, it was reported that the SRA domain and Dnmt1 cannot bind to the same hemi-methylated DNA at the same time, due to steric hindrance (Arita et al., 2008). In order to utilize the hemi-methylated DNA bound to the SRA domain, the SRA domain must release the DNA. To this end, the SRA binding to DNA was competed with the RFTS. As shown in **Figure 12A**, the RFTS domain significantly inhibited the DNA binding of the SRA in a dose-dependent manner. The result strongly suggests that the direct interaction between the RFTS and SRA domains forces to release the hemi-methylated DNA from the SRA domain to provide it to the catalytic center as a substrate for DNA methylation. The interaction between the RFTS and SRA domains may facilitate not only the removal of the RFTS domain from the catalytic pocket but also promotes handover of the substrate hemi-methylated DNA to the catalytic center.

It was reported that the cysteine residue at the catalytic center of Dnmt1 covalently traps F-DNA, in which the un-methylated cytosine in the anti-sense strand of hemi-methylated CpG was replaced with fluorocytosine (Brank et al., 2002). To evaluate directly the accession of short hemi-methylated DNA to the catalytic center, 12-bp F-DNA was synthesized. The DNA methylation reactions were performed for Dnmt1(291) and Dnmt1(602) using 12-bp F-DNA, of which 5' end was labeled with ^{32}P, in the absence and presence of the SRA domain. The reaction mixtures were subjected to SDS-polyacrylamide gel electrophoresis, and determined the radioactive bands associated with Dnmt1. As shown in **Figure 12B**, F-DNA scarcely bound to Dnmt1(291) and significantly increased the binding in the presence of the SRA domain. On the contrary, Dnmt1(602) that lacks the RFTS domain showed F-DNA-bound bands regardless of the presence of the SRA domain. The results clearly indicate that the interaction between the RFTS and SRA domains makes the short 12-bp DNA access to the catalytic center. Since the SRA domain with D474A and R496A mutations could enhance the DNA methylation of the 12-bp hemi-methylated DNA (see **Figure 11C**), it can be expected that the mutant SRA domain also can make

access the 12-bp DNA to the catalytic center of Dnmt1. To confirm this, I asked whether or not the SRA domain with D474A, R496A could promote the 12-bp F-DNA binding of Dnmt1(291). Under identical conditions, the SRA domain with D474A and R496A mutations enhanced the 12-bp F-DNA accession to the catalytic center of Dnmt1(291) (**Figure 12C**).

Because Dnmt1(291) exhibited a DNA-length dependent activity, and Dnmt1(291) showed significant DNA methylation activity towards 20-bp hemi-methylated DNA, it can be expected that Dnmt1(291) must significantly bind 20-bp hemi-methylated F-DNA. I performed binding experiment of Dnmt1(291) towards 20-bp hemi-methylated F-DNA. Expectedly, Dnmt1(291) showed significant binding activity to 20-bp hemi-methylated F-DNA even in the absence of the SRA domain. This was again observed for the mutant SRA domain with D474A and R496A (**Supplementary Figure S1**).

