STUDIES OF INTERACTING PROTEINS CONTROLLING DNA METHYLTRANSFERASE 1

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Contents

Summary

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Epigenetic mechanisms, including DNA methylation and histone modifications, control gene expression by modulating chromatin structure. DNA methylation is involved in genomic imprinting, X-chromosome inactivation and long-term transcriptional repression. DNA methylation patterns are established by *de novo* DNA methyltransferases Dnmt3a and Dnmt3b, and are propagated by the maintenance DNA methyltransferase Dnmt1.

In the present study, I aimed to identify novel interacting proteins and to characterize their functions in the regulation of Dnmt1 activity. For this purpose, I generated an ES cell line stably expressing GFP-Dnmt1. Using an immunoprecipitation approach in combination with mass spectrometry, I identified an ubiquitin specific protease Usp7 as a novel interaction partner. Interestingly, I could also show that Usp7 interacts with Uhrf1, a crucial cofactor of Dnmt1. To elucidate the role of Usp7 in the regulation of Dnmt1 *in vivo,* I established an ubiquitination and a protein stability assay. With these assays, I could show that Uhrf1 mediates the ubiquitination of Dnmt1 as well as its autoubiquitination, whereas Usp7 stabilizes Dnmt1 by removal of the ubiquitin chain from the modified Dnmt1. Taken together, Uhrf1 and Usp7 coordinately control the Dnmt1 abundance during the cell cycle.

Genetic ablation of *uhrf1* was reported to result in global hypomethylation in ES cells (ESCs) which is similar to that observed in *dnmt1*$^{-/-}$ ESCs. Uhrf1 is supposed to recruit Dnmt1 to hemimethylated CpG sites. To investigate the mechanism of Dnmt1 recruitment by Uhrf1, the domains involved in the interaction between Dnmt1 and Uhrf1 were mapped. Basing on the mapping result, several specific mutants of Dnmt1 and Uhrf1 were generated. Using a functional rescue approach, I could show that the interaction of Dnmt1 with Uhrf1 is necessary but not sufficient for the maintenance of DNA methylation. This observations lead to propose that Uhrf1 modulates the chromatin accessibility for recruiting Dnmt1 to substrate sites *in vivo*. To test this hypothesis, I established an *in vitro* DNA methylation assay to analyze Dnmt1 activity on chromatin substrates in the presence or absence of Uhrf1. A deletion mutant of Dnmt1 which lacks amino acids essential for the interaction with Uhrf1 is enzymatically active *in vitro*. Interestingly, this mutant is unable to methylate naked hemimethylated substrates when cell extracts were added to the methylation reaction. These results suggest that activation and inhibition of Dnmt1 occur *in vivo* and that Uhrf1 likely has multiple functions in the maintenance of DNA methylation.

In conclusion, this work provides novel insights into the mechanisms of Dnmt1 regulation by the interacting proteins Uhrf1 and Usp7.

Zusammenfassung

(Translation with the help of Zijing Gong, Zhuo Li, Daniel Smeets, Katharina Thanisch, Katrin Schneider, Andrea Rottach, Carina Frauer, Susanne Breitsameter)

Epigenetische Mechanismen, wie DNA-Methylierung oder postranslationale Modifikation von Histonen, kontrollieren Genexpression durch Veränderung der Chromatinstruktur. Die DNA-Methylierung spielt eine fundamentale Rolle in biologischen Schlüsselprozessen wie dem genomischen Imprinting, der X-Chromosomeninaktivierung und der langfristigen transkriptionellen Genrepression. Die Etablierung neuer Methylierungsmuster erfolgt durch die *de novo* DNA-Methyltransferasen Dnmt3a und Dnmt3b, während die *maintenance* DNA-Methyltransferase Dnmt1 diese Muster über sukzessive Zellteilungen hinaus erhält. Intra- und intermolekulare Wechselwirkungen gewährleisten hierbei eine strenge Regulation der Dnmt1-Aktivität.

Im Rahmen der vorliegenden Studie sollten neue mit Dnmt1 wechselwirkende Proteine identifiziert und bezüglich ihres Einflusses auf die Dnmt1-Aktivität untersucht werden. Dazu wurde eine embryonale Stammzelllinie (ES-Zellen) hergestellt, welche stabil GFP-Dnmt1 exprimiert. Durch eine mit Massenspektrometrie kombinierte Immunpräzipitation konnte Usp7 als neuer Interaktionspartner von Dnmt1 und dessen Kofaktor Uhrf1 identifiziert werden. Ubiquitinierungs- und Proteinstabilitäts-Tests in lebenden Zellen ergaben, dass Uhrf1 sowohl die Ubiquitinierung von Dnmt1 als auch seine Autoubiquitinierung vermittelt, während Usp7 Dnmt1-Proteine durch Entfernung von Ubiquitinresten stabilisiert. Die koordinierte Aktivität von Uhrf1 und Usp7 reguliert die Stabilität von Dnmt1 durch Ubiquitinierung beziehungsweise Deubiquitinierung, wodurch die Abundanz von Dnmt1-Molekülen während des Zellzyklus kontrolliert wird.

Interessanterweise zeigen *dnmt1* und *uhrf1* defiziente ES-Zellen gleichermaßen den Phänotyp einer genomischen Hypomethylierung. Darüber hinaus zeigt Dnmt1 eine diffuse Verteilung im Kern von *uhrf1*$^{-/-}$ ES-Zellen, was auf eine Rolle von Uhrf1 bei der Rekrutierung von Dnmt1 zu hemimethylierten CpG-Stellen hinweist. Zum näheren Verständnis des Mechanismus der Dnmt1-Rekrutierung durch Uhrf1, wurden daher die interagierenden Domänen ermittelt und eingegrenzt. Mittels spezifischer Dnmt1- und Uhrf1-Mutanten konnte die Rolle der Uhrf1-Dnmt1 Wechselwirkung für den Erhalt der DNA-Methylierung als notwendig, jedoch nicht ausreichend charakterisiert werden. Basierend auf diesen Ergebnissen postulieren wir, dass Uhrf1 die Zugänglichkeit des Chromatins *in vivo* moduliert

und die Rekrutierung von Dnmt1 zu hemimethylierter DNA ermöglicht. Mittels eines dafür entwickelten *in-vitro* DNA-Methylierungstests sollte die Aktivität von Dnmt1 an einer künstlich hergestellten Chromatinmatrix bestimmt werden, um diese Hypothese zu testen. Interessanterweise ist eine mutierte Form von Dnmt1, der die für die Wechselwirkung mit Uhrf1 entscheidenden Aninosäuren fehlt, in Gegenwart von Zellextrakten nicht in der Lage, die nackte hemimethylierte DNA zu methylieren, obwohl das isolierte Enzym aktiv ist. Diese Ergebnisse deuteten darauf hin, dass *in vivo* sowohl eine Aktivierung als auch eine Inhibition von Dnmt1 stattfindet und Uhrf1 verschiedene Funktionen bei der Erhaltung der DNA-Methylierung durch Dnmt1 hat.

Die vorliegende Studie trägt zum Verständnis der Regulationsmechanismen von Dnmt1 durch die Interaktionspartner Uhrf1 und Usp7 bei.

1. Introduction

1.1. DNA methylation in eukaryotic cells

In addition to the well known four bases, the genome of higher eukaryotes contains methylated cytosine residues, which predominately occur in the context of CpG dinucleotide sites. The CpG sites are distributed throughout the genome and highly concentrated at gene bodies, repetitive elements and promoter regions, so called as CpG island. Approximately 60-80% of CpG dinucleotides are methylated in mouse and human (Ehrlich et al., 1982; Gruenbaum et al., 1981). Cytosine methylation in DNA is established and maintained by a family of DNA methyltransferases (Dnmts), the *de novo* methyltransferases Dnmt3a and 3b and the maintenance methyltransferase Dnmt1. In the complex reaction, the target cytosine is flipped out of the DNA double helix and forms a covalent complex with the DNA methyltransferase through its C6 position. After transfer of a methyl group from the donor of S-Adenosyl-L-Methionine (AdoMet) to the C5 position of the nucleobase, the enzyme is released by β-elimination.

DNA methylation is an important epigenetic modification that in general provides long-term gene silencing (Bird, 2002). DNA methylation was implicated in numerous biological processes, including genomic imprinting, X chromosome inactivation and transposon silencing. DNA methylation controls gene expression in a direct and an indirect manner. The direct transcriptional repression mediated by DNA methylation is achieved by preventing transcriptional factors binding to their recognition sites. Most mammalian transcription factors are sensitive to CpG methylation in their recognition sequence, such as c-Myc, E2F and NF-kB (Prendergast et al., 1991; Di Fiore et al., 1999; Bednarik et al., 1991). The indirect mechanism of transcriptional repression involves methylation readers, the methyl CpG binding proteins (MBDs) or the SRA proteins (Uhrf1/2), which induce the formation of silent chromatin (Fuks, 2005; Fatemi and Wade, 2006; Bostick et al., 2007; Papait et al., 2007; Sharif et al., 2007).

Additionally, DNA methylation was shown to cooperate with dynamic histone modifications including methylation, ubiquitination, acetylation and phosphorylation, to regulate the expression of individual genes.

1.1.1. DNA methyltransferases

Dnmt3 family

Dnmt3a and 3b are two closely related *de novo* methyltransferases (Fig. 1) and responsible for generating DNA methylation patterns during gametogenesis and early embryonic development (Okano et al., 1999; Kaneda et al., 2004). They are highly conserved and have a similar organization with a C-terminal catalytic domain and an N-terminal regulatory domain. The catalytic domain takes a folding similar to bacterial DNA methyltransferases. In the regulatory domain, a proline-tryptophan-proline motif (PWWP) is involved in functional specialization of these enzymes. The PWWP domain was shown to bind the trimethylated histone H3K36 and target Dnmt3a and 3b to pericentromeric heterochromatin (Ge et al., 2004; Dhayalan et al., 2010). A crystal structure suggested that the PWWP domain of Dnmt3b can bind to DNA as well through its basic surface (Qiu et al., 2002). Hence, the methylation of major satellite repeats was abolished by disruption of the PWWP domain (Chen et al., 2004).

