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# **Role of the RFTS domain of Dnmt1 in maintenance DNA methylation**

**Ronald Garingalao Garvilles**

**2015**

Thesis Advisor:

Prof. Shoji Tajima

Laboratory of Epigenetics, Institute for Protein Research

The Department of Biological Sciences

Graduate School of Science

Osaka University

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## Abbreviations

ADCA-DN	autosomal dominant cerebellar ataxia, deafness and narcolepsy
5mC	5-methylcytosine
AdoMet	S-adenosyl-L-methionine
AdoHcy	S-adenosyl-L-homocysteine
ATRX	thalassemia/mental retardation syndrome X-linked
BAH	bromo-adjacent homology
BSA	bovine serum albumin
CARM1	coactivator-associated arginine methyltransferase 1
CC	coiled-coil
CDKL5	cyclin-dependent kinase-like 5
CoREST	co-repressor for element-1-silencing transcription factor
DMAP	Dnmt1-associated protein
DMR	differentially methylated region
Dnmt	DNA methyltransferase
Dnmt1	DNA methyltransferase 1
Dnmt3a	DNA methyltransferase 3a
Dnmt3b	DNA methyltransferase 3b
DOT1L	DOT1-like histone H3K79 methyltransferase
EdU	5-Ethynyl-2'-deoxyuridine
ESC	embryonic stem cells
Ezh2/PRC2	enhancer of zeste homolog 2/ Polycomb-repressive complex 2
F/F	flox/flox
FL	full-length
GMEM	Glasgow minimum essential medium
GST	glutathione-S-transferase
HAT	histone acetyltransferase
HBO1	histone acetyltransferase binding to ORC
HDAC	histone deacetylase
HP	heterochromatin protein
HSAN	hereditary sensory and autonomic neuropathy
IAP	intracisternal A-particle
ICF	instability centromere and facial anomalies
JHDM	Jumonji domain-containing histone demethylase
LINE	long interspersed nuclear elements
LOI	loss of imprinting

LSD	lysine specific demethylase
MBP	methyl-CpG binding proteins
MCM	mini-chromosome maintenance proteins
Mi-2/NuRD	Mi-2/nucleosome remodeling deacetylase
NTD	N-terminal independently folded domain
OHT	4-hydroxytamoxifen
OMIM	Online Mendelian Inheritance in Man
PBD	PCNA binding domain
PBDm	PCNA binding domain mutant
PBS	Dulbecco's phosphate buffered saline
PBS-T	PBS added with Triton X-100
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDB	protein data base
PHD	plant homeodomain
PRMT	protein arginine methyltransferase
PWWP	Pro-Trp-Trp-Pro motif
RFTS	replication foci targeting sequence
SDS	sodium dodecyl sulfate
Set1	SET domain-containing histone methyltransferase
SINE	short interspersed nuclear elements
SRA	SET and RING associated
TAP	tandem affinity purification
TKO	<i>Dnmt1</i> , <i>Dnmt3a</i> , <i>Dnmt3b</i> triple-knockout ESC
TTD	tandem tudor domain
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
Ubl	ubiquitin-like domain
Uhrf1	ubiquitin-like with PHD and ring finger domains 1

## Summary

In mammals, DNA methylation plays important roles in embryogenesis and terminal differentiation through the regulation of transcription-competent chromatin state. The methylation patterns are propagated to next generation during replication by maintenance DNA methyltransferase, Dnmt1, in co-operation with Uhrf1. In the N-terminal regulatory region, Dnmt1 contains proliferating cell nuclear antigen (PCNA)-binding and replication foci targeting sequence (RFTS) domains, which are thought to contribute to maintenance methylation during replication.

To determine the contributions of the N-terminal regulatory domains on the maintenance DNA methylation during replication, Dnmt1 lacking the RFTS and/or PCNA-binding domains were ectopically expressed in embryonic stem cells (ESC), and then the effects were analyzed. Deletion of both the PCNA-binding and RFTS domains did not significantly affect the global DNA methylation level. However, replication-coupled maintenance DNA methylation of the differentially methylated regions (DMRs) of three imprinted genes *Rasgrf1*, *Peg3* and *Kcnq1ot1/Lit1* was impaired in the cells expressing the Dnmt1 deleted both the PCNA-binding and RFTS domains, but not the PCNA-binding domain alone. I concluded that the RFTS domain is necessary and sufficient, and the PCNA-binding domain is dispensable for the replication-coupled maintenance DNA methylation.

On the other hand, in the absence of Uhrf1, which is a prerequisite factor for maintenance DNA methylation, the ESC expressing Dnmt1 deleted both the PCNA-binding and RFTS domains apparently maintained global DNA methylation level, whilst those expressed with full-length and the RFTS domain-containing Dnmt1 could not. This clearly indicates that the Dnmt1 lacking the PCNA-binding and RFTS domains could add DNA methylation to genome in replication-independent manner, which means adding aberrant DNA methylation. The addition of DNA methylation by the Dnmt1 lacking the PCNA-binding and RFTS domains was dependent on the DNA methylation activity of Dnmt1 as well as the presence of *de novo*-type DNA methyltransferase. I propose that the RFTS domain works as a safety valve by protecting the genome from replication-independent DNA methylation.

## 4. Background

### 4.1. Epigenetics

Epigenetics is a category of the study on gene expression change without changing DNA sequences, and the memory is mitotically and/or meiotically heritable (Shemer et al., 1996). The mechanisms governing epigenetics are complex and violate the classical Mendelian inheritance. Epigenetic mechanisms contribute to the phenomena such as embryonic development (Ehrlich, 2003), replication timing (Ting et al., 2004), chromatin stability (Robertson et al., 2004), and tissue specific gene expression (Ehrlich, 2003). In mammals, genomic imprinting (Ting et al., 2004) and X-chromosome inactivation (Chow and Brown, 2003) are also under the regulation of epigenetics. The posttranslational modifications of histones, which are the basic component of nucleosomes, and methylation of cytosine base, which is the constituent of DNA, are the major covalent chemical modifications that contribute to underlying molecular mechanism of epigenetics.

### 4.2. Modification of core histones

Basic structure of chromatin is nucleosome, which comprises two molecules each of core histones, histone H2A, H2B, H3, and H4, wrapped with 147 bp DNA. X-ray crystal structures of mono- and tetra-nucleosomes have been reported (Luger et al., 1997; Schalch et al., 2005), however, precise positions of the N-terminal and short C-terminal portions of the core histones in the nucleosome structure cannot be determined due to their flexible property. These flexible parts of core histones called “tail” are subject to acetylation, methylation, ubiquitylation, phosphorylation, sumoylation, and ADP-ribosylation (Vaquero et al., 2003). It was proposed that the combination of histone tail modifications contribute to gene expression, which is called “histone code hypothesis” (Jenuwein and Allis, 2001). Up to present, many concrete evidences supporting this hypothesis have been reported. In nuclei, chromatin state is not uniform; densely stained regions with DAPI are called heterochromatin, which tends to locate in periphery of nuclei and is transcriptionally inactive (Kalverda et al., 2008). Pericentric and telomere regions are constitutively in the state of heterochromatin. Rest of the region, which is less densely stained with DAPI, is called euchromatin, which is generally transcriptionally active (Saksouk et al., 2015).

#### 4.2.1. Histone acetylation

Histone acetylation is catalyzed by histone acetyltransferases (HATs) using acetyl-CoA as a co-factor. Acetyl group is added to  $\epsilon$ -amino group of lysine, leading to

cancellation of positive charge of lysine, and thus destabilizes the interaction of histone with DNA (Xhemalce et al., 2011). For this property, generally, histone tail acetylation enhances transcription activity. The acetyl group is removed by histone deacetylases (HDACs). Acetylation and deacetylation states of histone tail are dynamically regulated. In addition to gene expression regulation, histone acetylation affects replication. MCM (mini-chromosome maintenance) proteins, which are the licensing factor for replication, interacts with histone H4 acetylated at K5, K8, and K12 catalyzed by HBO1 (histone acetyltransferase binding to ORC) at the pre-replicative assembly to increase replication origin firing (Iizuka et al., 2006). Recently, H3 acetylated at K56 was reported to interact with Oct4 to induce pluripotency in mouse embryonic stem cells (ESC) (Tan et al., 2013).

HDACs are classified into four families. Among them, HDAC1 and 2, which are the members of class 1 HDAC family, play crucial role in histone tail deacetylation and gene silencing. HDAC1 and 2 are the component of Sin3, Mi-2/NuRD, and CoREST co-repressor complex. HDAC inhibitors are expected to be potential drugs for anti-cancer by increasing p21 expression (Halkidou et al., 2004, Huang et al., 2005, Hrzenjak et al., 2006).

#### 4.2.2. Histone methylation

Methylation modification is either on lysine or arginine residue. Lysine residues can be mono-, di-, or tri-methylated, and arginine residues mono- or di-methylated. As for arginine di-methylation modifications, two forms exist; symmetric and asymmetric methylation (Xhemalce et al., 2011). Different from acetylation, methylation of lysine residue is performed by position-specific methyltransferases using common co-factor S-adenosyl-L-methionine (AdoMet). Typical methyltransferases are Set1/Compass for histone H3K4, G9a, Suv39h, and SetDB1 for histone H3K9, Ezh2/PRC2 for histone H3K27, DOT1L for histone H3K79, and Suv4-20h for histone H4K20 (Binda, 2013).

Histone H3K4 tri-methylation is the mark for gene activation, histone H3K36 tri-methylation positively contribute to the elongation of transcription, and histone H3K79 di-methylation positively correlate with transcription (Kouzarides, 2007). Tri-methylation at histone H3K4 by Set9 activates transcription by inhibiting the recognition of nucleosome remodeling and deacetylase repressor complex (Mi2-NuRD) (Zegerman et al., 2002). The histone H3K36 methyltransferase, Set2, associate with the C-terminal domain-phosphorylated RNA polymerase II, which is the activated form of the enzyme, indicating that histone H3K36 methylation contributes to transcription elongation (Kizer et al., 2005). Histone H3K79 methylation is important during

embryonic development and conservation of heterochromatin structure at telomeres and centromere (Jones et al., 2008).

Methylation on histone H3K9, H3K27 and H4K20 generally are responsible in forming condensed heterochromatin, that is, repressed state (Kouzarides, 2007). Di- and tri-methylated K9 of histone H3 serve as marks for heterochromatin protein 1 (HP1) binding through its chromodomain, which forms and maintains heterochromatin state (Nishibuchi and Nakayama, 2014). X-chromosome inactivation was initially enriched with histone H3K27 tri-methylation (Rougeulle et al., 2004). *Hox* gene silencing by polycomb group proteins (PcG) via histone H3K27 tri-methylation is an important mechanism in regulating embryogenesis (Cao et al., 2002). Histone H4K20 di-methylation was reported to be recognized by the tudor domain of a cell cycle checkpoint protein Crb2, and related to DNA repair (Botuyan et al., 2006). In addition, H4K20 di-methylation may also play a role in replication as the modification was reported to interact with Orc1, a component of ORC (origin of replication complex) (Kuo et al., 2012).

In general, symmetrical and asymmetrical di-methylation of arginine residue of histone contribute to gene silencing and activation, respectively (Di Lorenzo and Bedford, 2011). PRMT1 methylates histone H4R3 and H2AR3 asymmetrically, PRMT5 methylates histone H3R8, H4R3, and H2AR3 symmetrically, PRMT6 methylates histone H3R2 symmetrically, PRMT7 methylates histone H4R3 and H2AR3 symmetrically, and CARM1 methylates histone H3R17 and R26 asymmetrically (Di Lorenzo and Bedford, 2011). Mono-methylation form of arginine generally is an intermediate on its way to di-methylation state.

Lysine-specific demethylase 1 (LSD1) (other name, KDM1) is the first identified lysine demethylase of histone H3K4 methylation (Shi et al., 2004). Importantly, LSD1 cannot demethylate tri-methylation but di- and mono-methylation states. Lysine demethylation catalyzed by LSD1 was reported to contribute in maintaining global DNA methylation and establishment of genomic imprinting during oogenesis in coordination with KDM1B, a histone H3K4 demethylase (Ciconne et al., 2009). On the other hand, LSD1-dependent demethylation of histone H3K9 methylation activates the transcription of androgen receptor-regulated genes (Metzger, 2005). Jumonji domain-containing proteins are another group of histone demethylase (JHDM) (Chen et al., 2011). JHDM1 is the first identified JHDM as histone H3K36 demethylase (Tsukada et al., 2006). Different from LSD1, JHDM can catalyze demethylation of tri-methylation state of lysine.

#### 4.2.3. Histone ubiquitylation

Ubiquitin comprising 76 amino acid residues is generally added onto the protein in poly-ubiquitylated state to mark the proteins to be degraded through proteasomes (Pickart, 2001). However, as for core histones, mono-ubiquitylation states exist. Mono-ubiquitylation on histone H2AK119 catalyzed by Bmi/Ring1A, a component of polycomb repressive complex 1 (PRC1), contributes to transcription repression (Cao et al., 2005). Another mono-ubiquitylation on histone H2BK120 by RNF20/RNF40 contributes to transcription activation (Zhu et al., 2005). In budding yeasts, deubiquitylation of H2BK123 by Ubp10 deubiquitylase contributes in telomere silencing (Emre et al., 2005; Gardner et al., 2005). Recently, it was reported that histone H3K23 is ubiquitylated, and is the prerequisite step for maintenance DNA methylation (Nishiyama et al., 2013).