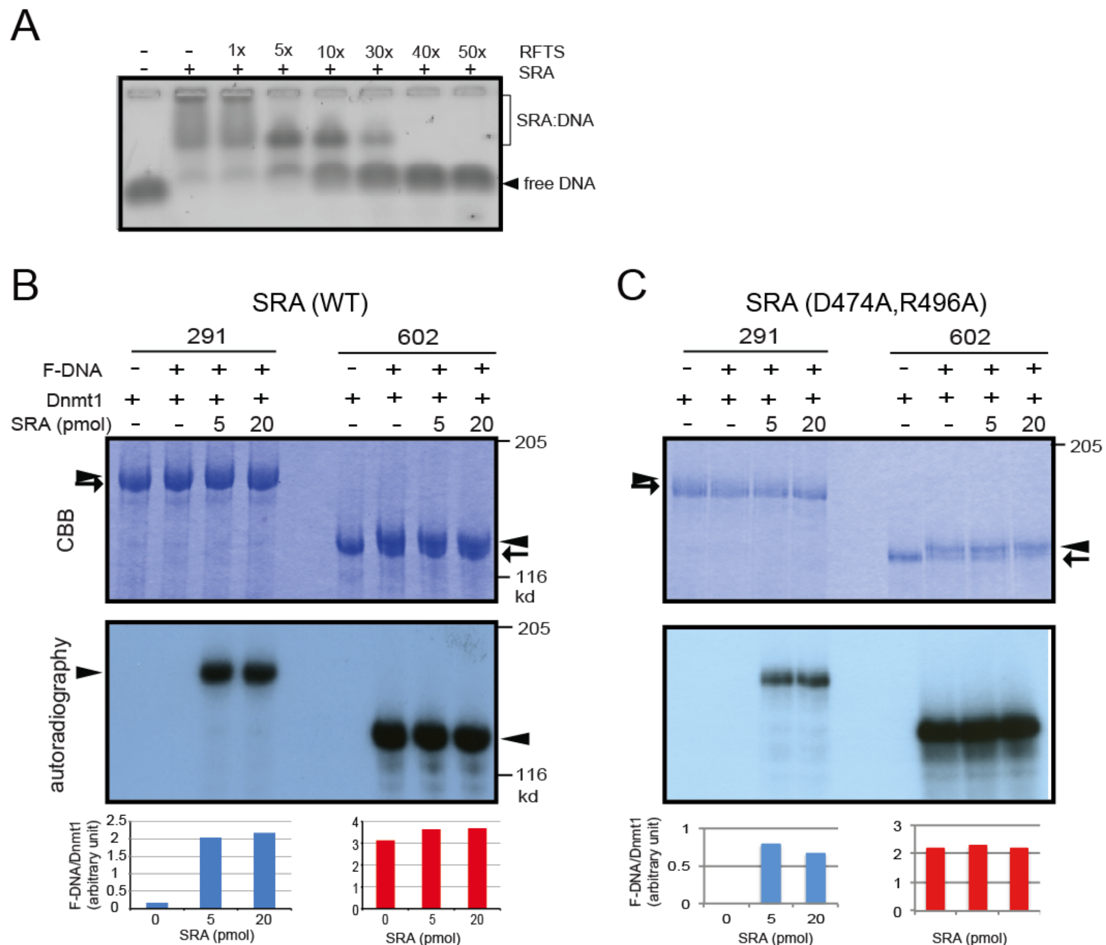


Figure 12. The RFTS and SRA interaction enables the catalytic pocket to access the DNA-1. A. The SRA binding to 12-bp hemi-methylated DNA was competed for by the RFTS in a dose-dependent manner. SRA (0.6 μM) and 12-bp hemi-methylated DNA (0.2 μM) binding was competed for with 1 to 50-fold higher concentrations of RFTS, and then analyzed by gel shift assaying. **B.** Dnmt1(291) or Dnmt1(602) was incubated with 12-bp ^{32}P-labeled F-DNA in the absence or presence of the SRA. **C.** Dnmt1(291) or Dnmt1(602) was incubated with 12-bp ^{32}P-labeled F-DNA in the absence or presence of the SRA (D474A, R496A), which is the DNA binding mutant of the SRA domain. After the reaction, samples were subjected to SDS-polyacrylamide gel electrophoresis, and the protein bands and radiolabeled F-DNA were visualized with CBB (upper panel) and autoradiography (middle panel), respectively. Arrows and arrowheads indicate free Dnmt1 and Dnmt1 bound to F-DNA, respectively. The radioactive bands visualized with a BAS2000 were quantitated and normalized as to those of the CBB-stained Dnmt1 bands (lower panel).

4. DISCUSSION

As described in the previous study, there exists an energy barrier in removing the RFTS domain from the catalytic pocket to provide a room for DNA (Takeshita et al., 2011). This energy barrier is due to the hydrogen bonds formed between the RFTS and catalytic domains. In the present study, DNA longer than 16-bp and the interaction of the RFTS of Dnmt1 with the SRA domain in Uhrf1 are shown to be contributing to the relocation of the RFTS from the catalytic pocket and making hemi-methylated DNA access to the catalytic center.

4.1. DNA length-dependent DNA methylation activity of Dnmt1

Dnmt1(602) could but Dnmt1(291) scarcely methylated 12-bp short hemi-methylated DNA (**Figure 8B**). Since Dnmt1(602) deleted the RFTS domain methylated 12-bp hemi-methylated DNA, the catalytic domain of Dnmt1(602) by itself is able to recognize 12-bp hemi-methylated DNA as methyl group acceptor. Contrarily, hemi-methylated DNA longer than 16-bp was able to be methylated even by Dnmt1(291). Dnnmt1(291) harboring the mutations in the RFTS domain involved in the hydrogen bonds with the catalytic domain showed significant DNA methylation activity towards 12-bp hemi-methylated DNA (**Figure 9B** and **C**), indicating that the impairment in the hydrogen bonds between the RFTS and catalytic domains cannot inhibit completely the accession of 12-bp hemi-methylated DNA to the catalytic center. Longer the hemi-methylated DNA, the DNA apparently removes the RFTS domain from the catalytic pocket for the DNA methylation activity. This is supported by the observation that the exogenously added RFTS domain effectively inhibited the DNA methylation activity of Dnmt1(602) not for 42-bp but short 12-bp hemi-methylated DNA as methyl group acceptor (**Figure 8C**). In addition, mutations of the RFTS in the residues involved in the hydrogen bond formation was less effective in inhibiting the DNA methylation activity (**Figure 9D**). Apparently, DNA methylation activity of Dnmt1(291) reached maximal when the DNA was longer than 30-bp (**Figure 8B**). It was reported that 12-bp hemi-methylated DNA is fitted into the catalytic pocket of Dnmt1 and thus DNA is not protruded from the Dnmt1 (Song et al., 2012). The present study suggests that hemi-methylated DNA longer than 16-bp can interact directly with the Dnmt1(291) to remove the RFTS domain from the catalytic pocket. Considering that Dnmt1(291-1620) can processively methylate hemi-methylated DNA (Vilkaitis et

al., 2005), the extra DNA sequence longer than 16-bp that is estimated to interact with Dnmt1's catalytic pocket may contribute to the processive methylation property and the initial loading onto a replication foci of Dnmt1 on genomic DNA.

In addition, I noticed that there was a gap between the enhancement of DNA methylation activity and F-DNA binding of the SRA domain with DNA binding mutant (see **Figures 11** and **12**). Although the enhancing by the mutant SRA domain is almost identical to that of the wild-type SRA domain but the F-DNA binding level was significantly less for the mutant SRA domain. This may indicate that Dnmt1 moving away from the already methylation site seems to be the rate limiting step. In order to move away from the methylation site, it can be speculated that Dnmt1 may require longer DNA length than 12-bp. Not only for the initial interaction, but also for the moving away, Dnmt1 may require long DNA for the methylation.