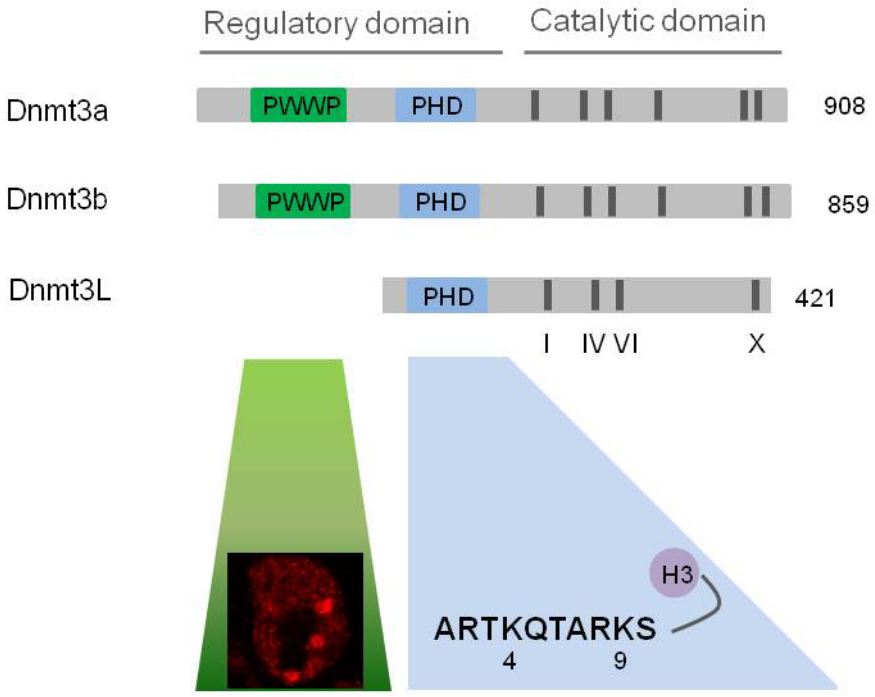

Figure 1. Schematic representation of the domain structure of the Dnmt3 family and the subcellular localization mediated by its individual regulatory subdomains.
Dnmt3 family harbors a conserved catalytic domain and a regulatory domain. The PWWP domain of Dnmt3a and 3b can recognize trimethylated histone H3K36 and target the proteins to heterochromatin (Ge et al., 2004; Dhayalan et al., 2010). The image shows the cellular localization of endogenous Dnmt3a stained by using an anti-Dnmt3a antibody. In addition, the functional PHD finger in the regulatory domain can bind to the N-terminal histone H3 only when lysine 4 was unmethylated (Jia et al., 2007; Otani et al., 2009).

Another functional domain in the N-terminal part is the plant homeodomain (PHD) finger，

which mediates the interactions with transcriptional repressors, histone deacetylases and histone methyltransferases (HMTs). As readers of histone modifications, the PHD fingers of Dnmt3a and 3b specifically recognize unmethylated histone H3K4 and might link DNA methylation and histone modifications (Otani et al., 2009).

Dnmt3a and *dnmt3b* are highly expressed in undifferentiated embryonic stem cells (ESCs) but down-regulated in differentiated somatic cells (Okano et al., 1998b) suggesting that Dnmt3a and 3b are highly active in ESCs and early embryos. Mice lacking *dnmt3a* died at about four weeks of age, indicating that Dnmt3a is not required for early development and may methylate a set of sequences critical for postnatal development. Dnmt3a specifically mediates the methylation of imprinted genes in germ cells. *Dnmt3a* conditional mutant females in germ line lack methylation of allele-specific expression at maternally imprinting genes, whereas the mutant males show hypomethylation at two of three paternally imprinting genes (Kaneda et al., 2004). Unlike *dnmt3a* null mice, ablation of *dnmt3b* is embryonic lethal at embryonic days E14.5-18.5, implicating that Dnmt3b plays an important role during early development. DNMT3b specifically methylates the centromeric minor satellite repeats in ESCs and is involved in a human immunodeficiency, centromeric instability, facial anomalies (ICF) syndrome, which is caused by mutations in the C-terminal domain of DNMT3b (Xu et al., 1999; Hansen et al., 1999). Loss of DNMT3b activity in ICF syndrome leads to a reduction of DNA methylation only at specific repeated sequences and CpG islands in the inactive X chromosome in females. In addition, Dnmt3a and 3b form a hetero-complex *in vivo* to mediate the silencing of transcription factors *oct4* and *nanog* during ESCs differentiation (Li et al., 2007).

The third member of Dnmt3 family is Dnmt3L, which is essential for the methylation of maternally imprinted genes (Hata et al., 2002; Bourc'his et al., 2001). Structurally, Dnmt3L only possesses a short N-terminal regulatory domain and a catalytic domain (Fig.1). Due to the lack of several conserved residues required for methyltransferase activity, Dnmt3L is not able to catalyze methyl group transfer. However, Dnmt3L interacts and colocalizes with Dnmt3a and 3b during early embryonic development and stimulates their activity (Hata et al., 2002; Xie et al., 2006). Additionally, Dnmt3a and 3b coordinately regulate Dnmt3L expression by methylating the promoter of *dnmt3l* (Hu et al., 2008). Crystallographic studies revealed that Dnmt3L recognizes the N-terminal tail of histone H3 only when lysine 4 is unmethylated and induces *de novo* DNA methylation by recruitment and activation of Dnmt3a2, a variant of Dnmt3a (Jia et al., 2007). *Dnmt3l* knockout mice failed to establish the maternal methylation imprints in oocytes, but DNA methylation of most sequences is not

affected, suggesting that other mechanisms may exist to regulate the activity of Dnmt3a and 3b (Bourc'his et al., 2001; Ooi et al., 2007).

Dnmt2

Dnmt2 is a relatively small protein and lacks the N-terminal regulatory domain present in Dnmt1 and Dnmt3 families. Dnmt2 was discovered by Okano *et al* in 1998, but no methyltransferase activity towards DNA was detected (Okano et al., 1998). However, a crystal structure of human DNMT2 complex containing S-adenosyl-L-homocysteine (AdoHcy) showed that Dnmt2 possesses DNA-binding property (Dong et al., 2001) and might have very weak DNA methyltransferase activity (Hermann et al., 2004). Most importantly, Dnmt2 was shown to methylate aspartic acid transfer RNA ($tRNA^{asp}$; Goll et al., 2006). Therefore it is controversial question whether Dnmt2 has DNA methyltransferase activity.

Dnmt1

Dnmt1 is the first discovered mammalian DNA methyltransferase and responsible for inheritance of DNA methylation patterns after DNA replication (Bestor et al., 1988). Although homozygous ESCs lacking *dnmt1* show a four-fold reduction in global DNA methylation, the mutant cells are viable and have no obvious abnormalities with growth rate and morphology (Li et al., 1992; Lei et al., 1996), suggesting that DNA methylation is not required for self proliferation of ESCs. However, *dnmt1* null embryos showed a delayed development and died at mid-gestation stage, implicating that DNA methylation is indispensable for early embryonic development.

Dnmt1 is a relatively large protein comprising an N-terminal regulatory domain in addition to the C-terminal catalytic domain, which contains all the conserved motifs for DNA methyltransferase activity. The N-terminal domain constitutes two thirds of the molecule, which contains different functional subdomains: a proliferating cell nuclear antigen (PCNA) binding domain (PBD), a heterochromatin targeting sequence (TS), a CXXC-type zinc finger domain and two Bromo-Adjacent Homology domains (BAH1 and BAH2, Fig. 2). The N-terminal domain of Dnmt1 is linked to the catalytic domain by seven glycine-lysine repeats, $(KG)_7$. PCNA targets Dnmt1 to replication sites *in vivo* through the interaction with the PBD domain (Chuang et al., 1997; Leonhardt et al., 1992). Dnmt1 is recruited to DNA repair sites by PCNA to restore DNA methylation patterns (Mortusewicz et al., 2005). The TS domain is required for recruitment of Dnmt1 to pericentric heterochromatin in late S phase and G2 phase (Easwaran et al., 2004) and mediates dimerization of Dnmt1 (Fellinger et al., 2009). Recently, the TS domain was reported to play a role in autoinhibition of Dnmt1 activity by

virtue of binding DNA (Syeda et al., 2011; Takeshita et al., 2011). The BAH domain might be involved in protein-protein interaction. Under the control of the N-terminal regulatory domain, Dnmt1 shows a preferential binding for hemimethylated DNA in contrast to Dnmt3 enzymes.

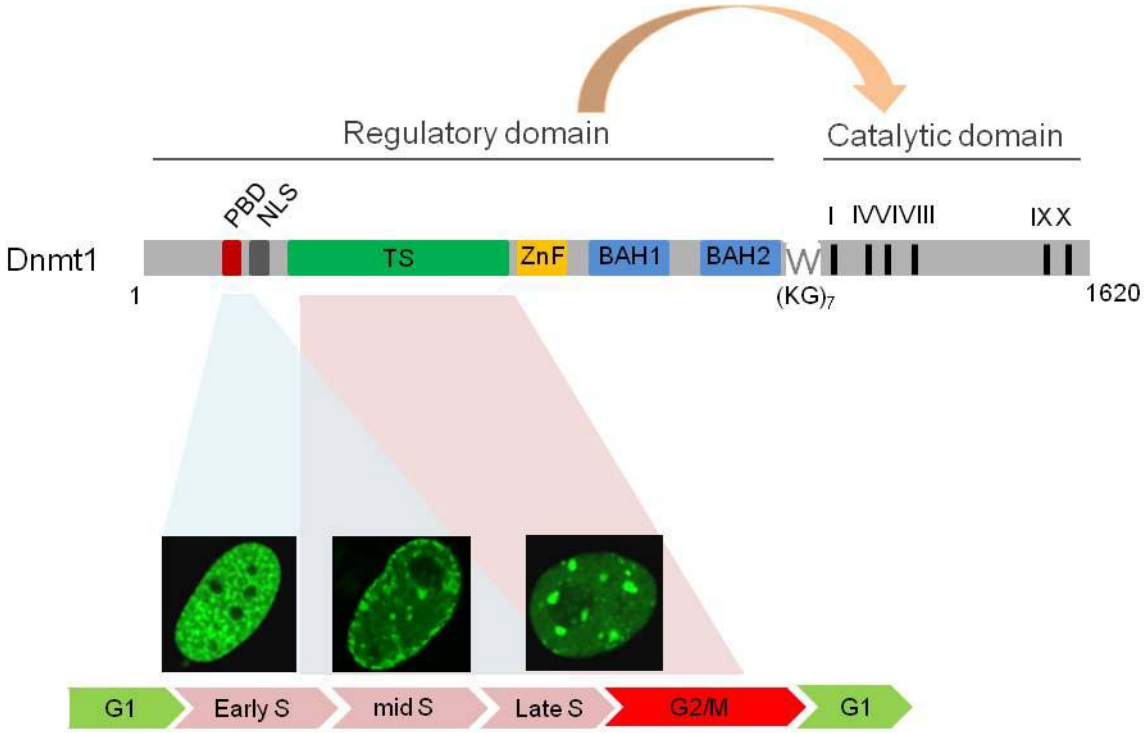

Figure 2. Schematic representation of the domain structure of Dnmt1 and subcellular localization mediated by its individual regulatory subdomains during the cell cycle
Dnmt1 comprises a regulatory domain and a catalytic domain. The regulatory domain harbors several functional subdomains: PBD (proliferating cell nuclear antigen binding domain); TS domain (the targeting sequences); ZnF domain (CXXC type zinc finger) and BAH domains (bromo-adjacent homology domains). The PBD-mediated interaction with PCNA targets Dnmt1 to the replication machinery during S phase while the TS domain targets Dnmt1 to pericentric heterochromatin during late S and G2 phases. Images show the localization of GFP-Dnmt1 during S and G2 phase. The ZnF preferentially binds to unmethylated CpG (Frauer et al., 2009). The methylation activity of Dnmt1 is regulated by the N-terminal regulatory domain.