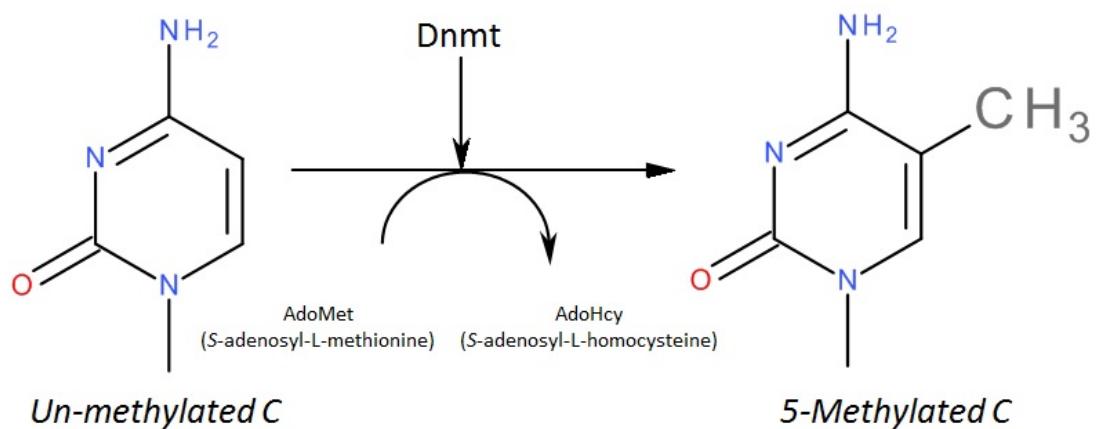
### 4.3. DNA methylation

#### 4.3.1. DNA methylation in genome

In vertebrates, DNA methylation is one of the most well-studied epigenetic mechanisms regulating development through gene expression, and is the possible targets for treating cancers (Razin and Riggs, 1980; Esteller, 2003). In mammals, majority of methylation is added to the 5<sup>th</sup> carbon of cytosine base in the CpG sequence, with AdoMet as the methyl group donor by DNA methyltransferase (Dnmt) (Cheng and Roberts, 2001), of which mechanism is similar to that of the bacterial DNA methyltransferase (Wu and Santi, 1987) (**Figure 1**). In mammalian somatic tissues, about 60 to 80% of the CpG sites are methylated (Ehrlich et al., 1982). Centromeric heterochromatin, telomere region, endo-parasitic sequences such as retrotransposons of Alu, IAP (intracisternal A particle), LINE (long interspersed nuclear elements), and the tissue-specific genes in non-expressing tissues are densely methylated (Bird, 1986). Approximately 60% of human genes are identified to have GC-rich regions near the promoter, referred to as CpG island promoter that drive house-keeping genes. Such the CpG islands are under methylated in contrast to the densely methylated tissue-specific non-CpG promoter (Saxonov et al., 2006; Deaton and Bird, 2011).

Higher eukaryotes increased their DNA methylation content during evolution especially on non-coding and repetitive elements in the genome. Experimental evidence suggests that these types of methylations are a kind of defense mechanism to silence inserted foreign DNA such as retrotransposons in the host genome (Yoder et al., 1997).

In plant genome, not only the CpG sequence but also the CpHpG and CpHpH sequences are methylated (Law and Jacobsen, 2010). Different from plants, the CpG



**Figure 1. Scheme of cytosine DNA methylation.** Addition of methyl group on to the 5<sup>th</sup> carbon of cytosine to form 5-methyl cytosine by DNA methyltransferase using AdoMet as methyl-group donor.

sequence is the major methylation site in vertebrates. DNA methylation in the promoter region negatively correlates with transcription activity. However, the methylation of gene body apparently positively correlates with gene expression (Jones, 1999). Function of the gene body methylation is poorly understood. Since gene body methylation seemed to be conserved among species (Feng et al., 2012), the DNA methylation should play important role. Gene body methylation is expected to repress activation of intragenic promoters that could alter genome integrity (Maunakea et al., 2010). Genome-wide studies identified that gene bodies are enriched with H3K36 tri-methylation, which is the mark for promoting transcription elongation (Hodges et al., 2009) and specifically interacts with the PWWP domain of Dnmt3a. Dnmt3a recruitment to gene body may stimulate *de novo* methylation of the gene body (Dhayalan et al., 2010). Gene body methylation may affect RNA splicing as RNA polymerase II pauses at the DNA methylation site during transcription elongation (Shukla et al., 2011).

#### 4.3.2. Diseases associated with defective methylation and mutant Dnmt

Several diseases have been implicated due to impaired methylation in the genome. Aberrant changes in the DNA methylation patterns can be the cause of epimutations (Jones and Baylin, 2002). Many cancers are associated with global hypomethylation and loci-specific hypermethylation (Wu et al., 2014). In some of the cancers, promoters of the tumor suppressor genes are hypermethylated (Ushijima, 2005). Genomic imprinting is a non-Mendelian inheritance system regulating expression of parental-specific alleles and chromosomal region by dictating their physical, genetic, and epigenetic characteristics. Aberration in imprinting centers such as deletion or inappropriate methylation leads to loss of imprinting (LOI), and is the cause for diseases (Nicholls, 2000). Impaired methylation of differentially methylated regions (DMRs) cause developmental defects and results in genetic disorders such as Beckwith-Wiedemann syndrome, Prader-Willi syndrome, or Angelman syndrome.

Although DNA methylation abnormality can be a cause for disease, not many of them are identified as to mutation of Dnmt. Recently, it was reported from different laboratories that the mutation in DNMT3A (*de novo*-type Dnmt described later) can be a cause for leukemia (Yamashita et al., 2010; Yang et al., 2015). DNMT3B (another *de novo*-type Dnmt) mutation is a cause for immunodeficiency, centromere instability and facial anomalies (ICF) syndrome (Okano et al., 1999; Hansen et al., 1999). Since DNMT1 is crucial for development and proliferation, it was thought that individuals with heritable mutation in DNMT1 (maintenance-type Dnmt) may not survive, and thus

such the disease has not been reported until recently. Surprisingly, neurodegenerative diseases caused by DNMT1 mutations are reported from two different groups. One is a point mutation in the Zn-finger motif region in the RFTS domain that causes the peripheral and central nervous system leading to hereditary sensory and autonomic neuropathy type 1 (HSAN1, Online Mendelian Inheritance in Man (OMIM) accession number, 614116) with dementia and hearing loss (Klein et al., 2011). This mutation is dominant and is due to hypomethylation by unstable DNMT1. Another disease mutation is also in the RFTS domain, which are at the contact surface of the RFTS and catalytic domains, resulting in autosomal dominant cerebellar ataxia, deafness and narcolepsy (ADCA-DN, OMIM accession number, 604121) (Winkelmann et al., 2012).

#### 4.3.3. Recognition of methylated DNA

The methylated DNA is specifically recognized by the proteins classified as methyl-CpG binding proteins (MBPs). These proteins act as readers of the methylated DNA. Three classes of the proteins are identified. One group contains common domain called MBD comprising about 60 amino acid residues. The main members are MeCP2, MBD1, MBD2, MBD3, and MBD4. Except for mammalian MBD3, which has lost its methylated DNA-binding activity, MeCP2, MBD1, MBD2, and MBD4 share common binding property towards methylated DNA (Saito and Ishikawa, 2002). MeCP2 is coded on X-chromosome and its mutation is a cause of Rett syndrome (Amir et al., 1999). MBD1 binds not only to methylated DNA through its MBD but also to un-methylated DNA via the Cys-rich domain (CXXC) (Jorgensen et al, 2004). MBD1 is proposed to contribute to the maintenance of histone H3K9 methylation during replication by collaborating with SetDB1, a histone H3K9 methyltransferase (Sarraf and Stancheva, 2004). MBD4 (MED1) may act as a member of base excision repair system as it contains thymine DNA glycosylase domain in the C-terminal region, which function as T:G mismatch glycosylase (Bellacosa et al., 1999). During evolution, mammalian MBD3 lost its methylated DNA-binding activity, however, it is an intrinsic component of co-repressor Mi2-NuRD complex containing HDAC, and thus participates in epigenetic gene silencing (Reese et al., 2007; Yildrim et al., 2011). Exceptionally, *Xenopus* MBD3 possesses methylated DNA-binding activity and its selective recognition of methylated CpG plays a crucial role in development (Iwano et al., 2004).

Uhrf1 (Np95), which is reported to be a prerequisite factor for maintenance DNA methylation (Sharif et al., 2007), specifically interacts with hemi-methylated DNA through its SET and RING associated (SRA) domain (Arita et al., 2008; Avvakumov et al., 2008; Hashimoto et al., 2008). Uhrf1 comprises ubiquitin-like (Ubl), tandem tudor

(TTD), plant homeodomain (PHD), SRA, and RING domains, which functions as ubiquitin ligase (Hashimoto et al., 2009). The SRA binds hemi-methylated CpG and flips out the methylated cytosine out of the double-stranded DNA. The TTD and PHD domains, which position at the N-terminal region, recognize histone H3R2 and H3K9 tri-methylation as well (Arita et al., 2012). Recognition of both DNA methylation and histone H3K9 tri-methylation by Uhrf1 suggests its contribution to cross talk between the two modifications (Rothbart et al., 2012). It was reported that the SRA domain of Uhrf1 also recognizes hydroxymethylcytosine (Frauer et al., 2011), however, our group has proven that the SRA domain cannot bind hydroxymethylcytosine (Otani et al., 2013).

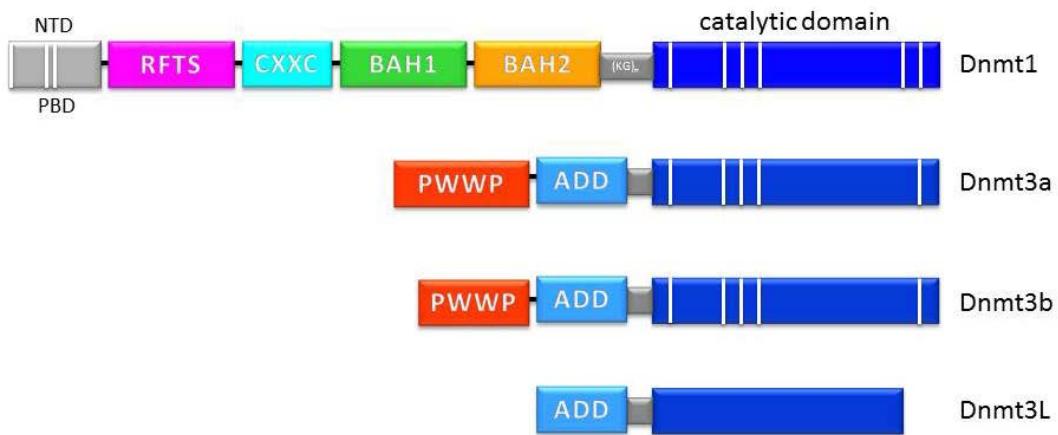
Another DNA-binding protein Kaiso (ZBTB33) binds to fully methylated CpG site through its Kruppel-like C<sub>2</sub>H<sub>2</sub> zinc finger motif (van Roy and McCrea, 2005). Removal of Kaiso proteins from *Xenopus* embryos induces abnormal development caused by activation of methylated genes (Ruzov et al., 2009). In addition, Kaiso is reported to be involved in mitotic spindle apparatus formation (Soubry et al., 2011).

## 5. Introduction

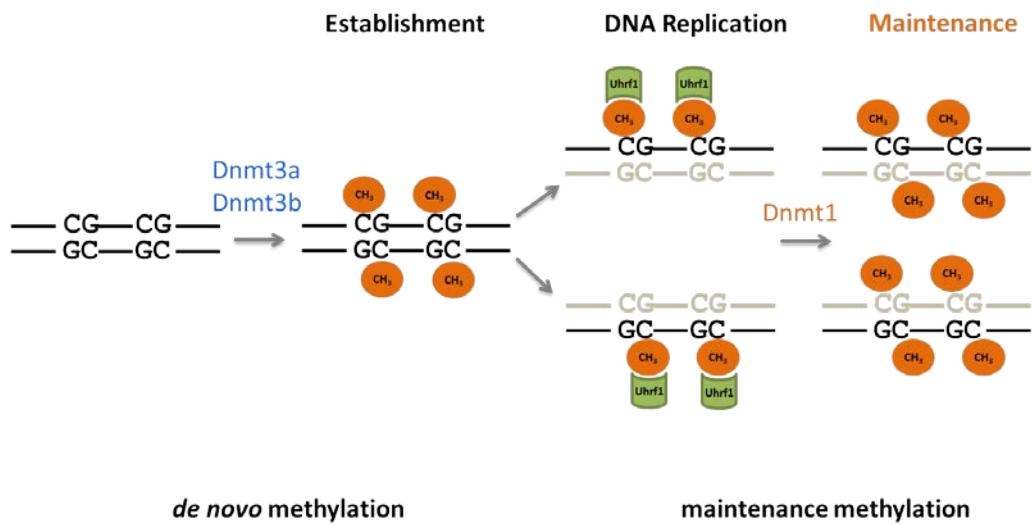
### 5.1. Establishment of DNA methylation patterns

In mammals, three DNA methyltransferases (Dnmt) are reported (**Figure 2**). DNA methylation patterns are established by Dnmt3a and Dnmt3b, which are called *de novo*-type DNA methyltransferases (Okano et al., 1999; Aoki et al., 2001), and the patterns are propagated to next generation by Dnmt1, which preferentially methylates hemi-methylated state. This step is called maintenance DNA methylation (Li et al., 1992) (**Figure 3**). Dnmt3a and Dnmt3b are closely related to each other. In addition to the C-terminal catalytic domain, both have common domains, the PWWP and the PHD-like ATRX-DNMT3-DNMT3L (ADD) domains in the N-terminal half (Okano et al., 1998). Since knockout of either *Dnmt3a* or *Dnmt3b* gene severely impairs development of mouse, *Dnmt3a* and *Dnmt3b* are responsible for distinct function. Actually, *Dnmt3b* is solely responsible for methylating centromeric minor satellite repeats, and the defect in methylating the region is the cause for ICF syndrome (Okano et al., 1999; Hansen et al., 1999). Mouse with both *Dnmt3a* and *Dnmt3b* knockout shows more severe defect on the development compared to a single mutation of *Dnmt3a* or *Dnmt3b* (Okano et al., 1999). Dnmt3b is highly expressed in implantation embryo and thus is responsible for global DNA methylation (Watanabe et al., 2002). As the embryogenesis proceeds, Dnmt3a2, which is an isoform of Dnmt3a lacking about 200 amino acid residues of the N-terminus (Chen et al., 2002), and Dnmt3b quickly decrease, of which phenomenon is also observed on the differentiation of ESC (Sato et al., 2006).

Dnmt3a and Dnmt3b possess *de novo*-type DNA methylation activity not only *in vivo* but also *in vitro*, which was proven by using recombinant Dnmt3a and Dnmt3b (Aoki et al., 2001; Suetake et al., 2003). Although it is minor, non-CpG methylation can be found in some cell types, such that pluripotent stem cells of ESC and induced pluripotent stem (iPS) cells (Ramsahoye et al., 2000; Lister et al., 2011; Guo et al., 2014), female growing oocytes (Tomizawa et al., 2011), and neurons (Kinde et al., 2015). In neurons, non-CpG methylation is accumulated during maturation, onto which *de novo* methyltransferase contributes (Guo et al., 2014). It is not surprising that those cells possess non-CpG methylation since *de novo*-type DNA methyltransferases, Dnmt3a and Dnmt3b, which methylate mainly the CpG sequences, possess weak methylation activity towards non-CpG sequences such as CpA and/or CpT (Aoki et al., 2001; Suetake et al., 2003). As pluripotent stem cells express high level of Dnmt3a and Dnmt3b (Watanabe et al., 2002), there are higher chance to methylate non-CpG sequences than other somatic cells. Importantly, however, during replication, non-CpG



**Figure 2. Schematic illustration of the family of mouse DNA methyltransferases (Dnmt).** Three Dnmt genes coding DNA methyltransferase with DNA methylation activity are identified; Dnmt1, Dnmt3a, and Dnmt3b. Dnmt3L (like) is a homologue of Dnmt3 but has no DNA methylation activity.