4.2. Significance of the interaction between the RFTS and the SRA

The RFTS domain is responsible in recruiting Dnmt1 to the replication region (Leonhardt et al., 1992), and interacts with Uhrf1 during replication (Sharif et al., 2007). Uhrf1 is the prerequisite factor for the maintenance DNA methylation *in vivo* (Sharif et al., 2007), and the removal of the RFTS from the catalytic pocket was dependent on the interaction of Dnmt1 with Uhrf1 at the replicating region (This work; Berkyurek et al., 2014). The interaction of the SRA domain of Uhrf1 with the RFTS domain of Dnmt1 to remove the RFTS from the catalytic pocket and providing hemi-methylated DNA to the catalytic center can be a fail-safe mechanism that contributes to the faithful inheritance of the methylation patterns to next generation. Since the SRA binds selectively to hemi-methylated DNA (Arita et al., 2008; Avvakumov et al., 2008; Hashimoto et al., 2008), the function of the SRA may not only be limited to the replacement of the RFTS domain from the catalytic pocket of the Dnmt1 but also to the handover step of hemi-methylated DNA. The RFTS and SRA interaction-dependent release of hemi-methylated DNA (**Figure 12A**) following the removal of the RFTS domain from the catalytic pocket is an important step of the handover of hemi-methylated DNA to the catalytic center of Dnmt1.

4.3. Proposed model and future aspects

Based on the present study, we propose that Dnmt1 loads onto the replication foci by DNA-length dependent activity and methylates DNA in a processive manner. Yet the detailed mechanisms of how a long DNA pushes the RFTS domain out of the catalytic pocket remain to be investigated. A structural study of a Dnmt1 fragment in complex with a long hemi-methylated DNA may provide answer to reveal the mechanism. As discussed earlier in this chapter, the CXXC domain might have a crucial role in this mechanism.

REFERENCES

Achour, M., Mousli, M., Alhosin, M., Ibrahim, A., Peluso, J., Muller, C. D., Schini-Kerth, V. B., Hamiche, A., Dhe-Paganon, S., and Bronner, C. (2013) Epigallocatechin-3-gallate up-regulates tumor suppressor gene expression via a reactive oxygen species-dependent down-regulation of UHRF1. Biochem. Biophys. Res. Commun. *430*, 208–212.

Antequera, F. and Bird, A. (1993) Number of CpG islands and genes in human and mouse. Proc. Natl. Acad. Sci. U. S. A. *90*, 11995–11999.

Aoki, A., Suetake, I., Miyagawa, J., Fujio, T., Chijiwa, T., Sasaki, H., and Tajima, S. (2001) Enzymatic properties of *de novo*-type mouse DNA (cytosine-5) methyltransferases. Nucl. Acids Res. *29*, 3506–3512.

Arita, K., Ariyoshi, M., Tochio, H., Nakamura, Y., and Shirakawa, M. (2008) Recognition of hemi-methylated DNA by the SRA protein UHRF1 by a base-flipping mechanism. Nature *455*, 818–821.

Arita, K., Isogai, S., Oda, T., Unoki, M., Sugita, K., Sekiyama, N., Kuwata, K., Hamamoto, R., Tochio, H., Sato, M., Ariyoshi, M., and Shirakawa, M. (2012) Recognition of modification status on a histone H3 tail by linked histone reader modules of the epigenetic regulator UHRF1. Proc. Natl. Acad. Sci. U. S. A. *109*, 12950–12955.

Avvakumov, G. V, Walker, J. R., Xue, S., Li, Y., Duan, S., Bronner, C., Arrowsmith, C. H., and Dhe-Paganon, S. (2008) Structural basis for recognition of hemi-methylated DNA by the SRA domain of human UHRF1. Nature *455*, 822–825.

Ball, M. P., Li, J. B., Gao, Y., Lee, J., and Leproust, E. (2009) Targeted and genome-scale methylomics reveals gene body signatures in human cell lines. Nat. Biotechnol. *27*, 361–368.

Bártová, E., Krejčí, J., Harničarová, A., Galiová, G., and Kozubek, S. (2008) Histone modifications and nuclear architecture: A review. J. Histochem. Cytochem. *56* , 711–721.

Bashtrykov, P., Jankevicius, G., Jurkowska, R. Z., Ragozin, S., and Jeltsch, A. (2014a) The UHRF1 protein stimulates the activity and specificity of the maintenance DNA methyltransferase DNMT1 by an allosteric mechanism. J. Biol. Chem. *289*, 4106–4115.

Bashtrykov, P., Rajavelu, A., Hackner, B., Ragozin, S., Carell, T., and Jeltsch, A. (2014b) Targeted mutagenesis results in an activation of DNA methyltransferase 1 and confirms an autoinhibitory role of its RFTS domain. ChemBioChem *15*, 743–748.

Bell, A. C. and Felsenfeld, G. (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature *405*, 482–485.

Bell, O., Tiwari, V. K., Thomä, N. H., and Schübeler, D. (2011) Determinants and dynamics of genome accessibility. Nat. Rev. Genet. *12*, 554–564.

Berkyurek, A. C., Suetake, I., Arita, K., Takeshita, K., Nakagawa, A., Shirakawa, M., and Tajima, S. (2014) The DNA methyltransferase Dnmt1 directly interacts with the SET and RING finger-associated (SRA) domain of the multifunctional protein Uhrf1 to facilitate accession of the catalytic center to hemi-methylated DNA. J. Biol. Chem. *289*, 379–386.

Bestor, T., Laudano, A., Mattaliano, R., and Ingram, V. (1988) Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells: The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. J. Mol. Biol. *203*, 971–983.

Brank, A. S., Van Bemmel, D. M., and Christman, J. K. (2002) Optimization of baculovirus-mediated expression and purification of hexahistidine-tagged murine DNA (cytosine-C5)-methyltransferase-1 in *Spodoptera frugiperda* 9 cells. Protein Expr. Purif. *25*, 31–40.

Cedar, H. and Bergman, Y. (2009) Linking DNA methylation and histone modification: Patterns and paradigms. Nat. Rev. Genet. *10*, 295–304.

Chen, T., Tsujimoto, N., and Li, E. (2004) The PWWP domain of Dnmt3a and Dnmt3b is required for directing DNA methylation to the major satellite repeats at pericentric heterochromatin. Mol. Cell. Biol. *24*, 9048–9058.