Dnmt1 is ubiquitously expressed and it has different translational start points that lead to different splice variants. In somatic cells, mouse Dnmt1 comprises 1620 amino acids. A short isoform of Dnmt1, which lacks the first 118 amino acids of the somatic form, is specifically expressed during oocytes growth and maturation, and also during preimplantation development (Carlson et al., 1992; Gaudet et al., 1998; Hirasawa et al., 2008; Howell et al., 2001). The oocyte specific variant of Dnmt1 (Dnmt1o) was shown to transiently translocate from cytoplasm to nucleus at the 8-cell stage during early embryonic development (Carlson et al., 1992; Cardoso and Leonhardt, 1999), where it maintains genomic imprinting. Dnmt1o protein is more stable than the somatic form Dnmt1 (Ding and Chaillet, 2002), possibly due to lacking the first 118 amino acids, which are suggested to comprise ubiquitination sites

(Agoston et al., 2005).

1.1.2. Dynamics of DNA methylation

DNA methylation is a stable but not irreversible epigenetic mark. Although the molecular mechanism of DNA demethylation is poorly understood and the enzymes are so far not identified, DNA methylation reprogramming was already observed during early mammalian embryogenesis and gametogenesis (Mayer et al., 2000; Monk et al., 1987). The first phase of methylation reprogramming occurs within 4-8 hours after fertilization, in which methylation patterns in the paternal genome are erased except for imprinted loci and repetitive elements. After implantation, DNA methylation patterns are reestablished by Dnmt3a and 3b. Most previous studies suggest that the genome wide demethylation observed after fertilization occurs actively, implicating the presence of demethylating enzymes that can actively remove either the methyl group from the methylated cytosine or the whole nucleotide. The second phase of demethylation occurs before implantation and is replication-dependent process, that is, by the lack of maintenance DNA methyltransferase following DNA replication and cell division (Morgan et al., 2005). Consistent with this observation, it was reported that the maternally contributed maintenance methyltransferase Dnmt1 is excluded from nucleus during early embryonic development (Carlson et al., 1992; Cardoso and Leonhardt, 1999).
Concerning the mechanism of demethylation, several mechanisms suggested: (1) direct removal of the methyl group from 5-methylcytosine (5mC), (2) direct removal of 5mC by a glycosylase, followed by DNA repair machinery to replace 5mC with cytosine, (3) deamination of 5mC to thymine, subsequent removal and repair. The deamination of 5mC catalyzed by the cytidine deaminase family (Activation Induced deaminase and Apolipo-protein B RNA-editing catalytic component-1) is the first step for DNA demethylation, in which an intermediate G:T mismatch is generated. Subsequently, the enzymes responsible for DNA repair are recruited to the mismatch sites. DNA demethylation through DNA repair mechanisms was first described in mammalian cells by Jost *et al*, who reported an enzymatic system which replace 5mC by cytosine (Jean Pierre Jost, 1993). Later, thymine DNA glycosylase (TDG) was proposed as the enzyme responsible for replacing the mismatch nucleotide with cytosine (Zhu et al., 2000). Moreover, Dnmt3a and 3b were found to directly participate in active demethylation of 5mC through deamination and interaction with TDG (Li and Zhou et al., 2007; Metivier et al., 2008). This interaction between DNA methyltransferases and TDG can stimulate glycosylase activity. It is surprising that Dnmt3a

and 3b possess two opposite enzymatic activities. Gadd45 (growth arrest and DNA damage inducible protein 45α) protein family is also involved in DNA demethylation by inducing the nuclear excision repair system (Rai et al., 2008; Jirichy and Menigatti, 2008; Schmitz et al., 2009; Barreto et al., 2007).

Recently, it was reported that the mammalian genome contains a novel modification, 5-hydroxymethylcytosine (5hmC) (Tahiliani et al., 2009), which results from oxidation of 5mC by Tet proteins. 5hmC is supposed to be an intermediate in the DNA demethylation process, which may directly be dealkylated resulting in cytosine (Guo et al., 2009; Liutkeviciute et al., 2009). Additionally, by tracking the paternal DNA methylation state in live zygotes that were treated with siRNA (Okada et al., 2010), the elongator complex component Elp3 (or KAT9) was supposed to participate in paternal genomic DNA demethylation.

1.2. Regulation of Dnmt1

The maintenance of DNA methylation is a complex process, including the recognition of hemimethylated DNA substrate, binding of methyl group donor, formation of a covalent complex, transfer of a methyl group to cytosine and release from the covalent complex. The characterization of Dnmt1 and the identification of its functional regulators were the major area of study in the past two decade.

1.2.1. Characterization and functional analysis of Dnmt1

According to bioinformatic analysis, Dnmt1 was proposed to have evolved by fusion of two or more ancestral genes for controlling DNA methyltransferase activity (Margot et al., 2000). Whereas the purified C-terminal domain of Dnmt3a and 3b still possess the ability to transfer methyl group to DNA substrate, the isolated C-terminal domain of Dnmt1 is not sufficient for enzymatic activity (Fig. 3a), neither alone nor in combination with other single domains (Fatemi et al., 2001). Using deletion analysis, a large part of the N-terminal domain (NTD) was reported to be required for enzymatic activity of Dnmt1 (Zimmermann et al., 1997; Aubol et al., 2003; Araujo et al., 2001; Margot et al., 2000). These results suggest that the enzymatic activity of Dnmt1 is under control of the N-terminal regulatory domain of the enzyme.

In contrast to prokaryotic DNA methyltransferases, Dnmt1 shows a preferential binding for the DNA substrates containing hemimethylated CpG sites under the regulation of the NTD. The preference rate was investigated by different groups with variable results ranging from 2 to 200 fold (Fatemi et al., 2001; Jeltsch et al., 2006), depending on the DNA template and conditions used in the assay. Recently, a novel non-radioactive assay for DNA methyltransferase activity and DNA binding was developed and showed an averaged preference rate of 15-fold for hemimethylated DNA (Frauer et al., 2009). Interestingly, the preferential binding of bacterial DNA methyltransferase HhaI for hemimethylated DNA was increased to 2.5-fold by fusing NTD of Dnmt1 (Pradhan et al., 2000). However, the binding affinity is not comparable with Dnmt1, suggesting that the region responsible for substrate recognition might reside not only in the regulatory domain, but also in the catalytic domain.

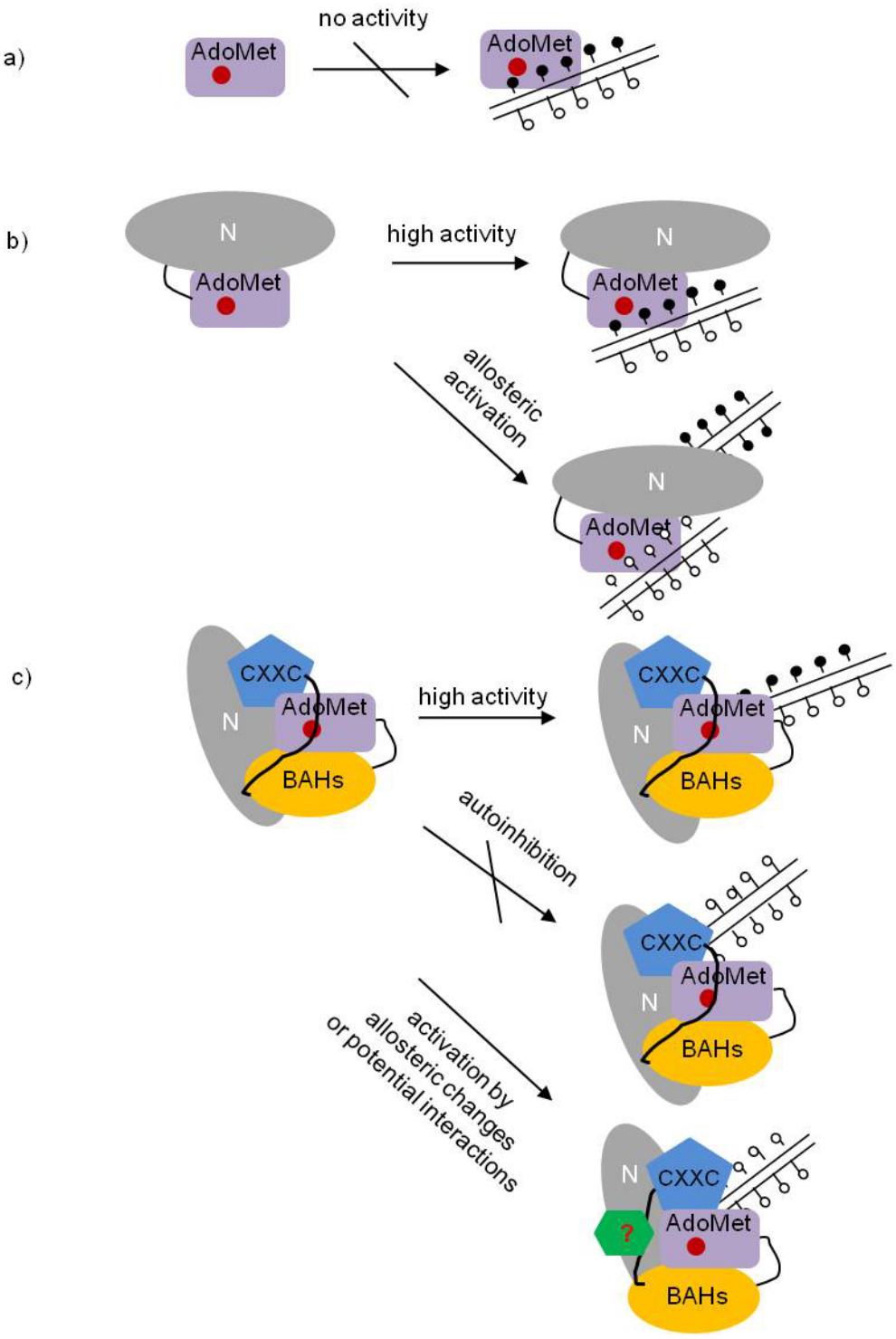

Figure 3. Regulation of Dnmt1 activity by its N-terminal regulatory domain
a) Isolated catalytic domain of Dnmt1 cannot methylate DNA substrates containing the hemimethylated CpG sites. b) Dnmt1 possesses a preferential binding and activity for the hemimethylated DNA under control of the N-terminal regulatory domain. In addition, Dnmt1 shows the methylation activity toward unmethylated DNA by activation of fully methylated DNA. c) The linker between the CXXC and BAH1 domains could hold the unmethylated DNA away from catalytic center to prevent *de novo* methyltransferase activity of Dnmt1. It can also be speculated that an unknown protein might reposition the linker to release the autoinhibition.