**Figure 3. Establishment and maintenance of DNA methylation patterns in mammals.** In mammals, global DNA methylation patterns are established in early stage of embryogenesis and in germ cells by Dnmt3a and Dnmt3b. Once the patterns are established, Dnmt1 propagate the patterns to next generation during replication with the aid of Uhrf1.

methylation sites are erased due to maintenance DNA methylation by Dnmt1, which methylates and maintains only the methylated CpG sequences (Vilkaitis et al., 2005). Since growing oocytes and neurons, in which the expression levels of *de novo*-type methyltransferases are not so high as those in ESC and iPS cells, are in post mitotic state and their life span is long, the added non-CpG methylations are not erased efficiently in a replication-dependent manner.

Dnmt3L (like), which was identified in 2000 (Aapola et al., 2000), has no catalytic motifs for DNA methylation activity. However, interestingly, *Dnmt3l*-knockout mice show defect in germ cell maturation (Bourc'his et al., 2001; Hata et al., 2002) due to decrease in global DNA methylation. Dnmt3L directly interacts with Dnmt3a or Dnmt3b through its C-terminal half and activate their DNA methylation activity (Suetake et al., 2004).

The PWP domain of Dnmt3b is reported to be the motif of DNA-binding (Qu et al., 2002) and, at the same time, the domain tethers Dnmt3 to heterochromatin (Ge et al., 2004; Chen et al., 2004). Interestingly, thymine DNA glycosylase (TDG), which is expected to catalyze the final demethylation step, interact with Dnmt3a through PWP and catalytic domains (Li et al., 2007). Recently, phosphorylation of PWP domain of Dnmt3a by CK2 was reported to impair DNA methylation status of Alu (SINE), indicating the role of PWP domain on heterochromatin formation (Deplus et al., 2014).

The ADD domain is reported to bind many factors such as co-repressor RP58 (Fuks et al., 2001a), oncogene c-myc (Brenner et al., 2005), histone H3K9 methylase Suv39h1 and heterochromatin protein 1 (HP1)  $\beta$  (Fuks et al., 2003b), histone H3K9 methylase Setdb1 (Li et al., 2006), histone H4R3 me2s (symmetrical methylation) (Zhao et al., 2009), histone H3K4 with no modification (Otani et al., 2009). The interaction with histone H3K4 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).

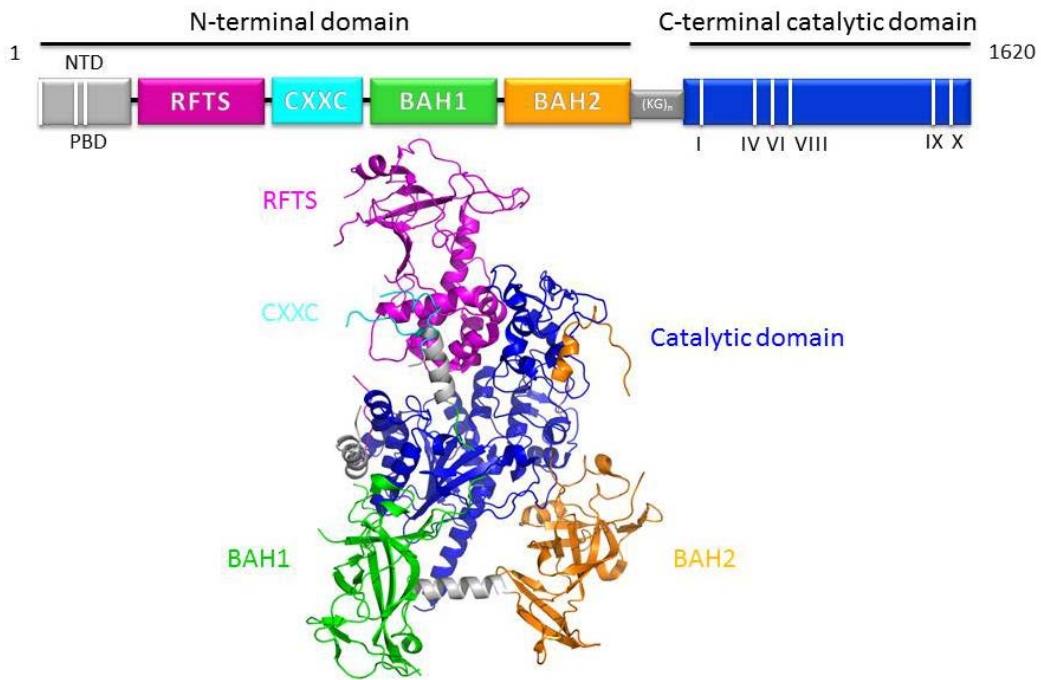
## 5.2. Maintenance DNA methylation

Once the methylation patterns are established, they are propagated to next generation through replication. Dnmt1 is responsible for this maintenance DNA methylation (Li et al., 1992) (**Figure 3**). Dnmt1 selectively methylates hemi-methylated state of DNA (Vilkaitis et al., 2005). For the recruitment of Dnmt1 to the replication foci, the replication foci target sequence (RFTS) (Leonhardt et al., 1992) and/or the PCNA

binding motif (Chuang et al., 1997) in the N-terminal independently folded domain (NTD) are reported to play crucial roles. As expected, production and decay of Dnmt1 is cell-cycle dependent; proliferating cells expresses high level of Dnmt1 protein and quickly decreases by terminal differentiation due to arrest of proliferation (Liu et al., 1996; Suetake et al., 1998).

In mouse oocyte, Dnmt1 isoform that lacks the N-terminal 118 amino acid residues is the dominant form (Mertineit et al., 1998). Surprisingly, Dnmt1 is enriched in the cytoplasmic compartment in mature oocyte and in early stage embryo till blastocyst except for at eight-cell stage, at which stage Dnmt1 is translocated into nuclei. The papers argue that this cytoplasmic localization is the cause for the global DNA demethylation during early stage embryogenesis (Mertineit et al., 1998; Grohmann et al., 2005). The differentially methylated regions (DMR) of the imprinted genes escape from this genome-wide wave of demethylation during early embryogenesis, and was thus once speculated the existence of a novel DNA methyltransferase(s). However, small amounts of maternal and zygotic Dnmt1 were turned out to be sufficient for maintenance of the DMR methylation of imprinted genes (Hirasawa et al., 2008). The oocyte-type short Dnmt1 found in mammals was not found in the oocyte of *Xenopus laevis*, an amphibian (Shi et al., 2001). However, similar to mouse, the oocyte Dnmt1 in *Xenopus laevis* is located not in germinal vesicles (nuclei) but mainly in cytoplasmic compartment (Kimura et al., 2002). This unusual localization of Dnmt1 in oocyte and early stage embryo is also observed in neurons. Although neurons are post mitotic, the expression level of Dnmt1 is similar to that in proliferating cells, but is localized to cytoplasm (Inano et al., 2000).

Mouse Dnmt1 comprises 1,620 amino acid residues composed of multi domains (**Figure 4**) (Takeshita et al., 2011). The catalytic domain that resides in the C-terminal one third of the molecule is responsible for DNA methylation. Two third of the N-terminal domain is the regulatory region. The very N-terminal 1-243 sequence forms independently folded domain (NTD) (Suetake et al., 2006). The NTD is reported to bind DMAP, which is a co-repressor (Rountree et al., 2000), Dnmt3a and 3b (Kim et al., 2002), Rb2 (Pradhan et al., 2002), PCNA (proliferating cell nuclear antigen) (Chuang et al., 1997), CDKL5 kinase (Kameshita et al., 2008), casein kinase (Sugiyama et al., 2010), and AT-rich DNA (Suetake et al., 2006). Because of these properties, the NTD is proposed to be the platform for the factors that regulate the function of Dnmt1 (Suetake et al., 2006). In 2011, our group solved the three-dimensional structure of Dnmt1 that lacks the NTD (Takeshita et al., 2011; PDB accession number, 3AV4). After the NTD, the RFTS domain, CXXC motif, two bromo-adjacent homology (BAH1 and BAH2)



**Figure 4. Crystal structure of mouse Dnmt1(291-1620)** (Takeshita et al., 2011; PDB accession number, 3AV4). The molecule is composed of multi-domains. The N-terminal domain of about 30 kDa folds independently. This domain works as a platform to bind many factors that may regulate the function of Dnmt1; factors such that DMAP1, PCNA, Rb, *de novo*-type Dnmt3s, several kinases, and DNA. RFTS domain brings Dnmt1 to replication foci. CXXC binds to DNA; this binding seems to be sequence or methylation state independent. Two tandem BAH domains follows. The function of these domains is not known. Then the C-terminal catalytic domain. The catalytic domain is highly homologous to bacterial DNA methyltransferases.

domains follow. The N-terminal region containing these domains is connected to the C-terminal catalytic domain with flexible KG-repeat. Each domain folds independently and interacts each other.

To our surprise, the RFTS domain plugs the catalytic pocket, to which the substrate hemi-methylated DNA must bind for DNA methylation activity. With such the RFTS positioning, substrate DNA cannot access to the catalytic center by steric hindrance. The RFTS domain is anchored to the catalytic pocket, of which position was stabilized by four hydrogen bonds between the RFTS and catalytic domains. Following the RFTS region is the CXXC motif that binds to un-methylated DNA, which is reported to contribute to an auto-inhibitory role by prohibiting the accession of un-methylated DNA to the catalytic center (Song et al., 2011). Function of the BAH1 and BAH2 domains is remained to be elucidated. But interestingly, lysine 1,097 of the BAH1 domain is methylated by Set7/9 and demethylated by LSD1, which are known as histone methylase and demethylase, respectively, and the demethylation activity of LSD1 stabilizes Dnmt1 and regulates the global DNA methylation level in mouse ESC (Wang et al., 2009).

For the propagation of DNA methylation, Dnmt1 requires factors for ubiquitylation and deubiquitylation at histone H3K23 (Nishiyama et al., 2013). Uhrf1, which is a prerequisite factor for maintenance DNA methylation *in vivo* (Sharif et al., 2007), possesses the Ring-finger motif responsible for an E3 ligase of ubiquitylation, and the SET and RING-associated (SRA) domain that specifically recognize hemi-methylated DNA (Arita et al., 2008; Avvakumov et al., 2008; Hashimoto et al., 2008). The SRA domain directly interacts with the RFTS domain of Dnmt1 to remove the RFTS domain from the catalytic pocket to make hemi-methylated DNA access to the catalytic center (Song et al., 2012; Berk'yurek et al., 2014).

### 5.3. Aim of the study

Dnmt1 is responsible for maintenance DNA methylation. Our group has elucidated the crystal structure of Dnmt1 lacking the NTD (Takeshita et al., 2011). A striking feature of the elucidated structure is that the RFTS domain is plugging the catalytic pocket of Dnmt1, indicating that the substrate DNA cannot access to the catalytic center *in situ*. The three dimensional structure of Dnmt1 provides us with the interaction information between the domains, and thus suggests where to introduce site-directed mutagenesis without affecting the whole structure of Dnmt1. To this end, I decided to ask the function of the RFTS domain in cells by expressing the mutant Dnmt1 in the ESC, of which endogenous *Dnmt1* gene can conditionally be deleted.

In the present study, I designed to ask the question whether or not the PCNA-binding motifs in the NTD and/or the RFTS domain are necessary for maintenance DNA methylation in the cells. The results clearly indicated that the NTD including the PCNA-binding motif was dispensable, but the RFTS domain was necessary for replication-coupled maintenance DNA methylation. Surprisingly, the RFTS domain was found to play important role in protecting genome from aberrant DNA methylation.

## 6. Materials and Methods

### Mouse Dnmt cDNA

Mouse Dnmt1 cDNA coding full-length, oocyte-type, 291-1620, and 602-1620 sequences with SV40 nuclear localization signal and myc-tag sequences at their N-termini, and mouse cDNA coding Dnmt3a2 with TAP tag (Puig et al., 2001) at the C-terminal end were cloned into pCAGIpuro, pCAGIblast, or pCAGIzeo, which were kind gifts from Dr. Niwa (RIKEN Center for Developmental Biology, Kobe, Japan). Each cDNA was constructed by PCR, and all the sequences were confirmed by the dideoxy method (Sanger et al., 1977).

### DNA methylation activity

Recombinants of the full-length Dnmt1, oocyte-type Dnmt1, Dnmt1(291-1620), Dnmt1(602-1620), and full-length Dnmt1 with the H168R mutation were prepared and their DNA methylation activities were determined as described elsewhere (Takeshita et al., 2011; Berkyurek et al., 2014). In brief, in a total volume of 25  $\mu$ l reaction buffer, 6.4 nM Dnmt1, 66 nM DNA, and 2.2  $\mu$ M [ $^3$ H]-S-adenosyl-L-methionine (10 Ci/mmol) (Perkin Elmer) were incubated at 37°C for 1 h, and then the activity was determined within a linear time-course range.

### Mouse ESC

Mouse ESC were cultured in Glasgow Minimum Essential Medium (Sigma Aldrich) supplemented with 15% (v/v) Knockout Serum Replacement (Invitrogen), 1% (w/v) fetal bovine serum (Intergen), 10 units/ml penicillin G, 100  $\mu$ g/ml streptomycin, 1 mM non-essential amino acids, 100  $\mu$ M sodium pyruvate, 0.1 mM  $\beta$ -mercaptoethanol, and 0.2% leukemia inhibitory factor, under a 5% CO<sub>2</sub> atmosphere at 37°C in dishes coated with gelatin. *Dnmt1* or/and *Uhrf1* gene conditional knockout ESC were prepared from mice embryos as described elsewhere (Sharif et al., 2007; Jackson-Grusby et al., 2001). *Dnmt1*, *Dnmt3a*, *Dnmt3b* triple-knockout ESC (TKO) was kindly provided by Dr.

Okano at RIKEN (Tsumura et al., 2006).

### Isolation of cells expressing ectopic Dnmt1 or Dnmt3a2

A plasmid harboring Dnmt1 or Dnmt3a2-TAP cDNA (50 µg) was linearized with ScaI, and transfected into ESC, in which the endogenous *Dnmt1* or/and *Uhrf1* gene was sandwiched between *loxP* sequences or TKO, with a Gene Pulser (BioRad) at 250 V and 500 µF according to the manufacturer's protocol. After the electroporation, the cells were cultured in the medium supplemented with 1.5 µg/ml puromycin, 20 µg/ml blasticidin S, or 100 µg/ml zeocin, and the resistant clones were isolated. Each clone was analyzed as to the expression of Dnmt1 or Dnmt3a2 by Western blotting.