Chu, J., Loughlin, E. A., Gaur, N. A., SenBanerjee, S., Jacob, V., Monson, C., Kent, B., Oranu, A., Ding, Y., Ukomadu, C., and Sadler, K. C. (2012) UHRF1 phosphorylation by cyclin A2/cyclin-dependent kinase 2 is required for zebrafish embryogenesis. Mol. Biol. Cell *23*, 59–70.

Chuang, L. S. H., Ian, H. I., Koh, T. W., Ng, H. H., Xu, G., and Li, B. F. L. (1997) Human DNA-(Cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. Science *277* , 1996–2000.

Clouaire, T. and Stancheva, I. (2008) Methyl-CpG binding proteins: Specialized transcriptional repressors or structural components of chromatin? Cell. Mol. Life Sci. *65*, 1509–1522.

Daskalos, A., Oleksiewicz, U., Filia, A., Nikolaidis, G., Xinarianos, G., Gosney, J. R., Malliri, A., Field, J. K., and Liloglou, T. (2011) UHRF1-mediated tumor suppressor gene inactivation in nonsmall cell lung cancer. Cancer *117*, 1027–1037.

Filion, G. J. P., Zhenilo, S., Salozhin, S., Yamada, D., Prokhortchouk, E., and Defossez, P. A. (2006) A family of human zinc finger proteins that bind methylated DNA and repress transcription. Mol. Cell. Biol. *26*, 169–181.

Frauer, C., Hoffmann, T., Bultmann, S., Casa, V., Cardoso, M. C., Antes, I., and Leonhardt, H. (2011) Recognition of 5-hydroxymethylcytosine by the Uhrf1 SRA domain. PLoS One *6*, e21306.

Fujita, N., Watanabe, S., Ichimura, T., Tsuruzoe, S., Shinkai, Y., Tachibana, M., Chiba, T., and Nakao, M. (2003) Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. J. Biol. Chem. *278* , 24132–24138.

Ge, Y. Z., Pu, M. T., Gowher, H., Wu, H. P., Ding, J. P., Jeltsch, A., and Xu, G. L. (2004) Chromatin targeting of *de novo* DNA methyltransferases by the PWWP domain. J. Biol. Chem. *279* , 25447–25454.

Gelato, K. A., Tauber, M., Ong, M. S., Winter, S., Hiragami-Hamada, K., Sindlinger, J., Lemak, A., Bultsma, Y., Houliston, S., Schwarzer, D., Divecha, N., Arrowsmith, C. H., and Fischle, W. (2014) Accessibility of different histone H3-binding domains of UHRF1 is allosterically regulated by phosphatidylinositol 5-phosphate. Mol. Cell *54*, 905–919.

Goll, M. G. and Bestor, T. H. (2005) Eukaryotic cytosine methyltransferases. Annu. Rev. Biochem. *74*, 481–514.

Goll, M. G., Kirpekar, F., Maggert, K. A, Yoder, J. A, Hsieh, C. L., Zhang, X., Golic, K. G., Jacobsen, S. E., and Bestor, T. H. (2006) Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science *311*, 395–398.

Guo, X., Wang, L., Li, J., Ding, Z., Xiao, J., Yin, X., He, S., Shi, P., Dong, L., Li, G., Tian, C., Wang, J., Cong, Y., and Xu, Y. (2015) Structural insight into autoinhibition and histone H3-induced activation of DNMT3A. Nature *517*, 640–644.

Hahn, M. A., Wu, X., Li, A. X., Hahn, T., and Pfeifer, G. P. (2011) Relationship between gene body DNA methylation and intragenic H3K9me3 and H3K36me3 chromatin marks. PLoS One *6*, e18844.

Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J., and Surani, M. A. (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech. Dev. *117*, 15–23.

Hark, A. T., Schoenherr, C. J., Katz, D. J., Ingram, R. S., Levorse, J. M ., and Tilghman, S. M. (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature *405*, 486–489.

Hashimoto, H., Horton, J. R., Zhang, X., Bostick, M., Jacobsen, S. E., and Cheng, X. (2008) The SRA domain of UHRF1 flips 5-methylcytosine out of the DNA helix. Nature *455*, 826–829.

Hashimoto, H., Horton, J. R., Zhang, X., and Cheng, X. (2009) UHRF1, a modular multi-domain protein, regulates replication-coupled crosstalk between DNA methylation and histone modifications. Epigenetics *4*, 8–14.

Hashimoto, H., Zhang, X., and Cheng, X. (2012) Excision of thymine and 5-hydroxymethyluracil by the MBD4 DNA glycosylase domain: Structural basis and implications for active DNA demethylation. Nucl. Acids Res. *40* , 8276–8284.

Hendrich, B. and Tweedie, S. (2015) The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends Genet. *19*, 269–277.

Hervouet, E., Lalier, L., Debien, E., Cheray, M., Geairon, A., Rogniaux, H., Loussouarn, D., Martin, S. A., Vallette, F. M., and Cartron, P. F. (2010) Disruption of Dnmt1/PCNA/UHRF1 interactions promotes tumorigenesis from human and mice glial cells. PLoS One *5*, e11333.

Hite, K. C., Adams, V. H., and Hansen, J. C. (2009) Recent advances in MeCP2 structure and function. Biochem. Cell Biol. *87*, 219–227.

Hotchkiss, R. D. (1948) The quantitative separation of purines, pyrimidine, and nucleosides by paper chromatography. J. Biol. Chem. *175*, 315-332.

Huang, H., Sabari, B. R., Garcia, B. A., Allis, C. D., and Zhao, Y. (2015) Snap shot: Histone modifications. Cell *159*, 458.

Huh, I., Zeng, J., Park, T., and Yi, S. V. (2013) DNA methylation and transcriptional noise. Epigenetics Chromatin *6*, 9.