In addition to methylate hemimethylated DNA, Dnmt1 has *de novo* methyltransferase activity *in vitro* (Gowher et al. 2005). Dnmt1 mediates *de novo* methylation of CpG islands in human

cells and is proposed to be responsible for erroneous hypermethylation in cancer cells (Jair et al., 2006). Regarding whether Dnmt1 could methylate unmethylated DNA, Bestor *et al* initially showed that the cleavage between N- and C-terminal domains caused a large stimulation of the initial velocity of methylation on unmethylated DNA without changing methylating rate of hemimethylated DNA, demonstrating that the NTD may be an intrinsic inhibitor of *de novo* methylation activity of Dnmt1 (Timothy H. Bestor, 1992). Furthermore, an allosteric activation mechanism to control *de novo* methyltransferase activity of Dnmt1 was suggested (Fig. 3b; Fatemi et al., 2001), in which Dnmt1 is stimulated to methylate unmodified cytosine by binding fully methylated DNA. The allosteric activation of Dnmt1 is mediated by its CXXC domain, suggesting that the binding of methylated DNA triggers a conformational change resulting in access of Dnmt1 to unmethylated DNA (Fatemi et al., 2001). Furthermore, the CXXC domain was observed to directly interact with the catalytic domain and might be act as an intrinsic inhibitor for controlling *de novo* methyltransferase activity of Dnmt1. However, complementation of *dnmt1*$^{-/-}$ ESCs with Dnmt1$^{\Delta CXXC}$ argues against the speculation that the CXXC domain function in restraining Dnmt1 methyltransferase activity on unmethylated DNA (Frauer et al., 2011; Song et al., 2010). A crystallographic study of Dnmt1-DNA complex revealed that Dnmt1 binds unmethylated DNA by the CXXC domain, but the linker between the CXXC and BAH1 domain blocks the catalytic center of the C-terminal domain and inhibits *de novo* methylation activity (Fig. 3c; Song et al., 2010). In addition, the loop of the BAH2 domain interacts with the target recognition domain (TRD), positioning the TRD in a retracted position and preventing it from access to DNA major groove (Song et al., 2010). Basing on the crystal structure studies, it can be speculated that the linker between the CXXC and BAH1 domains might be repositioned by potential interaction partners, which activate the *de novo* methylation activity of Dnmt1.

1.2.2. Regulation by posttranslational modifications

Posttranslational modifications often modulate the functions of target proteins by affecting their activity, localization, turnover and interactions with other proteins. So far most of the usual modifications were discovered on Dnmt1, including phosphorylation, methylation, acetylation, sumoylation and ubiquitination (Fig. 4a).

Dnmt1 can be phosphorylated at Ser140, Ser146 and Ser515 (Sugiyama et al., 2010; Goyal et al., 2007; Esteve et al., 2010). Phosphorylation of Dnmt1 at Ser515 is required for the intramolecular interaction between the N and C-terminal domains, which is essential for

enzymatic activity. In contrast to the modification of Ser515, phosphorylation of Dnmt1 at Ser146 by casein kinase 1δ/ε was reported to decrease DNA binding affinity. Recently, also sumoylation of human DNMT1 was observed (Lee et al., 2009). Sumoylated DNMT1 shows a stronger catalytic activity on genomic DNA compared with unmodified form *in vitro*.

Interestingly, the posttranslational modifications were shown to regulate Dnmt1 function in a cross talk manner during the cell cycle. One example is the control of DNMT1 abundance by a cross talk of methylation and phosphorylation during S phase and G2 phase (Fig. 4b). DNMT1 associates with a histone methyltransferase SET7, which catalyzes the addition of a methyl group to Lys142 (Esteve et al., 2010; Esteve et al., 2009). Methylation at Lys142 controls DNMT1 stability through an ubiquitination-dependent pathway. Furthermore, Ser143 of DNMT1 was identified to be phosphorylated by AKT1 kinase (Esteve et al., 2010). The methylation of Lys142 and phosphorylation of Ser143 exist in a mutually exclusive manner, and show different patterns during the cell cycle. Lys142 methylation enriches in the late S and G2 phases, whereas phosphorylated DNMT1 peaks in early and mid S phases. These results suggest that DNMT1 is stabilized by phosphorylation during early and mid S phase so that it can follow the fast DNA replication. After cells enter late S phase, DNMT1 degradation is activated by methylation of Lys142. These results reveal a dynamic regulation of DNMT1 protein level during the cell cycle, which is mediated by a cross-talk of methylation and phosphorylation. In addition, Dnmt1 was also observed to interact with a histone demethylase Lsd1, which catalyze the removal of a methyl group from Lys1096 and stabilize Dnmt1 (Wang et al., 2009).

In addition, a mechanism for controlling DNMT1 function by a cross talk of acetylation and ubiquitination was reported. DNMT1 stability is regulated by acetylation and ubiquitination during the cell cycle, which is mediated by the coordinated action of several DNMT1-associated proteins (Du et al., 2010; Bronner et al., 2011).

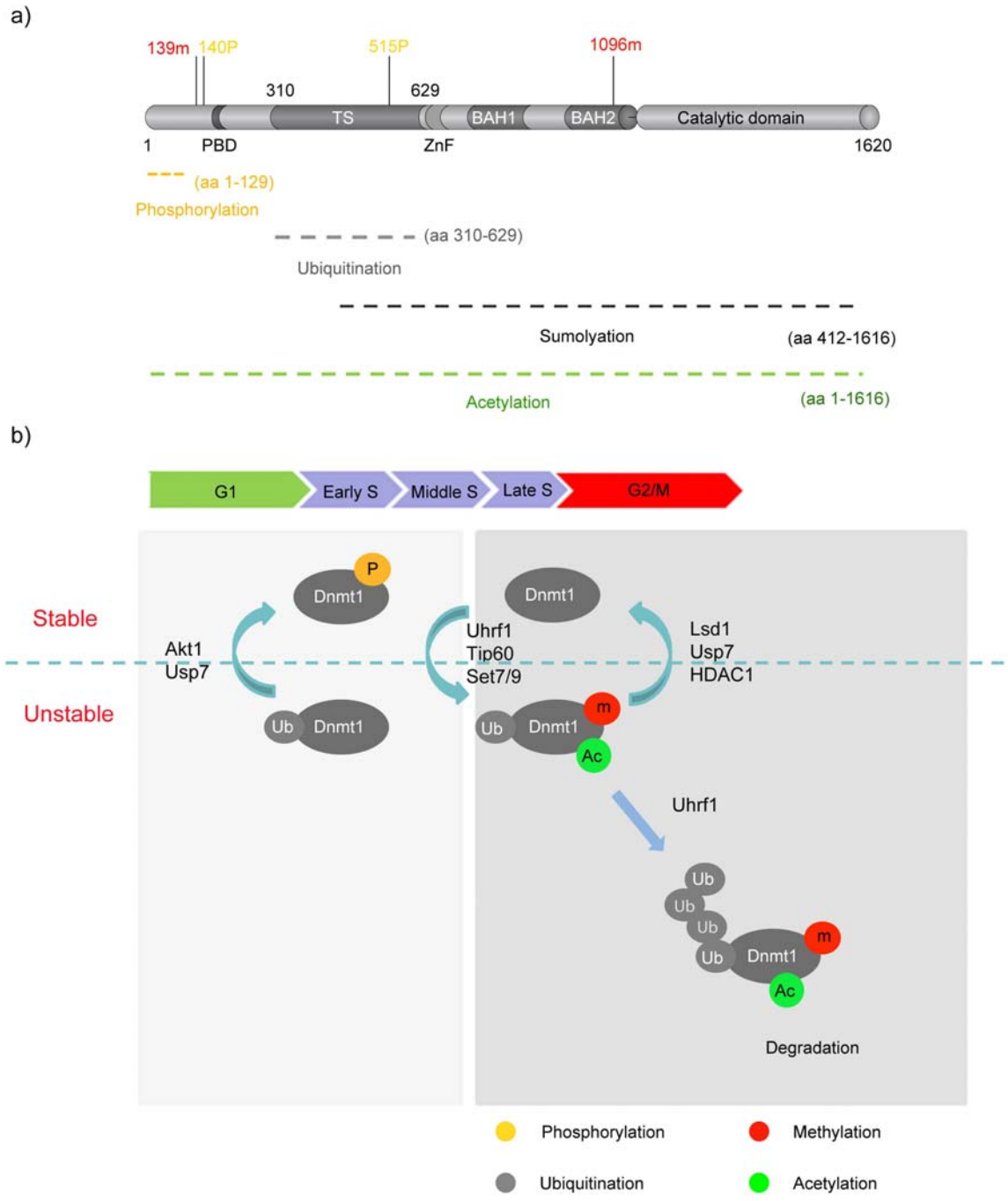

Figure 4. Dnmt1 abundance regulated by posttranslational modifications during the cell cycle
a) Posttranslational modifications of Dnmt1. Dnmt1 was shown to be modified by phosphorylation at lysine 140 (correspond to Lys143 in human DNMT1) and Lys515, by methylation at Lys139 (correspond to Lys142) and Lys1096. In addition, Dnmt1 was also reported to be modified by acetylation, sumoylation and ubiquitination. The acetylation, sumoylation and ubiquitination sites are so far not mapped, but were narrowed down to some regions of Dnmt1.
b) Dnmt1 may coordinately be controlled by Akt1, Usp7 and HDAC1 during early and mid S phases to get a stable form of phosphorylated Dnmt1. When cells enter late S phase, Dnmt1 in turn strongly interacts with Uhrf1, Tip60 and Set7/9 to activate its degradation through an ubiquitination pathway.