### Western blotting

The isolated clones were solubilized with 1% SDS, electrophoresed in a 7.5% polyacrylamide gel, and then electrophoretically transferred to a nitrocellulose membrane (Pall). The membrane was blocked with 5% (w/v) skimmed milk in Dulbecco's phosphate-buffered saline (PBS), and then incubated with the first antibodies in an antibody buffer comprising 1% (w/v) bovine serum albumin and 0.1% Triton X-100 in PBS. Anti-mouse Dnmt1 rabbit antibodies (Takagi et al., 1995), anti-mouse Uhrf1 rat monoclonal antibody clone Th-10a (MBL, Japan) (Muto et al., 1995), anti-myc monoclonal antibody clone 4A6 (Millipore), anti-tubulin mouse monoclonal antibody clone DM1A (Sigma Aldrich), and anti-mouse Dnmt3a rabbit antibodies (Sakai et al., 2004) were used as the first antibody, with incubation overnight at 4°C. After the incubation with the first antibodies, the membrane was washed and then incubated with antibodies conjugated with alkaline phosphatase, and visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Harland, 1991).

### Deletion of the endogenous *Dnmt1* and *Uhrf1* genes

To delete the endogenous *Dnmt1* or/and *Uhrf1* gene sandwiched with *loxP* sequences, cells were cultured in the presence of 0.8 µM 4-hydroxytamoxifen (OHT) (Sigma Aldrich) (Feil et al., 1997). Genome DNA before and after OHT treatment was PCR-amplified with the following primers. To determine *Dnmt1* deletion before and after OHT treatment, primers **a** and **b**, as follows were used. The amplification reaction comprised a cycle of denaturation at 94°C 2 min, and then 30 cycles of denaturation at 94°C 30 sec, annealing at 60°C for 30 sec, and extension at 72°C 1 min.

Primer **a**: 5'-GTAAGTCTGCCTTTCCCAGTT-3', and

Primer **b**: 5'- AAACCAGTATGTCTCGTGTCCCTTAC-3'

Successful deletion of the endogenous *Dnmt1* diminishes amplification of the 351 bp + loxP (34 bp) size fragment with the (a + b) primer set. The expression of the endogenous genes was monitored by Western blotting.

### **Detection of methylated DNA by dot blotting with anti-methylated cytosine antibody**

Genome DNA prepared as described elsewhere (Sambrook and Russel, 2001) was determined as to the DNA methylation level by dot blotting as described (Brown, 1993), except that the DNA was briefly sonicated in a Bioruptor (Cosmo Bio) before alkaline and heat treatment. The DNA blotted onto nitrocellulose membrane was immuno-detected with anti-methylated cytosine monoclonal antibody FMC-9 (MBL, Japan), followed by incubation with antibodies conjugated with alkaline phosphatase, and then visualized with nitroblue tetrazorium and 5-bromo-4-chloro-3-indolyl-phosphate (Harland, 1991). The DNA blotted onto nylon membrane was stained with 0.04% methylene blue in methanol.

### **Selective precipitation of methylated DNA fragments with the methylated DNA-binding domain of MBD1**

The methylated DNA-binding domain of MBD1 coding 1-75 (MBD1) was used to precipitate methylated DNA fragments (Ohki et al., 2001). The cDNAs of MBD1 and the point mutant with R22A, which cannot recognize methylated DNA, were subcloned into pET30a with the GST cDNA added at the 5' end. The recombinant proteins coding (His)<sub>6</sub>-GST-MBD1 and its R22A mutant were purified with Ni-chelate and glutathione-Sepharose columns (GE Healthcare). Methylated genome DNA fragments were selectively precipitated as described elsewhere (Morita et al., 2012), except for the use of magnet beads, MagneGST (Promega). In brief, DNA was fragmented in a sonicator M220 (Covaris) to obtain an average size of 150 bp (Taiwo et al., 2012). To the fragmented DNA in the buffer comprising 160 mM NaCl, 0.01% (w/v) Tween 20, and 10 mM Tris-HCl, pH 7.5, was added (His)<sub>6</sub>-GST-MBD1(1-75) or its R22A mutant, followed by incubation at 4°C overnight. After the incubation, a 1/10 volume of the mixture was saved for the determination of “input”. The rest of the mixture was washed four times with the same buffer. The input and washed beads were adjusted to 10 mM EDTA, 0.5% (w/v) SDS, and 50 mM Tris-HCl, pH 7.4, and then Proteinase K was added. The mixtures were incubated at 50°C for 3 h. After the digestion reaction, an aliquot of the supernatant fraction was determined in a fluorometer Quantus (Promega) according to the manufacturer's protocol. The relative level of specific binding was

determined by subtracting that on precipitation with the R22A mutant and then normalized as to the input. More than three different preparations of genomic DNA were precipitated, and the averages  $\pm$  S. D. were determined.

### Bisulfite sequencing

Genome DNA was treated with bisulfite using an EpiTect Bisulfite Kit (Qiagen) with an extended reaction time according to the manufacturer's protocol, and then amplified with EpiTaq DNA polymerase (Takara) using the following primer sets (Tomizawa et al., 2011) and conditions. For each of the PCR amplifications, treatment for 2 min at 98°C and 4 min at 72°C was performed initially and after the cycles, respectively.

*IAP gag*: 30 cycles of 98°C for 10 sec, 55°C 30 sec, and 72°C 30 sec with

FW primer: 5'-AAAACACCACAAACCAAAATCTTCTAC-3'

RV primer: 5'-TAAAACATATCCTCTAATCATTCTACTCA-3'

*Rasgrf1*: 40 cycles of 98°C for 10 sec, 60°C 30 sec, and 72°C 30 sec with

FW primer: 5'-GGAATTGGGGATTTAGAGAGTTATAAAAGT-3'

RV primer: 5'-CAAAAACAACAATAATAACAAAAACAAAAACAATAT-3'

*Peg3*: 35 cycles of 98°C for 10 sec, 61°C 30 sec, and 72°C 30 sec with

FW primer: 5'-TTTGTAGAGGATTTGATAAGGAGGTGTT-3'

RV primer: 5'-ATCTACAAACCTTATCAATTACCCTTAAAAA-3'

*Kcnq1ot1/Lit1*: 30 cycles of 98°C for 10 sec, 58°C 30 sec, and 72°C 60 sec with

FW primer: 5'-ATTTTGTTAGGTTATAGAAGTAGGG-3'

RV primer: 5'-TTAAAAATCACCAACATAAAATACTATAT-3'

The amplified DNA fragments were subcloned into pBlueScript SKII by TA cloning, and the nucleotide sequences were determined by dideoxy sequencing using the following primers and an ABI Prism 3100 DNA sequencer (Applied Biosystems).

FW primer: 5'-GTTTCCCAGTCACGACG-3'

RV primer: 5'-GAATTGTGAGCGGATAAC-3'

The results were analyzed with the software described elsewhere (Kumaki et al., 2008).

### Immuno-fluorescence observation of ectopically expressed Dnmt1

For ESC expressing Dnmt1 cultured on gelatin-coated cover glasses, the replicating DNA was labeled with Click-iT EdU Imaging Kit (Molecular Probes). In brief, half the culture medium was replaced with GMEM containing 2  $\mu$ M 5-ethynyl-2'deoxyuridine (EdU), followed by incubation at 37°C for 15 min, and then

fixing of the cells with 3.7% (v/v) formaldehyde at room temperature for 10 min. The specimens were washed three times, 2 min each, with 125 mM glycine, pH 7.0. The specimens were treated with 0.5% (w/v) Triton X-100 in PBS (PBS-T) at room temperature for 20 min to permeabilize the membrane, and then washed two times, 5 min each, with 125 mM glycine, pH 7.0. The following manipulations were performed in the dark. A Click-iT reaction cocktail containing Alexa Fluor 488 azide was added, followed by incubation at room temperature for 30 min, and then washing three times, 2 min each, with 125 mM glycine, pH 7.0. To this specimen, anti-mouse Dnmt1 antibodies (Takagi et al., 1995) in PBS containing 0.4% (w/v) SDS, 2% (w/v) Triton X-100, and 1% (w/v) bovine serum albumin (BSA) were added, followed by incubation at 4°C overnight. On the next day, the specimens were washed three times, 5 min each, with PBS-T, and then incubated at room temperature for 1 h with anti-rabbit antibodies conjugated with Alexa Fluor 546. After the incubation, the specimens were washed three times with PBS-T, mounted in 50% (v/v) glycerol, and then observed under a laser confocal microscope, Zeiss LSM510.

## 7. Results

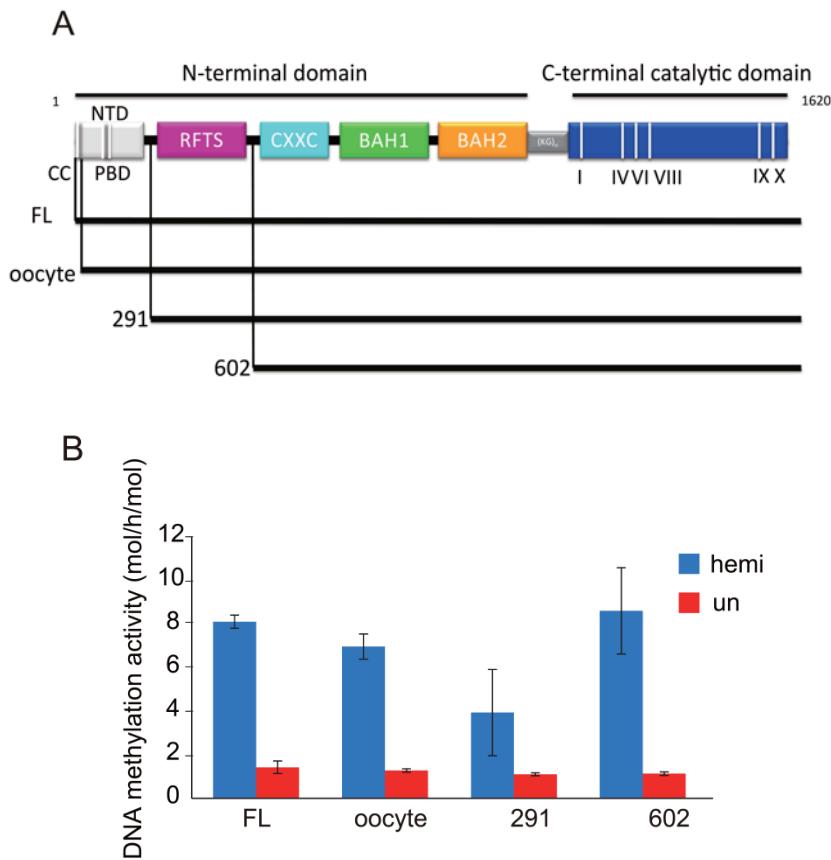
### 7.1. Dnmt1 that lacks the NTD and RFTS domain possesses equivalent DNA methylation activity and selectivity towards hemi-methylated DNA

Full-length Dnmt1 possesses multi-domains, which comprise the NTD containing the PBD (Suetake et al., 2006), the RFTS, the CXXC motif, and the two BAH domains, and the catalytic domain (**Figure 5A**). The catalytic domain with the CXXC and two BAH domains are reported to selectively methylate hemi-methylated DNA (Takeshita et al., 2011; Song et al., 2011; Bashtrykov et al., 2012). Recombinant Dnmt1 coding the full-length, oocyte-type (Mertineit et al., 1998), NTD-deleted {Dnmt1(291-1620)}, and both NTD and RFTS domain-deleted {Dnmt1(602-1620)} exhibited similar selectivity and specific activities towards hemi-methylated DNA (**Figure 5B**).

### 7.2. Dnmt1 that lacks the RFTS domain and/or NTD apparently maintains the global DNA methylation level of the genome

The PBD and RFTS domains are reported to be necessary for targeting replication foci for the maintenance DNA methylation in culture cells (Chuang et al., 1997; Leonhardt et al., 1992). These reports, however, did not confirm whether or not the PBD and/or RFTS domain are necessary for the maintenance DNA methylation in cells.

To examine the roles of the PBD and RFTS domain, stable clones expressing the



**Figure 5. DNA methylation activities of recombinant Dnmt1.**

**A.** Schematic illustration of the Dnmt1 structure. Dnmt1 is composed of the N-terminal independently folded domain (NTD) comprising a coiled-coil (CC) and the PBD, the RFTS domain, the CXXC motif, two BAH domains (BAH1 and BAH2), a KG-repeat (KG), and a catalytic domain for cytosine methyltransferases. Below the illustration the constructs used in the present study are indicated. **B.** The DNA methylation activities of full-length Dnmt1 (FL), oocyte-type Dnmt1 (oocyte), Dnmt1(291-1620) (291), and Dnmt1(602-1620) (602). The specific activities (mol/h/mol Dnmt1) towards hemi-methylated (blue, hm) and un-methylated (red, um) DNA are shown as averages  $\pm$  S. D. ( $n = 3$ ).

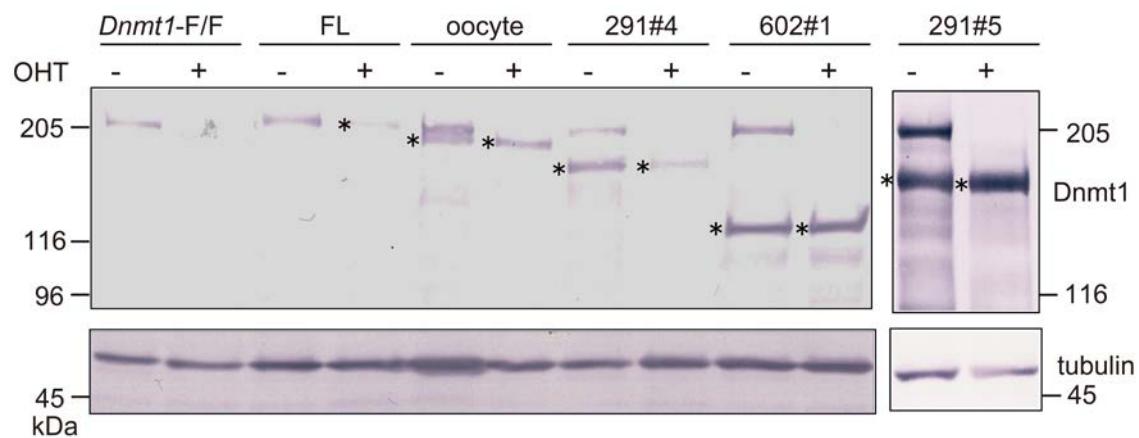
full-length Dnmt1, oocyte-type Dnmt1, Dnmt1(291-1620), and Dnmt1(602-1620) with a myc-tag at their N-termini in mouse ESC, in which the endogenous *Dnmt1* gene in the genome is sandwiched between *loxP* sequences (*Dnmt1*-F/F), were established. The clones expressing ectopic Dnmt1 at a similar level to that of endogenous Dnmt1 were used for further experiments (**Figure 6**). The endogenous *Dnmt1*-F/F was removed by 4-hydroxytamoxifen (OHT) treatment. Four days, not two days, treatment was sufficient to completely remove the endogenous *Dnmt1* (**Figure 7**).