Illingworth, R. S. and Bird, A. P. (2015) CpG islands - “A rough guide.” FEBS Lett. *583*, 1713–1720.

Inano, K., Suetake, I., Ueda, T., Miyake, Y., Nakamura, M., Okada, M., and Tajima, S. (2000) Maintenance-type DNA methyltransferase is highly expressed in post-mitotic neurons and localized in the cytoplasmic compartment. J. Biochem. *128* , 315–321.

Iwano, H., Nakamura, M., and Tajima, S. (2004) *Xenopus* MBD3 plays a crucial role in an early stage of development. Dev. Biol. *268*, 416–428.

Jjingo, D., Conley, A. B., Yi, S. V, Lunyak, V. V, and Jordan, I. K. (2012). On the presence and role of human gene-body DNA methylation. Oncotarget *3*, 462–474.

Kameshita, I., Sekiguchi, M., Hamasaki, D., Sugiyama, Y., Hatano, N., Suetake, I., Tajima, S., and Sueyoshi, N. (2008) Cyclin-dependent kinase-like 5 binds and phosphorylates DNA methyltransferase 1. Biochem. Biophys. Res. Commun. *377*, 1162–1167.

Kim, G. D., Ni, J., Kelesoglu, N., Roberts, R. J., and Pradhan, S. (2002) Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. EMBO J. *21*, 4183–4195.

Kim, S. W., Park, J. I., Spring, C. M., Sater, A. K., Ji, H., Otchere, A. A., Daniel, J. M., and McCrea, P. D. (2004) Non-canonical Wnt signals are modulated by the Kaiso transcriptional repressor and p120-catenin. Nat. Cell Biol. *6*, 1212–1220.

Kimura, H., Suetake, I., and Tajima, S. (1999) *Xenopus* maintenance-type DNA methyltransferase is accumulated and translocated into germinal vesicles of oocytes. J. Biochem. *125* , 1175–1182.

Kinde, B., Gabel, H. W., Gilbert, C. S., Griffith, E. C., and Greenberg, M. E. (2015). Reading the unique DNA methylation landscape of the brain: Non-CpG methylation, hydroxymethylation, and MeCP2. Proc. Natl. Acad. Sci. U. S. A. *112*, 6800-6806.

Kumaki, Y., Oda, M., and Okano, M. (2008) QUMA: Quantification tool for methylation analysis. Nucl. Acids Res. *36*, 170–175.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J., and Wilson, G. G. (1994) The DNA (cytosine-5) methyltransferases. Nucl. Acids Res. *22*, 1–10.

Law, J. A. and Jacobsen, S. E. (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and and animals. Nat. Rev. Genet. *11*, 204–220.

Leonhardt, H., Page, A. W., Weier, H. U., and Bestor, T. H. (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell *71*, 865–873.

Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., Nery, J. R., Lee, L., Ye, Z., Ngo, Q., Edsall, L., Antosiewicz, Bourget, J., Stewart, R., Routti, V., Millar, A. H., Thomson, J. A., Ren, B., and Ecker, J. R. (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature *462*, 315–322.

Maxwell, A. and Gellert, M. (1984) The DNA dependence of the ATPase activity of DNA gyrase. J. Biol. Chem. *259* , 14472–14480.

Meilinger, D., Fellinger, K., Bultmann, S., Rothbauer, U., Bonapace, I. M., Klinkert, W. E. F., Spada, F., and Leonhardt, H. (2009) Np95 interacts with *de novo* DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. EMBO Rep. *10*, 1259–1264.

Mertineit, C., Yoder, J. A., Taketo, T., Laird, D. W., Trasler, J. M., and Bestor, T. H. (1998) Sex-specific exons control DNA methyltransferase in mammalian germ cells. Development *125* , 889–897.

Monk, M., Boubelik, M., and Lehnert, S. (1987) Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development *99* , 371–382.

Moréra, S., Grin, I., Vigouroux, A., Couvé, S., Henriot, V., Saparbaev, M., and Ishchenko, A. A. (2012) Biochemical and structural characterization of the glycosylase domain of MBD4 bound to thymine and 5-hydroxymethyluracil-containing DNA. Nucl. Acids Res. *40* , 9917–9926.

Morgan, H., Sutherland, H., Martin, D., and Whitelaw, E. (1999) Epigenetic inheritance at the agouti locus in the mouse. Nat. Genet. *23*, 314–318.

Nishiyama, A., Yamaguchi, L., Sharif, J., Johmura, Y., Kawamura, T., Nakanishi, K., Shimamura, S., Arita, K., Kodama, T., Ishikawa, F., Koseki, H., and Nakanishi, M. (2013). Uhrf1-dependent H3K23 ubiquitylation couples maintenance DNA methylation and replication. Nature *502*, 249–253.

Otani, J., Nankumo, T., Arita, K., Inamoto, S., Ariyoshi, M., and Shirakawa, M. (2009) Structural basis for recognition of H3K4 methylation status by the DNA methyltransferase 3A ATRX-DNMT3-DNMT3L domain. EMBO Rep. *10*, 1235–1241.

Otani, J., Arita, K., Kato, T., Kinoshita, M., Kimura, H., Suetake, I., Tajima, S., Ariyoshi, M., and Shirakawa, M. (2013a) Structural basis of the versatile DNA recognition ability of the methyl-CpG binding domain of methyl-CpG binding domain protein 4. J. Biol. Chem. *288* , 6351–6362.

Otani, J., Kimura, H., Sharif, J., Endo, T.A., Mishima, Y., Kawakami, T., Koseki, H., Shirakawa, M., Suetake, I., and Tajima, S. (2013b) Cell cycle-dependent turnover of 5-hydroxymethyl cytosine in mouse embryonic stem cells. PLoS One *8*, e82961.