During early and mid S phase, DNMT1 shows strong interaction with histone deacetylase 1

(HDAC1) and the deubiquitinase HAUSP (herpes virus-associated ubiquitin-specific protease), which catalyze the removal of acetyl and ubiquitin groups from DNMT1, respectively, and stabilize DNMT1. In late S phase, the E3 ligase UHRF1 and acetyltransferase (HAT) Tip60 interact with DNMT1 counteracting the effect of HDAC1 and HAUSP, marking DNMT1 for proteasomal degradation. In addition, the expression analysis of Tip60 and HDAC1, as well as UHRF1 and HAUSP during the cell cycle, suggest that DNMT1 might be initially acetylated, subsequently triggering the degradation of DNMT1 through the ubiquitin pathway (Du et al., 2010).

1.2.3. Regulation of Dnmt1 by interacting factors

Dnmt1 has been extensively characterized for two decades. Several proteins were covered to associate with Dnmt1 and regulate its catalytic activity, substrate specificity and sequence targeting (Table 1). Interacting proteins link Dnmt1 to diverse biological pathways, like the cell cycle regulation, tumorigenesis, DNA repair and chromatin structure, implicating Dnmt1 might possess more functions in addition to DNA methylation. The identification and characterization of such these interacting factors will help to better understand the mechanism of maintenance of DNA methylation.

PCNA

Dnmt1 was first shown to associate with the replication machinery, coupling maintenance of genomic DNA methylation to DNA replication (Leonhardt et al., 1992). Later, a direct interaction between Dnmt1 and the replication processivity factor of proliferating cell nuclear antigen (PCNA) was observed and the interaction was mapped to the N-terminal part of Dnmt1, amino acids 163 to 174 (PBD domain, Chuang et al., 1997). PCNA is a cofactor of DNA polymerase delta and as a homotrimer it contributes to increase the processivity of leading strand synthesis during DNA replication (Bravo et al., 1987; Kelman and O'Donnell, 1995; Wyman and Botchan, 1995). DNA replication is highly processive taking about 0.035 second per nucleotide (Jackson et al., 1998), whereas the purified recombinant Dnmt1 show low turnover rates about 70-450 second per methyl group transfer by *in vitro* steady-state kinetic analysis (Pradhan et al., 1999). It is believed that the transient interaction of Dnmt1 with PCNA may enhance DNA methylation efficiency so that Dnmt1 can follow the fast and processive DNA replication machinery. However, the PBD-mediated association with the replication machinery is not strictly required for maintaining DNA methylation by Dnmt1 (Schermelleh et al., 2007; Spada et al., 2007).

Table 1 Dnmt1 interacting proteins and their proposed functions

Interacting proteins	*Proposed Functions*	*Reference*
Transcriptional repressors		
DMAP1	Involved in transcription repression and activation Component of the NuA4 histone acetyltransferase (HAT) complex	*Rountree et al. 2000; Xin et al. 2004; Muromoto et al., 2004*
PML-RAR	Transcriptional regulator of retinoic acid (RA) target genes; induces gene hypermethylation and silencing by recruiting Dnmt1	*Di Croce et al., 2002*
HESX1	Required for normal forebrain and pituitary development in human	*Sajedi et al., 2008*
mSin3a	A core component of a large multiprotein co-repressor complex	*Xin et al. 2004; Kimura et al., 2003*
Chromatin modifiers		
HDAC1/2	Histone deacetylases	*Fuks et al., 2000; Xin et al. 2004; Eden et al., 1998; Jung et al., 2007*
Lsd1	Histone demethylase	*Wang et al., 2008*
Suv39H1	H3K9me2/3 methyltransferase	*Fuks et al., 2003*
G9a	H3K9me1/2 methyltransferase	*Estève et al., 2006; Kim et al., 2009; Tachibana et al 2002; Peters et al., 2003*
Ezh2	H3K27me3/2 methyltransferase	*Taghavi et al., 2006; Vire et al., 2006; Wu et al., 2008; Shen et al., 2008*
(Methyl) CpG binding proteins		
MeCP2	Methylated CpG binding protein	*Hiromichi Kimura and Kunio Shiota, 2003*
MBD2/MBD3	Methylated CpG binding protein	*Tatematsu et al., 2000*
Uhrf1/2	Hemi-methylated CpG binding protein	*Bostick et al., 2007; Sharif et al., 2007; Arita et al., 2008; Awakumov et al., 2008; Qian et al., 2008*
CFP1	a component of Setd1A and Setd1B histone H3K4 methyltransferase complex; unmethylated CpG binding protein	*Butler et al., 2008*

Table 1 Dnmt1 interacting proteins and their proposed functions

Interacting proteins	*Proposed Functions*	*Reference*
Tumor suppressors		
p53	Tumor suppressor in many tumor types	*Esteve et al., 2005*
hNaa10p	Tumor suppressor Stimulate Dnmt1 activity	*Lee et al., 2010*
BRCA1	Breast cancer-associated gene 1	*Shukla et al., 2010*
DNA methyltransferases		
Dnmt3a	De novo DNA methylation	*Fatemi et al., 2002; Kim et al., 2002*
Dnmt3b	De novo DNA methylation	*Rhee et al., 2002; Kim et al., 2002*
Chromatin binding proteins		
HP1	Heterochromatin binding protein	*Fuks et al., 2003; Smallwood et al., 2007*
Cell cycle regulators and DNA replication factors		
PCNA	Targeting Dnmt1 to replication foci Associate with p21	*Chuang et al., 1997; Leonhardt et al., 1992*
pRb1/2	Cell cycle regulator	*Jung et al., 2007; Robertson et al., 2000*
E2F	Transcription factor Plays a role in controlling cell cycle entry	*Jung et al., 2007; McCabe et al., 2006; Robertson et al., 2000*
Chromatin remodeling factors		
Lsh	Chromatin remodeling	*Myant and Stancheva, 2008*
hSNF2H/Tip5	Chromatin remodeling	*Zhou and Grummt, 2005*
Others		
PARP1	poly ADP-ribose polymerase 1	*Reale et al., 2005; Zampieri et al., 2009*
p23	Chaperone	*Zhang and Verdine, 1996*
Annexin V	Anticoagulant protein	*Ohsawa et al., 1996*
Daxx	Suppression of B cell development and apoptosis	*Muromoto et al., 2004*
Akt1	Protein phosphorylation	*Esteve et al., 2010*

In addition, PCNA also plays a central role in DNA repair. PCNA serves as a loading platform for enzymes involved in DNA synthesis and chromatin assembly. In response to DNA damage, PCNA targets Dnmt1 to DNA damage sites for restoration of DNA methylation (Mortusewicz et al., 2005).

Histone methyltransferases: G9a, Ezh2 and Suv39H1

Mammalian heterochromatin binding protein 1 (HP1) and the major euchromatic histone methyltransferase G9a were identified as the proteins interacting with Dnmt1 (Jacobs et al.,

2002; Tachibana et al 2002; Peters et al., 2003). G9a is responsible for mono- and dimethylation of histone H3K9. *G9a* null ESCs reveal DNA hypomethylation at specific loci (Ikegami et al. 2007). Moreover, DNA methylation requires the lysine methyltransferase G9a but not its catalytic activity (Dong et al., 2008). The members of HP1 family were shown to directly interact with Dnmt1. This interaction results in a functional stimulation of Dnmt1 methyltransferase activity (Swallwood et al., 2007). Therefore, HP1 could be an adaptor to mediate communication between histone and DNA methyltransferases. In the pathway of G9a-HP1-Dnmt1, G9a might create a binding platform by methylation of histone H3K9 for HP1 α, β and γ, subsequently HP1 proteins are recruited, resulting in an increased DNA methylation by stimulation of Dnmt1 activity (Esteve et al., 2006). Dnmt1, G9a and HP1 form a potential positive feedback loop and functionally interacts each other to coordinate gene silencing (Fig. 5a and 5b).

Polycomb group (PcG)-mediated gene silencing is one of the two major epigenetic repression systems. The PcG protein Ezh2, a histone methyltransferase responsible for the methylation of histone H3K27, was shown to physically interact with all of the Dnmts. A remarkable reduction of DNA methylation at a number of CpG sites within the *MYT1* and *WNT1* promoters was observed by depletion of *ezh2*, suggesting that Ezh2 directly controls DNA methylation. The binding of Ezh2 promotes the association of Dnmts with chromatin templates. These results suggest a model in which PcG proteins recruit Dnmts to setup DNA methylation marks and subsequently recruit PcG proteins to maintain chromatin repression state (Vire et al., 2006; Wu et al., 2008; Shen et al., 2008).

In addition to association with G9a and Ezh2, also Suv39H1 interacts with Dnmt1 and HP1, forming a functional complex that mediates gene silencing (Fuks et al., 2003). Suv39H1, HP1 and Dnmt1 might form a positive feedback loop to repress gene expression.

HDCA1/2

Several transcriptional repressive complexes, including the methylated CpG binding proteins (MBDs) and the histone deacetylases (HDACs), are involved in DNA methylation. Since an interaction between Dnmt1 and MeCP2 was observed, Dnmt1 may play more roles in gene silencing in addition to its capacity to methylate hemimethylated CpG sites. A correlation between histone hypoacetylation and DNA hypermethylation at transcriptional inactive regions was reported (Eden et al., 1998), suggesting that DNA methylation contributes to gene repression by inducing decreased levels of chromatin acetylation.