The DNA methylation level of the genome DNA from the cells cultured for four days, which was just after complete deletion of endogenous *Dnmt1*, was determined by dot blotting (**Figure 8A**). The DNA prepared from the parent cells four days after the treatment with OHT showed a significantly reduced DNA methylation level due to global DNA demethylation. On the other hand, the genome DNA prepared from all the clones expressing ectopic Dnmt1 showed maintenance of the DNA methylation level, indicating that the NTD and RFTS domain are apparently dispensable for maintenance of the global genome methylation level in ESC. This was further confirmed by the precipitation method with the methylated DNA-binding domain of MBD1 (MBD1) after four- and ten-day cultivation in the presence of OHT (**Figure 8B**). Genome DNA of ESC ectopically expressing either of the constructs was significantly precipitated with the MBD1 even after ten days cultivation in the presence of OHT, indicating that the Dnmt1 construct with the NTD and RFTS domain deleted apparently possesses the ability to maintain the global DNA methylation level.

In general, retrotransposons in genome are densely methylated and thus silenced. The methylation level of intracisternal A particle (IAP), which is one of the retrotransposons, was determined (**Figure 9A**). The CpG sites in IAP *gag* were 88.5% methylated in the parent ESC, the level being reduced to 37.9, 31.8, and 28.8% after four, ten, and twenty days after treatment with OHT to delete the endogenous *Dnmt1*-F/F (*Dnmt1*-F/F+OHT). In ESC ectopically expressing full-length Dnmt1, oocyte-type Dnmt1, Dnmt1(291-1620), and Dnmt1(602-1620), the DNA methylation levels after 10 days treatment with OHT at the IAP-*gag* were 87.3, 93.2, 81.8 and 73.1%, respectively. This again supports that the deletion mutants apparently possess the ability to maintain the global genome methylation level. However, the recovery of the methylation level of the ESC expressing Dnmt1(602-1620) was slightly lower than those of the other Dnmt1 expressing ESC (**Figure 9B**).

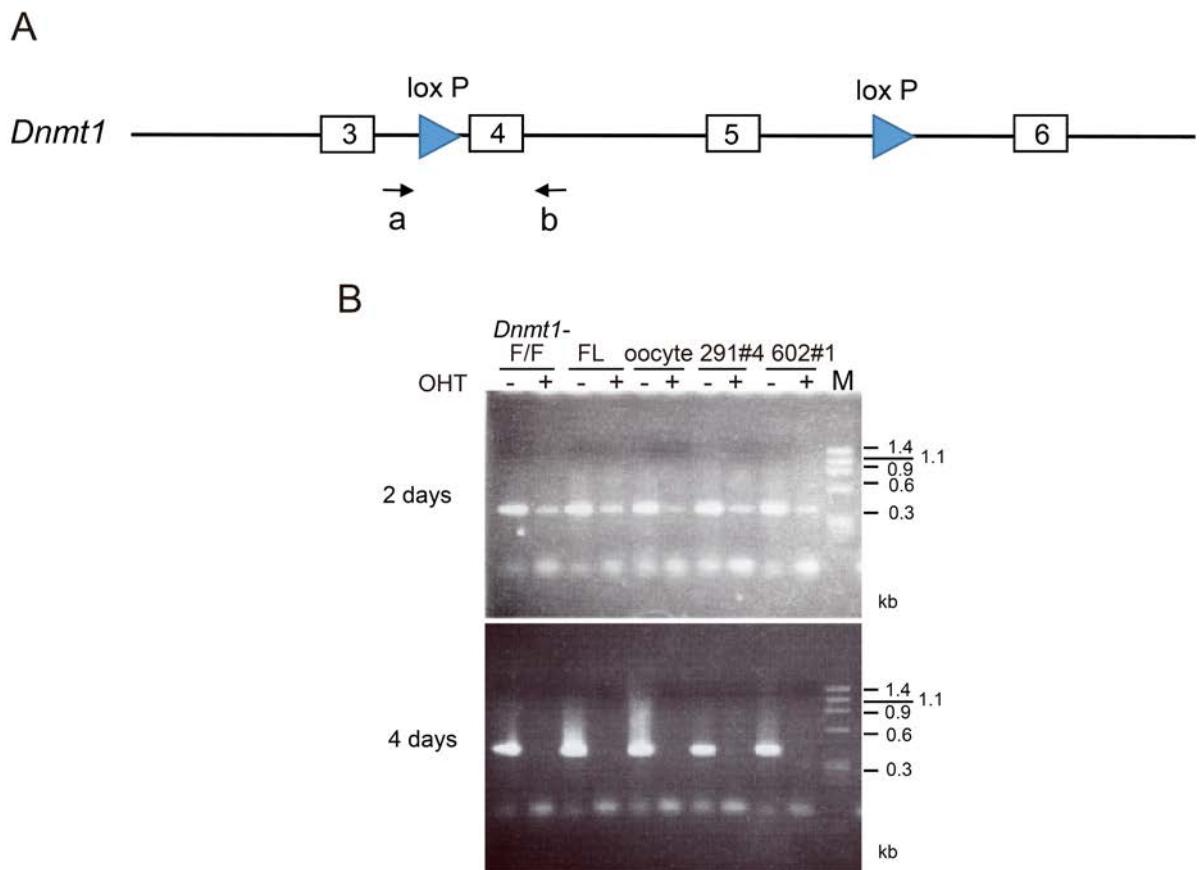
### 7.3. The RFTS is necessary for Dnmt1 to be localized to the replication foci

As it was reported that the RFTS domain is a prerequisite for Dnmt1 to be



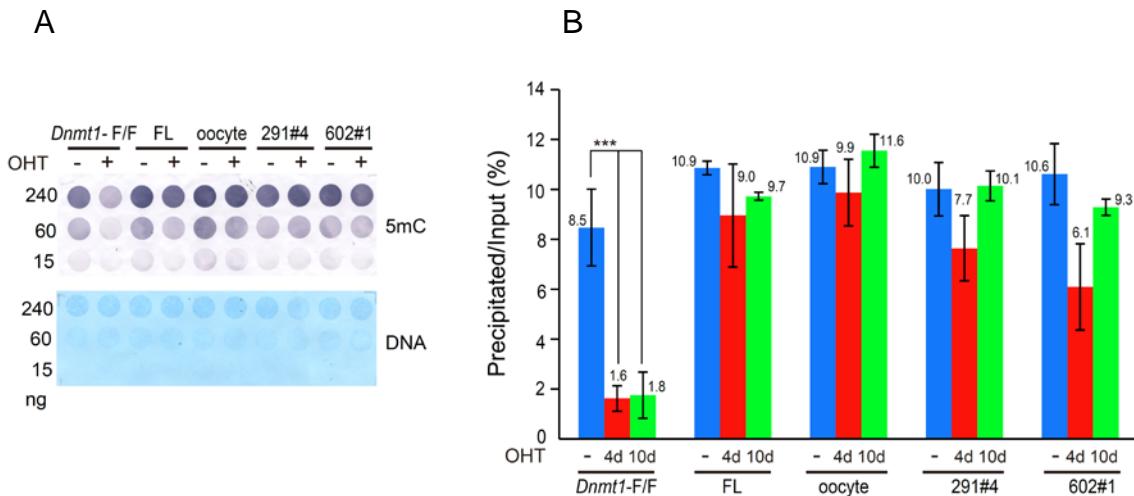
**Figure 6. Western blotting of ectopically expressed Dnmt1.**

Cell extracts of parent *Dnmt1*-F/F ESC, and cells ectopically expressing full-length Dnmt1 (FL), oocyte-type Dnmt1 (oocyte), Dnmt1(291-1620) (291) clones #4 and 5, or Dnmt1(602-1620) (602) before (-) and after (+) OHT-treatment were electrophoresed in SDS-polyacrylamide gels, and then Dnmt1 and tubulin were detected by Western blotting. Molecular size markers are shown at the left and right of the gel.



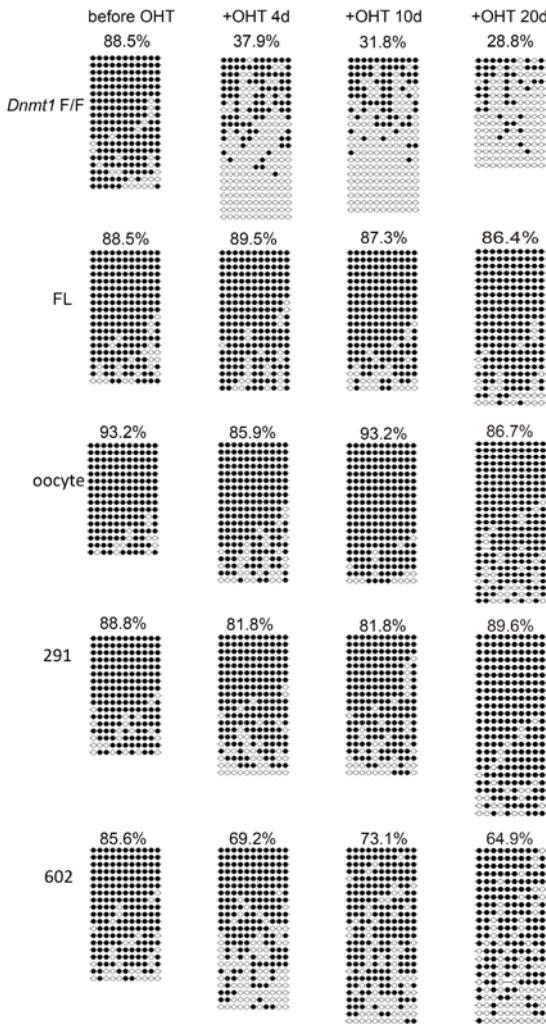
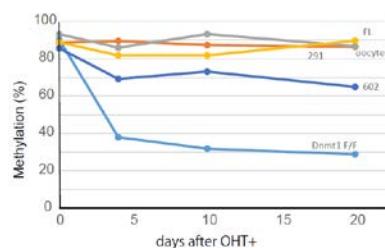
**Figure 7. Determination of the deletion of the endogenous *Dnmt1* gene by PCR amplification.**

**A.** Schematic illustration of the *loxP* inserted sites of the *Dnmt1* gene, and the positions of the primers a and b (arrows). The numbers in the boxes are exon numbers. **B.** PCR amplification of the endogenous *Dnmt1* gene of *Dnmt1*-F/F cells, and cells ectopically expressing full-length *Dnmt1* (FL), oocyte-type *Dnmt1* (oocyte), *Dnmt1*(291-1620) (291#4), and *Dnmt1*(602-1620) (602#1) before (-) and after (+) OHT treatment. PCR amplifications were performed after two (upper panel) and four days (lower panel) treatment with OHT. Four days, not two days, treatment of the ESC with OHT completely deleted the endogenous *Dnmt1* gene. Molecular size markers (M) are indicated at the right side of the gels.



**Figure 8. Global DNA methylation in ESC expressing ectopic Dnmt1.**

**A.** Genome DNA prepared from ESC expressing no ectopic Dnmt1 (*Dnmt1*-F/F), or full-length Dnmt1 (FL), oocyte-type Dnmt1 (oocyte), Dnmt1(291-1620) (291), or Dnmt1(602-1620) (602) treated without (-) or with OHT (+) for four days was immuno-detected with anti-methylated cytosine antibody (5mC, upper panel) or stained with methylene blue (DNA, lower panel). The amounts of DNA are shown at the left. **B.** Genome DNA prepared from the ESC treated without (-) or with OHT for four (4d) or ten days (10d) was fragmented by sonication, and then precipitated with MBD1. The precipitated DNA was normalized as to that of input DNA. The precipitated DNA is expressed as the averages  $\pm$  S. D. for more than three independently prepared genome preparations for before and after the OHT treatment, respectively. All the ESC expressing ectopic Dnmt1 showed significant methylation levels compared to that without ectopic Dnmt1 after the OHT treatment ( $p < 0.001$ ) except for the ESC expressing Dnmt1(602-1620), which was  $p < 0.01$ . \*\*,  $p < 0.01$  and \*\*\*,  $p < 0.001$ .

**A****B****Figure 9. DNA methylation analyses of the *gag* of IAP.**

**A.** The methylation state of the genome DNA prepared from ESC expressing no Dnmt1 (*Dnmt1* F/F), full-length Dnmt1 (FL), oocyte-type Dnmt1 (oocyte), Dnmt1(291-1620) (291), or Dnmt1(602-1620) (602) four, ten, or twenty days after addition of OHT. The methylation states of the *gag* of IAP are shown with that of the parent cells before (*Dnmt1*-F/F) and after the OHT treatment (*Dnmt1*-F/F+OHT). Each horizontal line indicates the CpGs in one analyzed clone. Each circle indicates one CpG site, methylated (filled circles) or un-methylated (open circles). The percentages of methylation are indicated at the top. **B.** Methylated CpG percentages are plotted.