Pichler, G., Wolf, P., Schmidt, C. S., Meilinger, D., Schneider, K., Frauer, C., Fellinger, K., Rottach, A., and Leonhardt, H. (2011) Cooperative DNA and histone binding by Uhrf2 links the two major repressive epigenetic pathways. J. Cell. Biochem. *112*, 2585-2593.

Pradhan, S. and Kim, G. D. (2002) The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity. EMBO J. *21*, 779–788.

Qiu, C., Sawada, K., Zhang, X., and Cheng, X. (2002) The PWWP domain of mammalian DNA methyltransferase Dnmt3b defines a new family of DNA-binding folds. Nat. Struct. Biol. *9*, 217–224.

Rajakumara, E., Wang, Z., Ma, H., Hu, L., Chen, H., Lin, Y., Guo, R., Wu, F., Li, H., Lan, F., Shi, Y. G., Xu, Y., Patel, D. J., and Shi, Y. (2011) PHD finger recognition of unmodified histone H3R2 links UHRF1 to regulation of euchromatic gene expression. Mol. Cell *43*, 275–284.

Raskó, T., Finta, C., and Kiss, A. (2000) DNA bending induced by DNA (cytosine-5) methyltransferases. Nucl. Acids Res. *28*, 3083–3091.

Reik, W. (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature *447*, 425–432.

Rothbart, S. B., Krajewski, K., Nady, N., Tempel, W., Xue, S., Badeaux, A. I., Barsyte-Lovejoy, D., Martinez, J. Y., Bedford, M. T., Fuchs, S. M., Arrowsmith, C. H., and Strahl, B. D. (2012) Association of UHRF1 with H3K9 methylation directs the maintenance of DNA methylation. Nat. Struct. Mol. Biol. *19*, 1155–1160.

Rottach, A., Leonhardt, H., and Spada, F. (2009a) DNA methylation-mediated epigenetic control. J. Cell. Biochem. *108*, 43–51.

Rottach, A., Frauer, C., Pichler, G., Bonapace, I. M., Spada, F., and Leonhardt, H. (2009b) The multi-domain protein Np95 connects DNA methylation and histone modification. Nucl. Acids Res. *38*, 1796–1804.

Rougier, N., Bourc'his, D., Gomes, D. M., Niveleau, A., Plachot, M., Pàldi, A., and Viegas-Péquignot, E. (1998) Chromosome methylation patterns during mammalian preimplantation development. Genes Dev. *12* , 2108–2113.

Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000) DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat. Genet. *25*, 269–277.

Di Ruscio, A., Ebralidze, A. K., Benoukraf, T., Amabile, G., Goff, L. A., Terragni, J., Figueroa, M. E., De Figueiredo Pontes, L. L., Alberich-Jorda, M., Zhang, P., Wu, M., D`Alo, F., Melnick, A., Leone, G., Ebralidze, K. K., Pradhan, S., Rinn, J. L., and Tenen, D. G. (2013) DNMT1-interacting RNAs block gene-specific DNA methylation. Nature *503*, 371–376.

Sakai, Y., Suetake, I., Shinozaki, F., Yamashina, S., and Tajima, S. (2004) Co-expression of *de novo* DNA methyltransferases Dnmt3a2 and Dnmt3L in gonocytes of mouse embryos. Gene Expr. Patterns *5*, 231–237.

Sandelin, A., Carninci, P., Lenhard, B., Ponjavic, J., Hayashizaki, Y., and Hume, D. A. (2007) Mammalian RNA polymerase II core promoters: Insights from genome-wide studies. Nat. Rev. Genet. *8*, 424–436.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. *74*, 5463–5467.

Sarraf, S. A. and Stancheva, I. (2015) Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. Mol. Cell *15*, 595–605.

Sato, N., Kondo, M., and Arai, K. (2006) The orphan nuclear receptor GCNF recruits DNA methyltransferase for Oct-3/4 silencing. Biochem. Biophys. Res. Commun. *344*, 845–851.

Sharif, J., Muto, M., Takebayashi, S., Suetake, I., Iwamatsu, A., Endo, T. A, Shinga, J., Mizutani-Koseki, Y., Toyoda, T., Okamura, K., Tajima, S., Mitsuya, K., Okano, M., and Koseki, H. (2007) The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. Nature *450*, 908–912.

Shirane, K., Toh, H., Kobayashi, H., Miura, F., Chiba, H., Ito, T., Kono, T., and Sasaki, H. (2013) Mouse oocyte methylomes at base resolution reveal genome-wide accumulation of non-CpG methylation and role of DNA methyltransferases. PLoS Genet. *9*, e1003439.

Song, J., Rechkoblit, O., Bestor, T. H., and Patel, D. J. (2011) Structure of DNMT1-DNA complex reveals a role for autoinhibition in maintenance DNA methylation. Science *331*, 1036–1040.

Song, J., Teplova, M., Ishibe-Murakami, S., and Patel, D. J. (2012) Structure-based mechanistic insights into DNMT1-mediated maintenance DNA methylation. Science *335*, 709–712.

Steele, R. E. and Rae, P. M. M. (1980) Ordered distribution of modified bases in the DNA of a dinoflagellate. Nucl. Acids Res. *8*, 4709–4726.

Suetake, I., Miyazaki, J., Murakami, C., Takeshima, H., and Tajima, S. (2003) Distinct enzymatic properties of recombinant mouse DNA methyltransferases Dnmt3a and Dnmt3b. J. Biochem. *133*, 737–744.

Suetake, I., Shinozaki, F., Miyagawa, J., Takeshima, H., and Tajima, S. (2004) DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. J. Biol. Chem. *279*, 27816–27823.

Suetake, I., Hayata, D., and Tajima, S. (2006) The amino-terminus of mouse DNA methyltransferase 1 forms an independent domain and binds to DNA with the sequence involving PCNA binding motif. J. Biochem. *140*, 763–776.