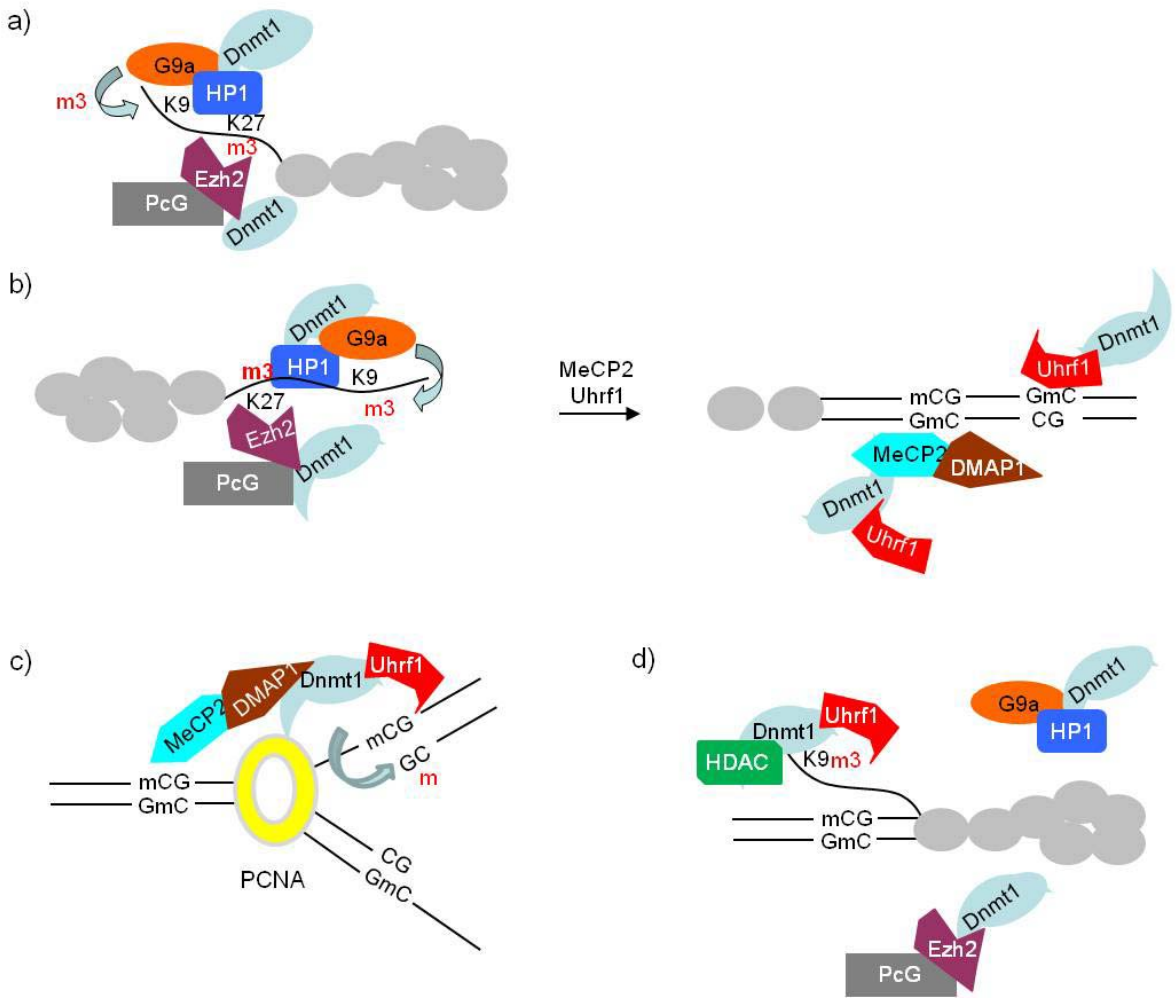

Figure 5. The proposed function of Dnmt1 interacting proteins in DNA methylation and the maintenance and spread of chromatin structure
a) Histone methyltransferases G9a and Ezh2 in G9a-HP1-Dnmt1 and Ezh2-HP1-Dnmt1 complexes, respectively, might establish methylation of histone H3K9 and H3K27 to maintain and spread higher order chromatin structure. b) The methylation marks setup by G9a and Ezh2 recruit chromatin binding proteins like Uhrf1 to target site and modulate the accessibility of chromatin for MBD proteins and DNA methyltransferases. c) During DNA replication, PCNA, MeCP2 and Uhrf1 are supposed to play a role in recruiting Dnmt1 to the replication fork in order to methylate the newly synthesized hemimethylated CpG site. d) After DNA replication, HDAC proteins could join the Dnmt1-Uhrf1 complex to spread the repressive chromatin states together with the histone methyltransferases, G9a and Ezh2.

Indeed, Dnmt1 directly interacts with HDAC1/2 (Fuks et al., 2000; Robertson et al., 2000; Rountree et al., 2000). A complex containing Dnmt1, HDCA2 and a Dnmt1 association protein DMAP1 was shown to localize at replication foci (Rountree et al., 2000). Whereas DMAP1 associates with Dnmt1 throughout S phase, HDCA2 only joins the complex in late S phase at heterochromatin regions, which might promote local chromatin condensation by establishing a hypoacetylation status of heterochromatin after DNA replication (Fuks et al., 2000). These observations lead to a speculation that at least two transcriptional repressive complexes might exist. In early S phase, when the euchromatin is duplicated, Dnmt1 is

targeted to replication foci by PCNA and forms a transcriptional repressive complex with DMAP1 (Fig. 5c). HDCA2 is recruited to this complex when cells enter to late S phase (Fig. 5d), when the heterochromatic regions containing highly methylated DNA and hypoacetylated histones are replicated. The selective interaction of DNMT1 with HDAC2 during S phase may be critical for maintaining these two epigenetically distinct compartments of the genome (Rountree et al., 2000).

Chromatin binding proteins: MBDs and Uhrf1/2

MBD proteins possess DNA binding affinity, specifically for fully methylated CpG dinucleotides (Jorgensen et al., 2004). MBDs contribute to transcriptional gene repression and organization of chromatin via association with HDACs (Wade, 2001). Moreover, MeCP2 is supposed to function in recruiting Dnmt1 to its target sites (Hiromichi Kimura and Kunio Shiota, 2003). MeCP2 associates with Dnmt1 through its transcription repressor domain (TRD), which is also required for recruitment of HDACs via a co-repressor mSin3a. Therefore, HDAC proteins are excluded from the Dnmt1-MeCP2 complex. Considering these observations, it is proposed that DNA methylation might be sequential process to mediate transcriptional repression. MeCP2 may contribute to DNA methylation by recruiting Dnmt1 to target sites (Kimura et al., 2003). Subsequently HDACs accumulate at heterochromatin to maintain and spread the silent chromatin state by deacetylating histones.

Uhrf1, also known as ICBP90 in human or Np95 in mouse, was reported in DNA-Dnmt1 complex. It interacts and colocalizes with Dnmt1 throughout S phase. Uhrf1 shows a weak preferential binding for hemimethylated CpG sites, which is mediated by its SRA domain. In addition, the tandem Tudor domain of Uhrf1 was shown to bind repressive chromatin mark, histone H3K9me3, possibly linking DNA methylation and histone modification (Rottach et al., 2009). Deletion of *uhrf1* leads to genomic hypomethylation in ESCs and embryos (Uemura et al., 2000; Bostick et al., 2007; Papait et al., 2007; Sharif et al., 2007; Achour et al., 2008), suggesting that Uhrf1 is an essential cofactor of Dnmt1. After Dnmt1 transferring methyl group to cytosine, Uhrf1 might also recruit histone modifiers such as HDAC1 (Unoki et al., 2004), the histone methyltransferase G9a and *de novo* DNA methyltransferases Dnmt3a and 3b.

***De novo* methyltransferase: Dnmt3a and 3b**

In general Dnmt3a and 3b are *de novo* methyltransferases which is required for establishment of DNA methylation patterns during early embryonic development, whereas Dnmt1 is responsible for maintaining DNA methylation patterns after replication. However, this categorical distinction does not precisely reflect their biological function, since human

DNMT1 was shown to form a complex with *de novo* methyltransferase DNMT3a and DNMT3b to regulate gene expression (Kim et al., 2002). In addition, Dnmt3a and 3b are required for proper maintenance of DNA methylation in somatic and ESCs (Liang et al., 2002; Chen et al., 2003; Dodge et al., 2005). The maintenance methyltransferase Dnmt1 was also observed to participate in *de novo* DNA methylation through cooperation with *de novo* methyltransferases, either Dnmt3a or Dnmt3b. Dnmt1 activity is enhanced on DNA template that is pre-incubated with Dnmt3a *in vitro*, suggesting that pre-existing methylated DNA might activate *de novo* activity of Dnmt1 (Fatemi et al., 2002). Besides DNMT3a, DNMT1 was also reported to cooperate with DNMT3b for silencing genes in human cancer cells (Rhee et al., 2002).

Recently, a crystal structure of a Dnmt1 fragment reveals that the TS domain inserts into the catalytic center of C-terminal domain, suggesting that it could mediate the autoinhibition of Dnmt1 (Syeda et al., 2011; Takeshita et al., 2011). Therefore, it is likely that Dnmt1 is activated by releasing the autoinhibition by either allosteric conformational changes or functional interacting proteins.

Many Dnmt1 interacting proteins were reported so far, which link DNA methylation and chromatin structure. However, additional interacting proteins may exist that reveal novel functions in regulation of Dnmt1. Although it is proposed that DNMT1 and DNMT3s cooperate to mediate gene silencing in human tumor cells, it remain elusive whether Dnmt1 possesses *de novo* methyltransferase activity. Likely, the characterization of new proteins interacting with Dnmt1 will shed light on the mechanism of aberrant hypermethylation in tumor cells.

1.3. Ubiquitination and De-ubiquitination

Ubiquitin is a small protein with 76 amino acids and highly conserved in all eukaryotes. Ubiquitin conjugation to substrate proteins is a highly ordered process (Fig. 6), in which at least three enzymes: an ATP-dependent ubiquitin-activating enzyme (E1); an intermediate ubiquitin-conjugating enzymes (E2) and a terminal ubiquitin ligase (E3) are required (Fang et al., 2004; Pickart et al., 2004; Raymod J. Deshaies and Claudio A.P. Joazeiro, 2009). In some cases, ubiquitin elongation factors, also named as E4 enzymes, are involved in the formation of multiubiquitin chains (Koegl et al., 1999). Ubiquitin is activated and transferred to the cysteine residue in an E1 active site, which requires ATP as an energy source. Then the ubiquitin peptide is passed on to the second enzyme, E2. Generally the E2 enzyme interacts with a specific E3 partner, which transfers the ubiquitin to target protein by generating an isopeptide bond between a lysine of substrate and the C-terminal glycine of ubiquitin. E2 enzymes share many conserved residues in their catalytic domain, whereas E3 ubiquitin ligases only share a few conserved motifs. Therefore, the substrate specificity of ubiquitination is mainly determined by the E3 ligase, although E2 enzymes can also play a role in substrate selection (Laney et al., 1999). There are two main classes of E3 ligases, the proteins with the E6-AP carboxyl terminus (HECT domain) (Huibregtse et al., 1995) and proteins with a really interesting new gene (Ring) finger domain (Jackson et al., 2000). E3 ligases can modify target proteins by mono-ubiquitination via Lys6, Lys11, Lys29, Lys48 and Lys63 residues of ubiquitin. In general, Lys48-linked polyubiquitination marks protein for degradation by 26S proteasome (Baumeister et al., 1998), whereas Lys63-linked monoubiquitination serves as signal in intracellular trafficking, DNA repair and signal transduction pathways (Hicke L et al., 2005).