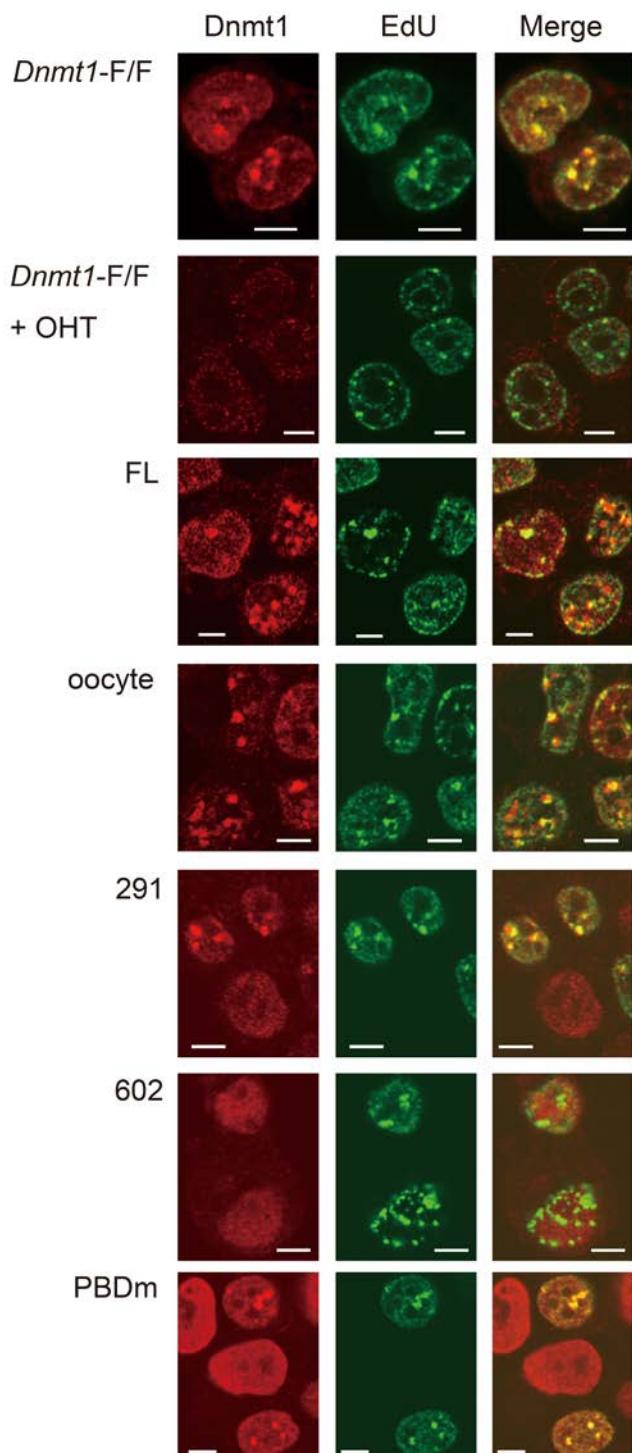
localized to the replication region (Leonhardt et al., 1992), and that PCNA, which is known to be a platform for the replication machinery, also contributes to the localization of Dnmt1 to the replication region (Chuang et al., 1997), I did not expect that the PBD and RFTS domain was dispensable for maintaining the global DNA methylation level. Therefore, I examined the localization of the Dnmt1 used in the present study at replication foci (**Figure 10**). Expectedly, Dnmt1(602-1620) was not co-localized with EdU, which is incorporated at the replication time and thus accumulates at replication foci. All the other Dnmt1 containing the RFTS domain were co-localized with EdU. This is consistent with the previous report that the RFTS is necessary for Dnmt1 to be localized at replication foci (Leonhardt et al., 1992).

This raised the questions whether or not the apparent retention of global methylation by ectopically expressed Dnmt1(602-1620) was the result of replication-coupled maintenance DNA methylation property of Dnmt1, and whether or not the PBD contributes to the maintenance methylation during replication.

#### 7.4. The RFTS domain, not the PBD, is necessary for replication-coupled maintenance DNA methylation

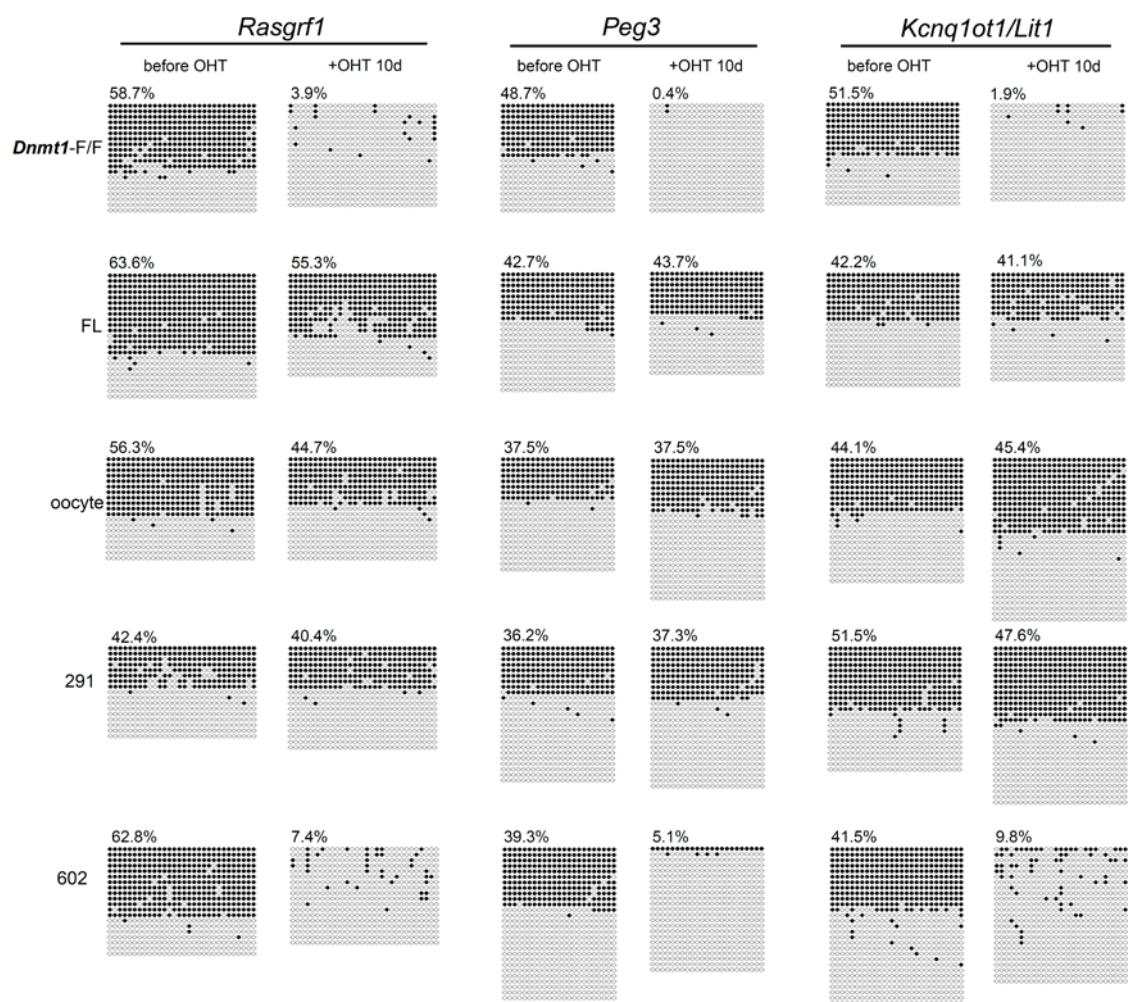
To examine replication-coupled maintenance DNA methylation, I monitored the differentially methylated regions (DMR) of imprinted genes. Imprinted genes show mono-allelic expression due to complete or null DNA methylation at the DMR in male- or female-germ cell-derived loci. The methylation patterns of DMR of imprinted genes cannot be restored once the methylation patterns are erased (Tucker et al., 1996; Borowczyk et al., 2009). Therefore, the DMR of an imprinted gene is an ideal indicator for evaluating the replication-coupled maintenance DNA methylation by ectopically expressed Dnmt1 after deletion of the endogenous *Dnmt1*-F/F.

For the DMR of three imprinted genes, *Rasgrf1* (Plass et al., 1996), *Peg3* (Kuroiwa et al., 1996), and *Kcnq1ot1/Lit1* (Engemann et al., 2000), the methylation state was determined (**Figure 11**). Expectedly, ESC expressing the full-length Dnmt1 or oocyte-type Dnmt1, which is specifically expressed in oocytes and at an early stage of embryogenesis, maintained the DMR methylation judging from that about half of the subcloned DMR of *Rasgrf1*, *Peg3*, and *Kcnq1ot1/Lit1* exhibited almost full methylation after deletion of the endogenous *Dnmt1* gene, which is an indication of mono-allelic DMR methylation. Dnmt1(291-1620), which lacks the PBD but contains the RFTS domain, maintained the full methylation of DMR in about half the clones tested for all three imprinted genes. On the contrary, with Dnmt1(602-1620), which lacks both the PBD and RFTS domain, DNA methylation in the DMR was lost.



**Figure 10. Localization of Dnmt1 at the replication region in ESC.**

ESC (*Dnmt1*-F/F), and ESC expressing no Dnmt1 (*Dnmt1*-F/F+OHT), ectopic full-length Dnmt1 (FL), oocyte-type Dnmt1 (oocyte), Dnmt1(291-1620) (291), Dnmt1(602-1620) (602), or full-length Dnmt1 with the mutation of H168R (PBDm) treated with OHT were labeled with EdU, and detected the EdU (green) and Dnmt1 (red), and the images were merged. White bars indicate 5  $\mu$ m.



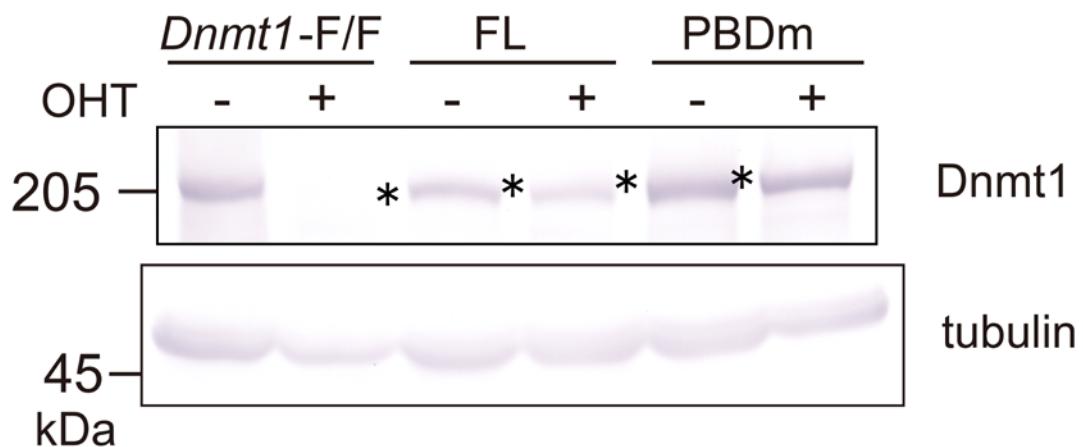
**Figure 11. DNA methylation analysis of three imprinted genes.**  
 The DNA methylation state of the DMRs of *Rasgrf1*, *Peg3*, and *Kcnq1ot1/Lit1*.

The results clearly indicate that the PBD is dispensable for the replication-coupled maintenance DNA methylation at the DMR in imprinted genes. To further test this, full-length Dnmt1 with a mutation of H168R in the PBD, this mutant Dnmt1 being reported to be localized to the replication region (Takebayashi et al., 2007) but not to interact with PCNA (Iida et al., 2002), was introduced into *Dnmt1*-F/F ESC (**Figure 12**). The DNA methylation activity of the recombinant Dnmt1 with the mutation (PBDm) was similar to that of the wild-type Dnmt1 (**Figure 13A**). Ten days after addition of OHT to delete the endogenous *Dnmt1*-F/F, the global DNA methylation level was determined by dot blotting with anti-methylated cytosine antibody and precipitation assaying with MBD1. The DNA methylation level did not show any difference from that of the parent ESC or expressed wild-type Dnmt1 (**Figure 13B and C**). Furthermore, the apparent DNA methylation level at IAP and the DNA methylation patterns at the DMR of imprinted genes in the cells expressing the mutant Dnmt1 were maintained (**Figure 13D**). In addition, as reported, Dnmt1 with the H168R mutation was localized to the replication region (**Figure 10**). These results indicate that Dnmt1 binding to PCNA is dispensable for maintenance DNA methylation. It can also be concluded that the RFTS is a prerequisite domain for the replication-coupled maintenance DNA methylation *in vivo*.

## 7.5. The RFTS domain protects the genome from replication-independent DNA methylation

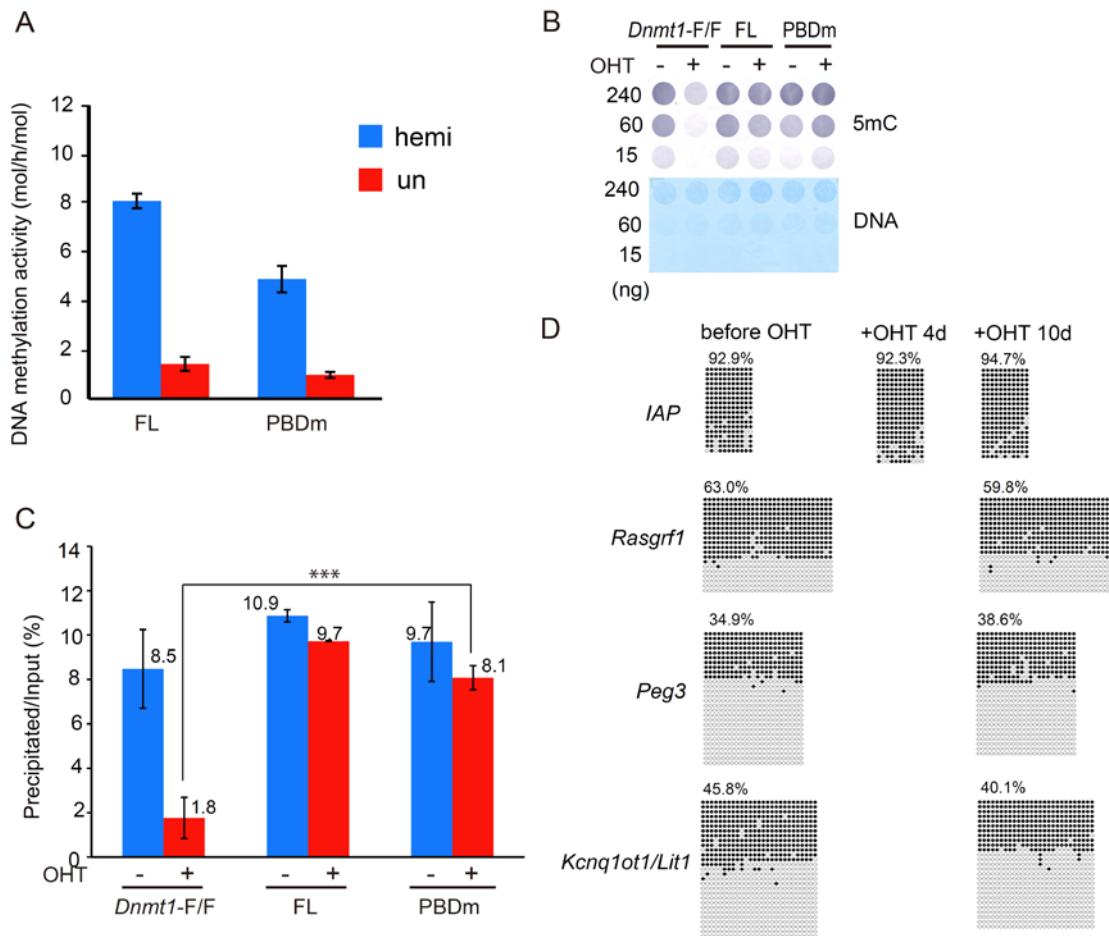
Dnmt1(602-1620) lacking the RFTS domain expressed in ESC apparently could maintain the global DNA methylation level, however, it was not co-localized to the replicating region (**Figure 10**) (Leonhardt et al., 1992), and could not maintain the DMR methylation in the imprinted genes (**Figure 11**). It was reported that Uhrf1, which binds specifically to hemi-methylated DNA that appears just after the replication, is a prerequisite factor for the DNA methylation during replication (Sharif et al., 2007), and directly interacts with the RFTS to remove it from the catalytic pocket (Berkyurek et al., 2014). Judging from these findings, the apparent maintenance of the global DNA methylation level by Dnmt1(602-1620) may not co-operate with Uhrf1.