Sugiyama, Y., Hatano, N., Sueyoshi, N., Suetake, I., Tajima, S., Kinoshita, E., Kinoshita-Kikuta, E., Koike, T., and Kameshita, I. (2010) The DNA-binding activity of mouse DNA methyltransferase 1 is regulated by phosphorylation with casein kinase 1delta/epsilon. Biochem. J. *427*, 489–497.

Suzuki, M. M. and Bird, A. (2008) DNA methylation landscapes: Provocative insights from epigenomics. Nat. Rev. Genet. *9*, 465–476.

Syeda, F., Fagan, R. L., Wean, M., Avvakumov, G. V., Walker, J. R., Xue, S., Dhe-Paganon, S., and Brenner, C. (2011) The replication focus targeting sequence (RFTS) domain is a DNA-competitive inhibitor of Dnmt1. J. Biol. Chem. *286*, 15344–15351.

Szyf, M., Bozovic, V., and Tanigawa, G. (1991) Growth regulation of mouse DNA methyltransferase gene expression. J. Biol. Chem. *266*, 10027–10030.

Tajima, S. and Suetake, I. (1998) Regulation and function of DNA methylation in vertebrates. J. Biochem. *123* , 993–999.

Takeshita, K., Suetake, I., Yamashita, E., Suga, M., Narita, H., Nakagawa, A., and Tajima, S. (2011) Structural insight into maintenance methylation by mouse DNA methyltransferase 1 (Dnmt1). Proc. Natl. Acad. Sci. U. S. A. *108*, 9055–9059.

Thomas, J. O., and Kornberg, R. D. (1975) An octamer of histones in chromatin and free in solution. Proc. Natl. Acad. Sci. U. S. A. *72*, 2626–2630.

Unoki, M., Brunet, J., and Mousli, M. (2009) Drug discovery targeting epigenetic codes: The great potential of UHRF1, which links DNA methylation and histone modifications, as a drug target in cancers and toxoplasmosis. Biochem. Pharmacol. *78*, 1279–1288.

Ushijima, T., Watanabe, N., Okochi, E., Kaneda, A., Sugimura, T., and Miyamoto, K. (2003) Fidelity of the methylation pattern and its variation in the genome. Genome Res. *13*, 868–874.

Ushijima, T., Watanabe, N., Shimizu, K., Miyamoto, K., Sugimura, T., and Kaneda, A. (2005) Decreased fidelity in replicating CpG methylation patterns in cancer cells. Cancer Res. *65*, 11–17.

Vilkaitis, G., Suetake, I., Klimašauskas, S., and Tajima, S. (2005) Processive methylation of hemimethylated CpG sites by mouse Dnmt1 DNA methyltransferase. J. Biol. Chem. *280*, 64–72.

Wang, L., Zhao, Y., Bao, X., Zhu, X., Kwok, Y. K., Sun, K., Chen, X., Huang, Y., Jauch, R., Esteban, M. A., Esteban, M.A., Sun, H., and Wang, H. (2015) LncRNA Dum interacts with Dnmts to regulate Dppa2 expression during myogenic differentiation and muscle regeneration. Cell Res. *25*, 335–350.

Watanabe, D., Suetake, I., Tada, T., and Tajima, S. (2002) Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. Mech. Dev. *118*, 187–190.

Weber, M., Davies, J. J., Wittig, D., Oakeley, E. J., Haase, M., Lam, W. L., and Schubeler, D. (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat. Genet. *37*, 853–862.

Winkelmann, J., Lin, L., Schormair, B., Kornum, B. R., Faraco, J., Plazzi, G., Melberg, A., Cornelio, F., Urban, A. E., Pizza, F., Poli, F., Grubert, F., Wieland, T., Graf, E., Hallmayer, J., Strom, T. M., and Mignot, E. (2012) Mutations in DNMT1 cause autosomal dominant cerebellar ataxia, deafness and narcolepsy. Hum. Mol. Genet. *21*, 2205–2210.

Wu, B. K., Mei, S. C., and Brenner, C. (2014) RFTS-deleted DNMT1 enhances tumorigenicity with focal hypermethylation and global hypomethylation. Cell Cycle *13*, 3222–3231.

Wyatt, G. R. (1951) Recognition and estimation of 5-methylcytosine in nucleic acids. Biochem. J. *48*, 581–584.

Yang, N. and Xu, R. M. (2012) Structure and function of the BAH domain in chromatin biology. Crit. Rev. Biochem. Mol. Biol. *48*, 211–221.

Yoon, H.G., Chan, D. W., Reynolds, A. B., Qin, J., and Wong, J. (2003) N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding Protein Kaiso. Mol. Cell *12*, 723–734.

Zhang, Y., Jurkowska, R., Soeroes, S., Rajavelu, A., Dhayalan, A., Bock, I., Rathert, P., Brandt, O., Reinhardt, R., Fischle, W., and Jeltsch, A. (2010) Chromatin methylation activity of Dnmt3a and Dnmt3a/3L is guided by interaction of the ADD domain with the histone H3 tail. Nucl. Acids Res. *38*, 4246–4253.