Ubiquitination is a highly dynamic process and can be reversed by removal of the ubiquitin moiety from ubiquitin-conjugated proteins by deubiquitylating enzymes (DUBs). DUBs constitute a super family comprising at least five major classes based on sequence and structural similarity: the ubiquitin-specific protease (USP, UBP), ubiquitin C-terminal hydrolase (UCH), ovarian tumor (OTU), Machado-Joseph disease (MJD), and the jab1/MPN domain-associated metalloisopepetidase (JAMM) class. The USP, UCH, OUT and MJD families are cysteine peptidases, while the JAMM family is zinc metalloisopeptidases. In addition to the function of processing ubiquitin precursors, the major role of DUBs is to remove the ubiquitin chain from ubiquitin-conjugated proteins, leading to protein

stabilization by rescue them from proteasomal degradation (Wilkinson et al.; 1997). Several previous studies showed that DUBs participate in the control of histone ubiquitination, which is involved in numerous biological important pathways, including cell growth, development, chromatin structure and transcriptional regulation (Kim et al., 2003; DiAntonio et al., 2001).

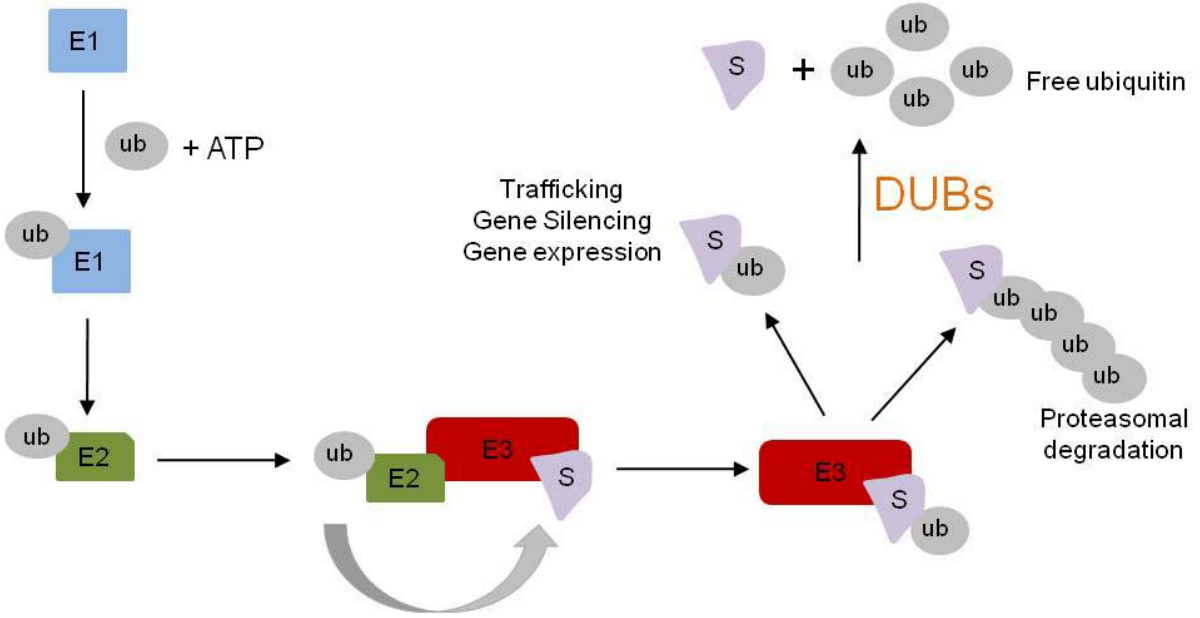

Figure 6. Ubiquitin pathway and its proposed functions
Ubiquitin (ub) is activated by E1 enzyme in the presence of ATP to form a high-energy of ub-adenylate, followed by transfer of ub to the catalytic cysteine of the E1. The ub is then transferred to the E2 conjugating enzyme via a transthiolation reaction. The ub is ligated to a substrate with the aid of an E3 ligase enzyme. The DUBs (deubiquitination enzymes) can remove ub from substrates (Kerscher et al. 2006). Ubiquitination is involved in numerous biological important pathways, including cell growth, development and transcriptional regulation.

The E1, E2, E3, 26S proteasome and DUBs form a complex ubiquitin proteasomal network to balance protein abundance in the cells. Proteins are tagged by a polyubiquitin chain via E1, E2 and E3, and subsequently are recognized and degraded by the 26S proteasome. DUBs can remove the ubiquitin chains to prevent degradation. The ubiquitin proteasome pathway is a major system responsible for removing intracellular proteins, especially mis-folded proteins in eukaryotes.

1.3.1. Uhrf1, a Ring-type E3 ligase for ubiquitination

As mentioned above, Uhrf1 is a crucial cofactor of Dnmt1 for maintenance of DNA methylation (Bostick et al., 2007; Sharif et al., 2007). Uhrf1 harbors a Ring domain at its C-terminal part. *In vitro*, Uhrf1 endows an ubiquitin E3 ligase activity, which is required for the growth regulation of tumor cells (Jenkinis et al., 2005). In addition, Uhrf1 is an E3 ubiquitin ligase for histone core particles *in vitro* (Citterio et al., 2004), although the functional role of this biochemical activity is still unclear. Recently, Uhrf1 was also shown to mediate the ubiquitination of Dnmt1, controlling Dnmt1 stability in coordination with acetylase Tip60 and histone deacetylase HDAC1 (Du et al., 2010; Achour et al., 2009).

In addition, a PHD finger of Cys4-His-Cys3 motif (four cysteines, one histidine and three cysteines) resides between the tandem Tudor and SRA domains. Generally, the PHD fingers comprise approximately 50-80 amino acids and present in more than 100 human proteins. Crystal structures reveal that the PHD fingers resemble similar to the Ring fingers, which typically have a C3HC4 motif and like the PHD fingers coordinate two zinc ions. The crucial difference between PHD and Ring fingers is in their surface areas. The Ring domain contains a conserved alpha-helix for E2 enzymes binding, which is absent from the PHD finger. However, the PHD fingers of MEKK and MIR were shown to function as ubiquitin E3 ligases (Lu et al., 2002). Later, structural analysis showed that the zinc finger of MIR looks like a Ring rather than a PHD finger and that the PHD finger of MEKK was misclassified as the PHD finger (Coscoy et al., 2003). Therefore it is still not fully understood whether the PHD finger can also mediate ubiquitination.

The biological function of PHD fingers was extensively investigated in previous studies. Basing on the binding specificity for histone modifications, the PHD finger family can be divided into several subgroups. The PHD finger of Dnmt3L was shown to mediate the protein binding to unmethylated H3K4 (Jia et al., 2007). In contrast, the PHD finger of nucleosome remodeling factor (NURF) can specifically recognize trimethylated histone H3K4 (Wysocka et al., 2006) and target the NURF complex to *Hox* gene promoters during development to activate transcription. The PHD finger of ICBP90 was shown to preferentially bind trimethylated H3K9 (Karagianni et al., 2008). However, we observed that the PHD finger of Uhrf1 does not possess a preferential binding for a peptide containing H3K9me3, but it can stimulate the binding affinity of H3K9me3 by the Tudor domain (Pichler et al., 2011). Interestingly, Papait *et al* observed that the PHD domain of Uhrf1 has a function in large-scale reorganization of pericentric heterochromatin (Papait et al., 2008).

1.3.2. Usp7, a ubiquitin specific protease

Ubiquitin specific protease 7 (Usp7) is one of the deubiquitylating enzymes (DUB) and belongs to the ubiquitin-specific protease (USP or UBP) family. It was originally identified as protein interacting with the ICP0 of herpes simplex virus, so also named Herpes virus-associated ubiquitin-specific protease (Hausp) (Everett et al., 1997; Holowaty et al., 2003). Usp7 plays an important role in the regulation of stress response pathways, epigenetic silencing and the progress of infections by DNA viruses (Fig. 7) (van der Horst et al., 2006; van der Knaap et al., 2005; Khoronenkova et al., 2010).

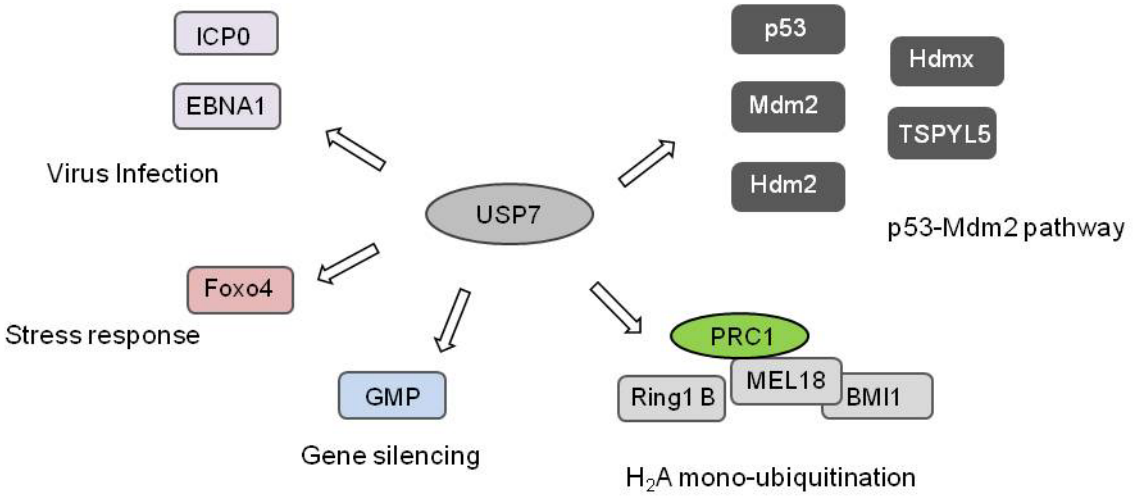

Figure 7. Proteins directly and functionally interact with Usp7
Usp7 interacts with various proteins involved in diverse pathway, like the p53-Mdm2 pathway and gene silencing.
(ICP0: Everett et al., 1997; Holowaty et al., 2003; EBNA1: Holowaty et al., 2003; Foxo4: Van der Horst et al., 2006; GMP: van der Knaap et al., 2005; p53: Li et al., 2002; Mdm2: Meulmeester et al., 2005; Tang et al., 2010; TSPYL5: Epping et al., 2011; Ring1B: de Bie P et al., 2010)