To determine whether or not Dnmt1(602-1620) co-operates with Uhrf1, the expression plasmids of Dnmt1 were transfected into ESC with *Dnmt1* and *Uhrf1* double conditional knockout (Double F/F), and the endogenous *Dnmt1*-F/F and *Uhrf1*-F/F were deleted after isolation of the clones ectopically expressing Dnmt1. For the clones expressing ectopic Dnmt1(602-1620) (**Figure 14**), the global methylation levels showed significant level of DNA methylation (**Figure 15A and B**), the DNA methylation level



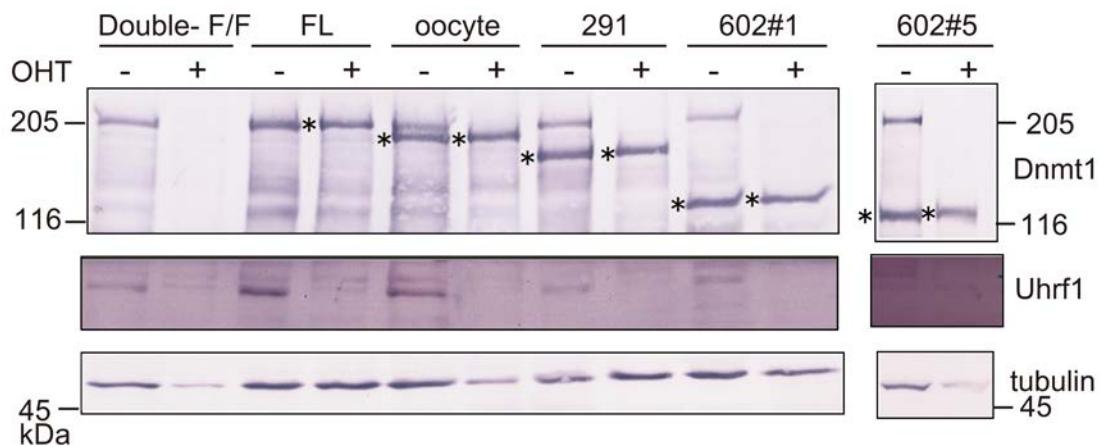
**Figure 12. Western blotting of ectopically expressed Dnmt1 with H168R.**

Cell extracts of parent *Dnmt1*-F/F ESC, and cells ectopically expressing full-length Dnmt1, and the mutant with H168R (PBDm) were electrophoresed and immuno-detected. Asterisks indicate ectopically expressed Dnmt1. Molecular size markers are shown at the left of the gel.



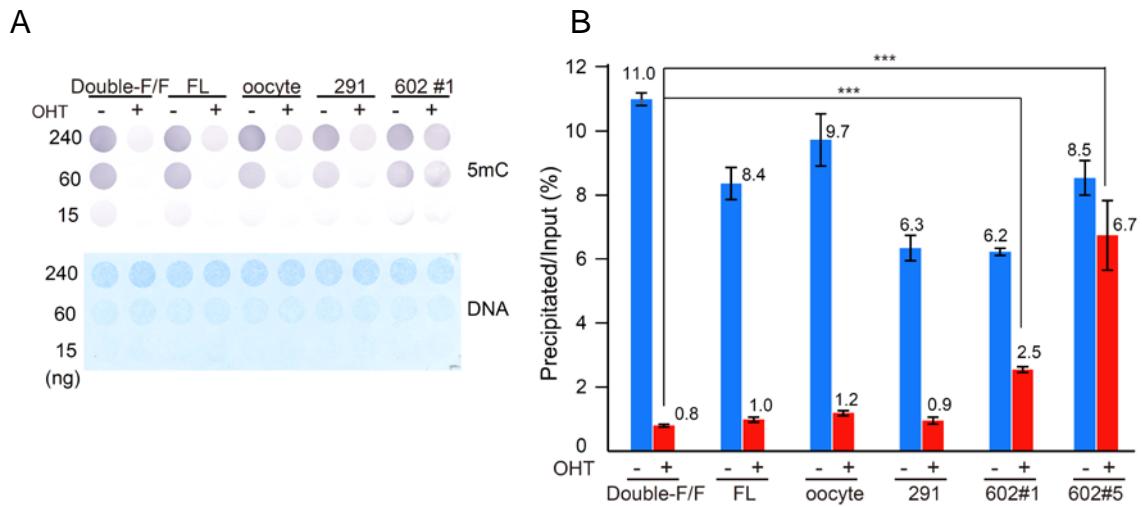
**Figure 13. DNA methylation of ESC expressing full-length Dnmt1 with a mutation in the PBD.**

**A.** DNA methylation activity of the recombinant full-length Dnmt1 (FL) and that with the H168R mutation (PBDm) was determined. The specific activities (mol/h/mol Dnmt1) towards hemi-methylated (hm) and un-methylated (um) DNA are shown as averages  $\pm$  S. D. (n=3). **B.** Genome DNA prepared from the cells before and after deletion of the endogenous *Dnmt1* gene with OHT for ten days was immuno-blotted with anti-methylated cytosine antibody (5mC, upper panel) or stained with methylene blue (DNA, lower panel). The amounts of DNA are shown at the left. **C.** Genome DNA prepared as in B was sonicated and precipitated with MBD1, and then quantitated, averages  $\pm$  S. D. (n = 3) being shown. The values for the parent genome before and after the OHT-treatment were taken from Figure 8B. \*\*\*, P<0.001. **D.** Genome prepared as in B was analyzed as to the methylation state at the *IAP* and DMR of three imprinted genes, *Rasgrf1*, *Peg3*, and *Kcnq1ot1/Lit1* in PBDm cells. The percentages of methylation are indicated at the top.



**Figure 14. Western blotting of ectopically expressed Dnmt1 in *Dnmt1*-F/F and *Uhrf1*-F/F double knockout ESC.**

Cell extracts of parent *Dnmt1*-F/F and *Uhrf1*-F/F ESC (Double F/F), and Double F/F ESC ectopically expressing full-length Dnmt1 (FL), oocyte-type Dnmt1 (oocyte), Dnmt1(291-1620) (291), or Dnmt1(602-1620) (602) clones #1 or 5, before (-) and after (+) OHT-treatment were electrophoresed and immuno-detected. Asterisks indicate ectopically expressed Dnmt1. Molecular size markers are shown at the left and right of the gel.



**Figure 15. Global DNA methylation of *Dnmt1* and *Uhrf1* double-knockout ESC expressing ectopic *Dnmt1*.**

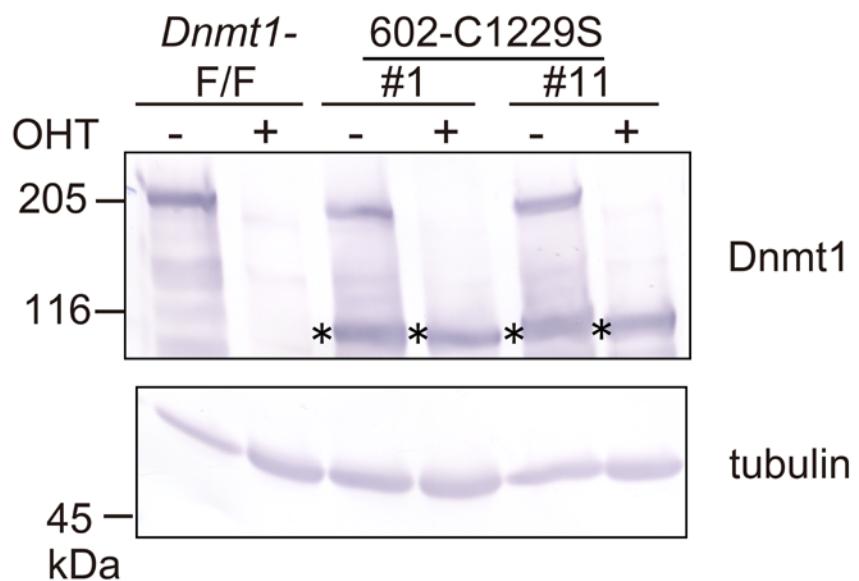
**A.** Genome DNA prepared from *Dnmt1* and *Uhrf1* conditional double-knockout ESC expressing either no ectopic *Dnmt1* (Double F/F), or full-length *Dnmt1* (FL), oocyte-type *Dnmt1* (oocyte), *Dnmt1*(291-1620) (291), or *Dnmt1*(602-1620) clone #1 (602#1) treated without (-) or with OHT (+) for ten days was immuno-blotted with anti-methylated cytosine antibody (5mC, upper panel) or stained with methylene blue (DNA, lower panel). The amounts of DNA are shown at the left. **B.** The genome DNA in panel A and *Dnmt1*(602-1620) clone #5 (602#5) were fragmented by sonication, and then precipitated with MBD1. The precipitated DNA was quantitated and averages  $\pm$  S. D. are shown. \*\*\*, p<0.001.

being significantly higher than in ESC with Double F/F or expressing the full-length Dnmt1, oocyte-type Dnmt1, or Dnmt1(291-1620) after deletion of the endogenous *Dnmt1* and *Uhrf1* genes with OHT. This finding indicates that Dnmt1(602-1620) lacking the RFTS domain may methylate the genome independent of replication, that is, the RFTS domain inserted into the catalytic pocket of Dnmt1(291-1620) acts as a safety valve ensuring replication-coupled maintenance DNA methylation in the genome.

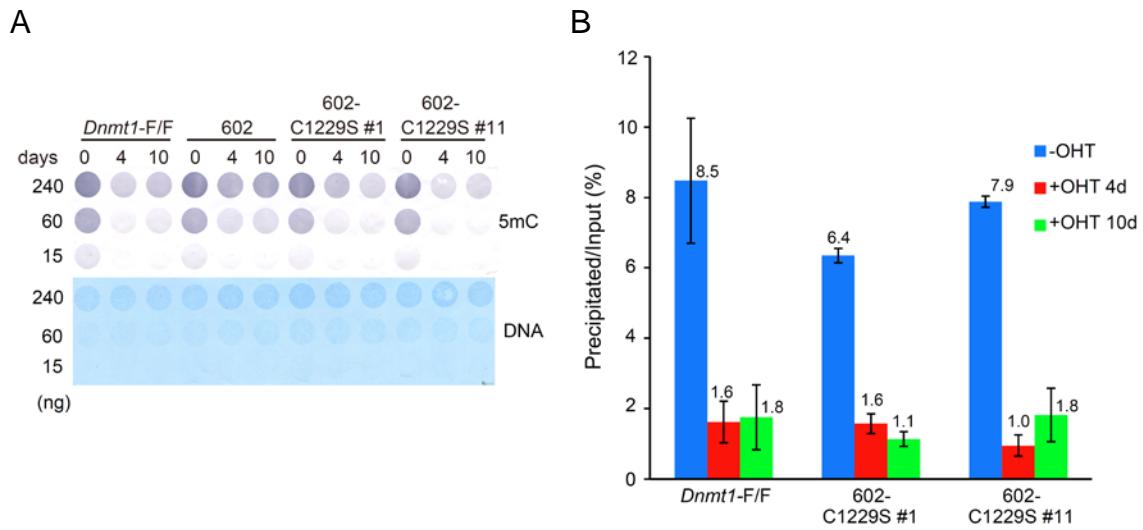
Since the *Dnmt1* and *Uhrf1*-double knockout ESC are expressing Dnmt3a and Dnmt3b, there is a possibility that the apparent global methylation may be due not to the DNA methylation activity of Dnmt1(602-1620) that lacks the NTD and RFTS domain, but to that of the Dnmt3a or/and Dnmt3b. Dnmt1(602-1620) might be just recruiting Dnmt3a or/and Dnmt3b to the target sites. To examine this possibility, I isolated the ESC clones expressing catalytically dead Dnmt1(602-1620) mutant, in which the cysteine at 1229 was replaced with serine (Takebayashi et al., 2007) (**Figure 16**). The ESC expressing the catalytically dead mutant Dnmt1 could not restore the global DNA methylation (**Figure 17A and B**), indicating that the DNA methylation activity of Dnmt1(602-1620) was necessary for the apparent global methylation.

#### 7.6. *De novo* methylation activity is necessary for the replication-independent DNA methylation by Dnmt1 that lacks the NTD and RFTS domain

As for the replication-independent methylation by Dnmt1(602-1620) that lacks the NTD and RFTS domain, two possibilities should be considered; Dnmt1(602-1620) itself created and maintained global methylation, or just fully methylated the hemi-methylated CpG produced by *de novo* methyltransferases, Dnmt3a2 and/or Dnmt3b. To examine this, Dnmt1(602-1620) was expressed in the *Dnmt1*, *Dnmt3a*, and *Dnmt3b* triple knockout (TKO) ESC (**Figure 18**), and then the global genome methylation levels were determined. Identical to the TKO cells expressing full-length Dnmt1 or Dnmt1(291-1620), the DNA methylation in the cells expressing Dnmt1(602-1620) was below the detection level on anti-methylated cytosine antibody reactivity and MBD1 precipitation analysis (**Figure 19A and B**). Furthermore, the expression of TAP-tagged Dnmt3a2 in the cells expressing Dnmt1(602-1620) (**Figure 19B**) led to significant recovery of the global DNA methylation level. These results indicate that the aberrant DNA methylation observed in Double F/F ESC expressing Dnmt1(602-1620) was due to the replication-independent maintenance DNA methylation by Dnmt1(602-1620) at the hemi-methylated CpG sites, which may have been created by *de novo*-type DNA methyltransferases, Dnmt3a2 or Dnmt3b.