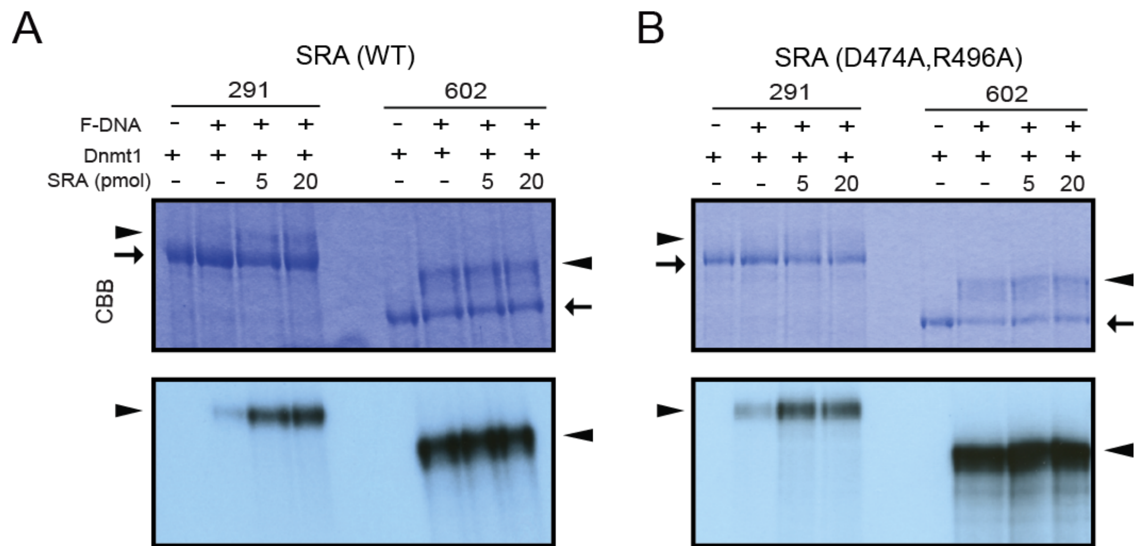
Zhou, T., Xiong, J., Wang, M., Yang, N., Wong, J., Zhu, B., and Xu, R. M. (2015) Structural basis for hydroxymethylcytosine recognition by the SRA domain of UHRF2. Mol. Cell *54*, 879–886.

Zhu, B., Zheng, Y., Angliker, H., Schwarz, S., Thiry, S., Siegmann, M., and Jost, J. P. (2000) 5-methylcytosine DNA glycosylase activity is also present in the human MBD4 (G/T mismatch glycosylase) and in a related avian sequence. Nucl. Acids Res. *28*, 4157–4165.

ACKNOWLEDGEMENTS

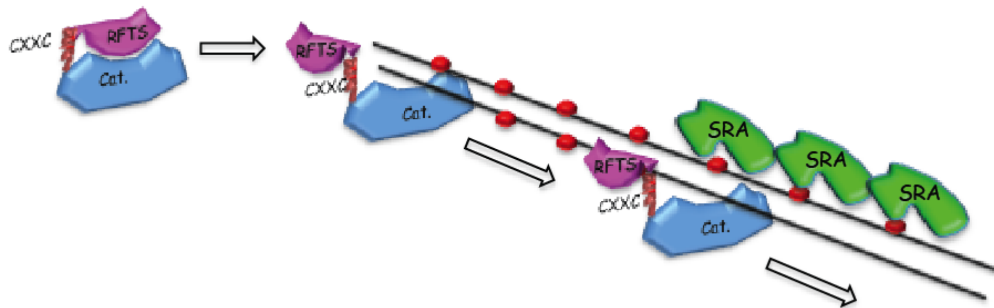
I would like to express my profound gratitude to my supervisor Prof. Dr. Shoji Tajima (Laboratory Epigenetics, IPR, Osaka University) for his patience, scientific discussions, support on conducting research and moral support during my stay in a foreign country. I consider myself fortunate. I am also deeply indebted to Drs. Isao Suetake and Hironobu Kimura for scientific discussions. I would like to thank Ms. Shinohara and Yamagami for their great effort in preparation of baculovirus expression system and protein purification. Also, a very special thanks goes to members of Laboratory of Epigenetics for their friendship and support.

Additionally, I would like to thank to Prof. Dr. Akira Shinohara (IPR, Osaka University) and Prof. Dr. Toshiharu Hase for reviewing my thesis.

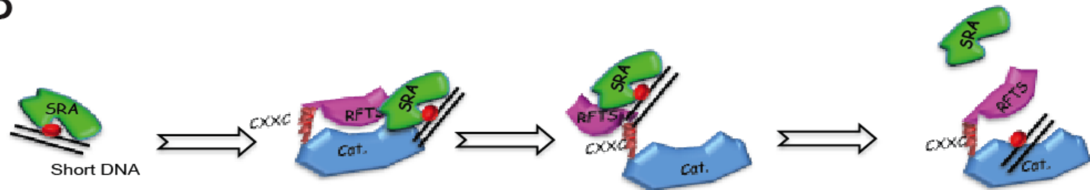


Supplementary Figure-S1. The RFTS and SRA interaction enables the catalytic pocket to access the DNA-2. A. Dnmt1(291) or Dnmt1(602) was incubated with 20-bp ^{32}P-labeled F-DNA in the absence or presence of the SRA. **B.** Dnmt1(291) or Dnmt1(602) was incubated with 12-bp ^{32}P-labeled F-DNA in the absence or presence of the SRA (D474A, R496A), which is the DNA binding mutant of the SRA domain. After the reaction, samples were subjected to SDS-polyacrylamide gel electrophoresis, and the protein bands and radiolabeled F-DNA were visualized with CBB (upper panel) and autoradiography (middle panel), respectively. Arrows and arrowheads indicate free Dnmt1 and Dnmt1 bound to F-DNA, respectively. The radioactive bands visualized with a BAS2000 were quantitated and normalized as to those of the CBB-stained Dnmt1 bands (lower panel).

A



B



Supplementary Figure-S2. Proposed model of interaction between Dnmt1 and Uhrf1. A. Dnmt1 is loaded on the replication foci by its DNA-length dependent function and prosessively methyltes DNA. **B.** The SRA domain of Uhrf1 binds to a hemi-methylated CpG and interacts with the RFTS domain of Dnmt1 by keeping Dnmt1 in open conformation. Then, the SRA domain of Uhrf1 hands over the hemi-methylated CpG to the catalytic pocket of Dnmt1.