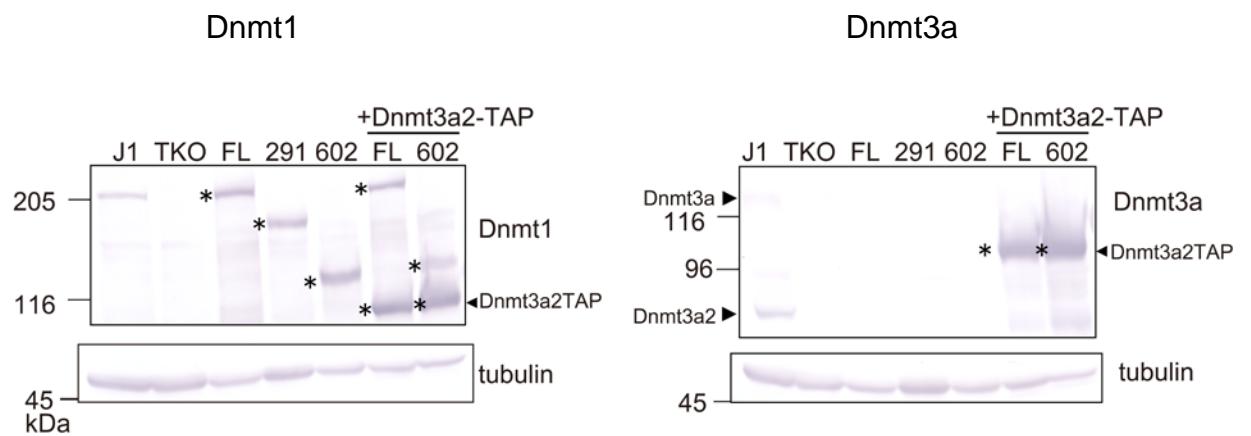


**Figure 16. Western blotting of ectopically expressed Dnmt1 with C1229S in ESC.** Cell extracts of parent *Dnmt1*-F/F ESC and cells ectopically expressing *Dnmt1*(602-1620) (602) with C1229S #1 (602-C1229S #1), or C1229S #11 (602-C1229S #11), before (-) and after (+) OHT-treatment were electrophoresed and immuno-detected. Asterisks indicate ectopically expressed *Dnmt1*. Molecular size markers are shown at the left of the gel.

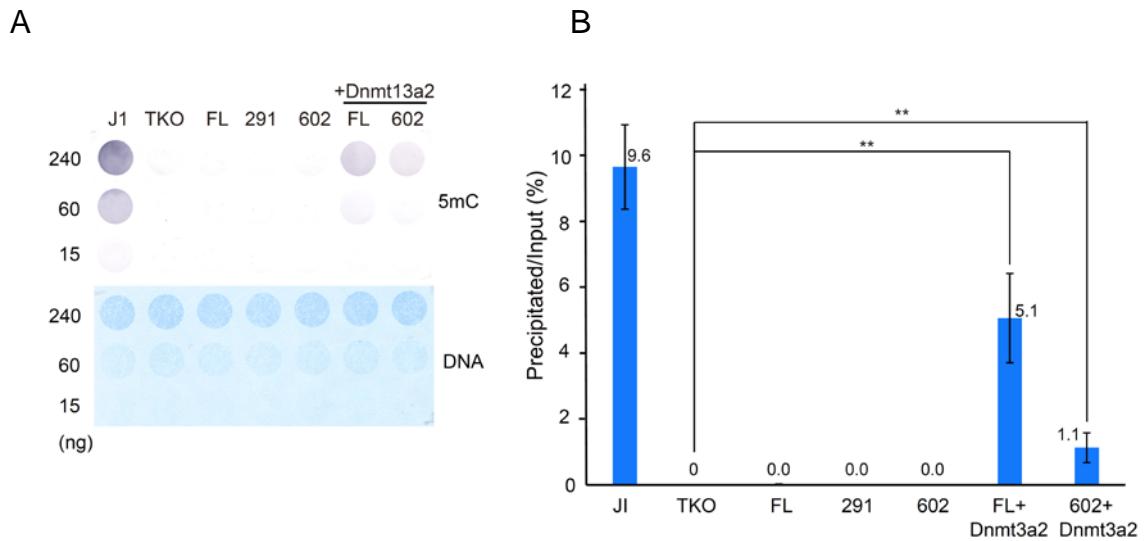


**Figure 17. Global DNA methylation of the genome prepared from the ESC expressing catalytically dead *Dnmt1*(602-1620).**

**A.** Genome DNA prepared from *Dnmt1* conditional-knockout ESC expressing either no ectopic *Dnmt1* (*Dnmt1*-F/F), *Dnmt1*(602-1620), *Dnmt1*(602-1620) with C1229S clones #1 (602-C1229S #1), or clone #11 (602-C1229S #11) treated without (0), or with OHT for four days (4), or ten days (10) was immuno-blotted with anti-methylated cytosine antibody (5mC, upper panel), or stained with methylene blue (DNA, lower panel). The amounts of DNA are shown at the left. **B.** Genome DNA prepared from *Dnmt1* conditional-knockout ESC expressing either no ectopic *Dnmt1* (*Dnmt1*-F/F), or *Dnmt1*(602-1620) with C1229S clones #1 (602-C1229S #1), or clone #11 (602-C1229S #11) treated without (blue) or with OHT for four days (red) or ten days (green) was fragmented by sonication, and then precipitated with MBD1. The precipitated DNA was quantitated and averages  $\pm$  S. D. are shown.



**Figure 18. Western blotting of ectopically expressed Dnmt1 and Dnmt3a2 in TKO ESC.**  
 Cell extracts of parent ESC (J1), TKO cells, and TKO cells ectopically expressing full-length Dnmt1 (FL), Dnmt1(291-1620) (291), Dnmt1(602-1620) (602), full-length Dnmt1 and Dnmt3a2-TAP(FL+Dnmt3a2), and Dnmt1(602-1620) and Dnmt3a2-TAP (602+Dnmt3a2) were electrophoresed and Dnmt1 (left panel) and Dnmt3a (right panel) were immuno-detected. Asterisks indicate ectopically expressed Dnmt1 or Dnmt3a2TAP. Molecular size markers are shown at the left of the gel.



**Figure 19. Global DNA methylation of TKO ESC expressing ectopic Dnmt1.**

**A.** Genome DNA prepared from parent ESC (JI), TKO ESC (TKO), TKO ESC expressing full-length Dnmt1 (FL), Dnmt1(291-1620) (291), Dnmt1(602-1620) (602), full-length Dnmt1 and Dnmt3a2-TAP (FL+Dnmt3a2), or (602-1620) and Dnmt3a2-TAP (602+Dnmt3a2) was immuno-blotted with anti-methylated cytosine antibody (5mC, upper panel) or stained with methylene blue (DNA, lower panel). The amounts of DNA are shown at the left. **B.** Genome DNA prepared from JI, TKO ESC (TKO), TKO ESC expressing no ectopic Dnmt1 (TKO), or full-length Dnmt1 (FL), Dnmt1(291-1620) (291), Dnmt1(602-1620) (602), full-length Dnmt1 and Dnmt3a2-TAP (FL+Dnmt3a2), or Dnmt1(602-1620) and Dnmt3a2-TAP (602+Dnmt3a2) was fragmented by sonication, precipitated with MBD1, and then analyzed. The value obtained for TKO was considered as no DNA methylation and subtracted from each measurement. Averages  $\pm$  S. D. are shown.

## 8. Discussion

In the present study, the contribution of the NTD containing the PBD and the RFTS domain to the DNA methylation in ESC was examined. It was found that the RFTS domain, not the PBD, is necessary for the replication-coupled maintenance DNA methylation. Importantly, the RFTS domain also protects the genome DNA from replication-independent DNA methylation.

### 8.1. The RFTS domain is necessary for replication-coupled maintenance DNA methylation

The recombinant Dnmt1(602-1620), which lacks the NTD and RFTS domain, selectively methylates hemi-methylated DNA, and its specific activity is similar to that of the full-length, oocyte-type, or Dnmt1(291-1620) (Figure 5B). However, because the RFTS domain is responsible for the tethering of Dnmt1 to the replication region (Leonhardt et al., 1992; see Figure 10), Dnmt1(602-1620) could not perform replication-coupled maintenance DNA methylation at the DMR of imprinted genes, this process being dependent on the existence of Uhrf1 at replication foci (Sharif et al., 2007). Recently, it was reported that the SRA domain of Uhrf1 interacts directly with the RFTS domain (Berkyurek et al., 2014; Bashtrykov et al., 2014a), which plugs the catalytic pocket of Dnmt1, to remove the domain from the catalytic pocket (Berkyurek et al., 2014).

It was reported that the 255-291 and 191-324 sequences of Dnmt1, which code the sequences between the NTD and the RFTS domain (Takeshita et al., 2011), and part of the NTD (Suetake et al., 2006), respectively, are necessary for the maintenance methylation in ESC (Borowczyk et al., 2009). This observation is distinct from our present results. Because the 1-248 sequence of the NTD is protease-resistant (Suetake et al., 2006), and the 291-356 sequence in the crystal structure of Dnmt1(291-1620) could not be deduced (Takeshita et al., 2011), possibly due to its flexible structure, deletion of the 255-291 and 191-324 sequences may change the relative positions of the NTD and RFTS domain and/or destroy the three-dimensional structure of either domain. This could be the reason for the lack of recovery of the maintenance methylation with the reported deletion constructs. Different from the study by Borowczyk et al. (Borowczyk et al., 2009), the constructs used in the present study were able to form an active structure *in vitro*, and thus the requirement solely of the RFTS domain for replication-coupled maintenance methylation in ESC is conclusive.

### 8.2. The PBD is not necessary for maintenance DNA methylation

PCNA encircling DNA binds DNA polymerase  $\delta$  and many factors contributing to replication is a prerequisite factor for replication (Moldovan et al., 2007). Dnmt1 binds PCNA through the PBD in the NTD (Chuang et al., 1997). In addition, the N-terminal domain containing PCNA binding domain is suggested to contribute in complete maintenance of DNA methylation (Egger et al., 2006). Recently, it was proposed that the interaction of PCNA with Dnmt1 facilitates tethering of Dnmt1 to replication sites to ensure propagation of DNA methylation (Schneider et al., 2013). However, the present study indicates that the NTD containing the PBD is dispensable for both the maintenance of global DNA methylation patterns and those of imprinted genes in ESC (**Figures 8 and 11**). The difference between our present results and those reported by others may be partly due to the expression level of the ectopically expressed Dnmt1. We conclude that the interaction of PCNA with the PBD in Dnmt1 is not necessarily required for the replication-coupled maintenance DNA methylation.

### 8.3. The RFTS domain protects the genome from replication-independent DNA methylation

Uhrf1 is a necessary factor for replication-coupled maintenance methylation, as the *Uhrf1*-knockout ESC exhibits a decreased global methylation level (Sharif et al., 2007). From this, it was expected that the ectopically expressed Dnmt1 constructs could not maintain DNA methylation in the *Dnmt1* and *Uhrf1* double-knockout ESC. However, Dnmt1(602-1620), which lacks the RFTS domain, showed a significant level of apparent global methylation (**Figure 15A and B**). This clearly indicates that the methylation of the genome by Dnmt1(602-1620) does not need the co-operation of Uhrf1. That is to say, the RFTS domain protects the genome from replication-independent DNA methylation by plugging the catalytic pocket. The masking of the catalytic pocket by the RFTS domain is important for preventing the access of DNA to the catalytic center (Takeshita et al., 2011; Bashtrykov et al., 2014b). Hemi-methylated DNA is passed onto Dnmt1 through interaction of the RFTS domain with Uhrf1 at the replication region only during replication (Berkyurek et al., 2014). The RFTS domain's position and interaction with Uhrf1 governs the precise timing and location of the maintenance methylation. Anchoring of the RFTS domain in the catalytic pocket is stabilized by four hydrogen bonds with the catalytic domain (Takeshita et al., 2011). Recently, patients with autosomal dominant cerebellar ataxia, deafness and narcolepsy due to point mutations at the interacting surface between the RFTS and catalytic domains were reported (Winkelmann et al., 2012). In addition it was also reported that the RFTS-deleted Dnmt1 enhances tumorigenicity (Wu et al., 2014). The

mutations may affect the protective role of the RFTS domain by disturbing the proper insertion of the domain into the catalytic pocket.

#### 8.4. *De novo* methylation activity is necessary for fixing the replication-independent DNA methylation

ESC with the *Dnmt1* gene deleted still expressed *de novo*-type DNA methyltransferase Dnmt3a2, which is an isoform lacking the N-terminal 218 residues, and Dnmt3b at high levels (Chen et al., 2002; Watanabe et al., 2002). This suggests that the genome is continuously undergoing *de novo* methylation. Nevertheless, methylation on the genome was abolished after four-day culture in *Dnmt1*-knocked out ESC (see **Figure 8**). The methylation marks formed by Dnmt3a2 and Dnmt3b are reported to be specifically hydroxylated through Tet activity and to be removed during replication because hemi-hydroxymethyl CpG is not a good substrate for the maintenance methylation by Dnmt1 and Uhrf1 (Otani et al., 2013). Judging from this, most of the *de novo* sites methylated by Dnmt3a2 and Dnmt3b are not fixed on the genome even in the presence of Dnmt1.

On the other hand, the ectopic expression of Dnmt1(602-1620), which lacks the NTD and RFTS domain, apparently maintained the global methylation level of the genome only when Dnmt3a2 and/or Dnmt3b were expressed (**Figures 15 and 19**). It was strongly suggested that Dnmt1(602-1620) may fix *de novo* methylated sites, which are created by *de novo*-type DNA methyltransferases Dnmt3a2 and Dnmt3b, by fully methylating the hemi-methylated sites, as Dnmt1(602-1620) still possesses selective methylation activity towards hemi-methylated DNA (see **Figure 5B**). It is thus rational to speculate that Dnmt1(602-1620), which lacks the NTD and RFTS domain, may establish and maintain the DNA methylation introduced by Dnmt3a2 and/or Dnmt3b at any times throughout the cell cycle.

The global DNA methylation level was much higher for the ESC expressing full-length Dnmt1 and Dnmt3a2 than those expressing Dnmt1(602-1620) and Dnmt3a2. Since the NTD is reported to bind Dnmt3a and Dnmt3b (Kim et al., 2002), in the present study, it is possible that full-length Dnmt1 efficiently coupled with the *de novo* DNA methylation activity of Dnmt3a2 in a replication-coupled manner.

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