Usp7 comprises 1103 amino acids harboring a catalytic domain, a N- and a C-terminal domain. Basing on the bioinformatic analysis, four ubiquitin-like domains reside in the C-terminal part (Fig. 8; Zhu et al., 2007), which are supposed to mediate protein-protein interactions. The C-terminal domain of Usp7 is required for interaction with a regulatory protein ICP0, which plays a critical role in the replication-latency balance of herpes simplex virus (Everett et al., 1997; Holowaty et al., 2003; Daubeuf et al., 2009). A NMR structure of the first Ubl domain was solved (PDB 2KVR), showing a folding similar to ubiquitin-like domains. In addition, the C-terminal domain of Usp7 is critical for deubiquitination activity (Ma et al., 2010). Usp7 also binds to another viral protein, the EBNA1 protein of Epstein-Barr virus (EBV), through the N-terminal tumor necrosis factor-receptor associated factor

(TRAF)-like domain (Holowaty et al., 2003). To better understand the molecular basis for the interaction between Usp7 and its substrates, several crystal structures containing the TRAF domain and EBNA1 peptides were solved (Saridakis et al., 2005). The TRAF domain uses an eight-stranded beta sandwich fold to create a surface groove, which provides a docking site for EBNA1 peptides (PDB 2FOJ).

Using an affinity purification approach, Usp7 was co-precipitated with p53 (Li et al., 2002). Usp7 directly binds to p53 through its TRAF-like domain, suggesting that Usp7 has an important role in p53 pathway. Over-expression of Usp7 stabilizes p53 and induces p53-dependent growth repression and apoptosis. The ablation of *usp7* expression was reported to have an opposite effect on the protein stability of p53. In addition to p53, other members of regulatory proteins in p53-Mdm2 pathway were also reported to interact with Usp7, such as Mdm2, MDMX and DAXX (Meulmeester et al., 2005; Tang et al., 2010), suggesting a central role of Usp7 in p53-Mdm2 pathway.

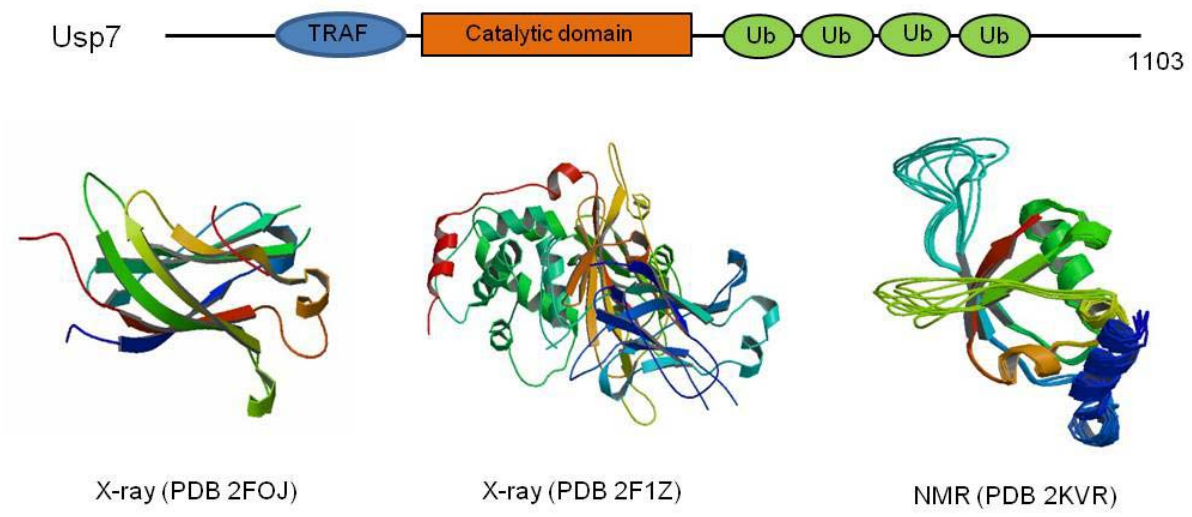

Figure 8. Schematic representation of Usp7 and solved structures
Usp7 comprises 1103 amino acids, harboring a tumor necrosis factor-receptor associated factor (TRAF)-like domain, a catalytic domain and four ubiquitin like domains (Ub). The crystal structure of TRAF-like domain was solved (PBD 2FOJ). The structures of the N-terminal domain comprising both TRAF and catalytic domain (PDB 2F1Z) and the first ubiquitin-like domain (PDB 2KVR) are also available in the PDB database.

Besides the regulation of protein stability, Usp7 is also involved in the modulation of chromatin structure and gene silencing. Usp7 binds to heterochromatin in Drosophila, catalyzing the removal of ubiquitin from monoubiquitinated histone H2B, which is important for the epigenetic silencing of homeotic genes. Moreover, the deubiquitination reaction of H2B is greatly stimulated through the association of Usp7 with Guanosine 5'-monophosphate

synthetase (GMP) (van der Knaap et al., 2005). Recently, Usp7 was reported to associate with DNA containing hemimethylated CpG sites by a novel SILAC-based DNA-protein interaction screening approach (Mittler et al., 2009). With this screening system, they confirmed that two hemimethylated CpG binding proteins Uhrf1 and Dnmt1 were co-precipitated with a DNA probe. Besides these two methylated CpG binding proteins, Usp7 was identified in the bound fraction, suggesting that Usp7 might be a component of Dnmt1-Uhrf1 complexes.

To elucidate the physiological functions of Usp7 *in vivo*, Kon *et al* generated the *usp7* knockout mice (Kon et al., 2010). Mice lacking *usp7* show an interesting phenotype with early embryonic lethality between embryonic days 6.5 (E6.5) and E7.5, implicating that Usp7 is essential for early embryonic development in mice. The *usp7* knockout embryos exhibit no recognizable structures and ESCs are greatly reduced in numbers compared with the wild type embryos. These findings suggest that embryonic lethality is caused by abnormal proliferation and developmental arrest.

1.3.3. Transcriptional regulation by ubiquitination and deubiquitination

In eukaryotic cells, gene expression is modulated at different levels from the transcription step to posttranslational modification. Ubiquitination participates in the transcriptional regulation either through proteasome dependent or proteasome independent mechanism, which involves the modulation of gene expression by histone ubiquitination.

Although transcriptional regulation and ubiquitin-mediated proteolysis are in general two distinct biological processes, several studies have directly connected these two events in the control of gene expression **(**Li et al., 2007; Xu et al., 2004; Liao et al., 2010; Zhang et al., 2003**)**. Ubiquitin was shown to control messenger RNA synthesis, a process that depends on RNA polymerase II and transcription factors. For example, the large subunit of RNA polymerase II Rpb1 is ubiquitinated by a HECT domain ubiquitin E3 ligase Wwp2 and subsequently degraded by the 26S proteasome (Li et al., 2007). Furthermore, Wwp2 was also reported to promote degradation of the transcription factor Oct4 and play a role in controlling the pluripotence of human ESCs (Xu et al., 2004; Liao et al., 2010).

In addition to acetylation, methylation and phosphorylation, histones can also be modified by ubiquitination. Previous studies were reported that all the core histones can be the substrate of ubiquitination. Ubiquitinated H2A was the first identified (Goldknopt et al., 1975). Later the ubiquitination site of H2A was mapped to a highly conserved residue lysine 119 (Nickel BE

et al., 1989). Furthermore, H2B was also reported to be modified by the addition of ubiquitin to its C-terminal region at lysine 120 (both in mammals and *S. cerevisiae*) (West et al., 1980; Thorne et al., 1987). The enzymes responsible for ubiquitination of histone H2B were first identified in *S. cerevisiae*: Rad6 (E2) and Bre1 (E3) (Robzyk et al., 2000; Hwang et al., 2003; Wood et al., 2003). In *S. cerevisiae*, the H2B ubiquitination can be reversed by deubiquitinases Ubp8 and Ubp10. In addition to histone H2A and H2B, ubiquitination of histone H3 was also observed (Chen et al., 1998; Dover et al., 2002; Liu et al., 2005; Wang et al., 2006), but it is not as prevalent as the ubiquitination of H2A and H2B. The ubiquitination sites of histone H3 are so far not determined.

Evidence from previous studies suggests that histone ubiquitination may contribute to gene activation (Shema et al., 2008; Espinosa, 2008). For example, Henry *et al* showed that the ubiquitin E3 ligase Bre1 and the deubiquitinase Ubp8, which are the subunits of the coactivator complex SAGA, mediate the ubiquitination and deubiquitination of H2B that contributing to transcriptional control in yeast (Fig. 9a, Henry et al., 2003). Moreover, the ubiquitin conjugation enzyme Rad6 and E3 ligase Bre1 were shown to associate with the transcription elongation complex in yeast cells (Xiao et al., 2005). The methylation levels of histone H3K4 and H3K36, which correlate with transcriptional active chromatin, are altered in yeast mutant with disrupted ubiquitination or deubiquitination of H2B. Furthermore, the Rad6 protein, which is a major E2 for the monoubiquitination of H2B in yeast, was shown to play an important role in transcriptional activation (Kao et al., 2004). To investigate the biological functions of H2B monoubiquitination, Minsky *et al* generated an antibody specific for ubiquitinated H2B and mapped the global distribution of H2B monoubiquitination in the genome. Using chromatin immunoprecipitation experiments on human cells, followed by microarray analysis they showed the ubiquitinated H2B preferentially associates with the highly expressed genes in transcribed regions (Minsky et al., 2008), suggesting that H2B ubiquitination correlates with transcriptionally active chromatin.

Although most studies suggest a positive correlation between transcription and histone ubiquitination, an opposite effect was also pointed out. Ring1A and Ring1B are two components of polycomb repressive complex 1 (PRC1) and contribute to the ubiquitination of H2A *in vivo* (Wang et al., 2004; de Napoles et al., 2004).

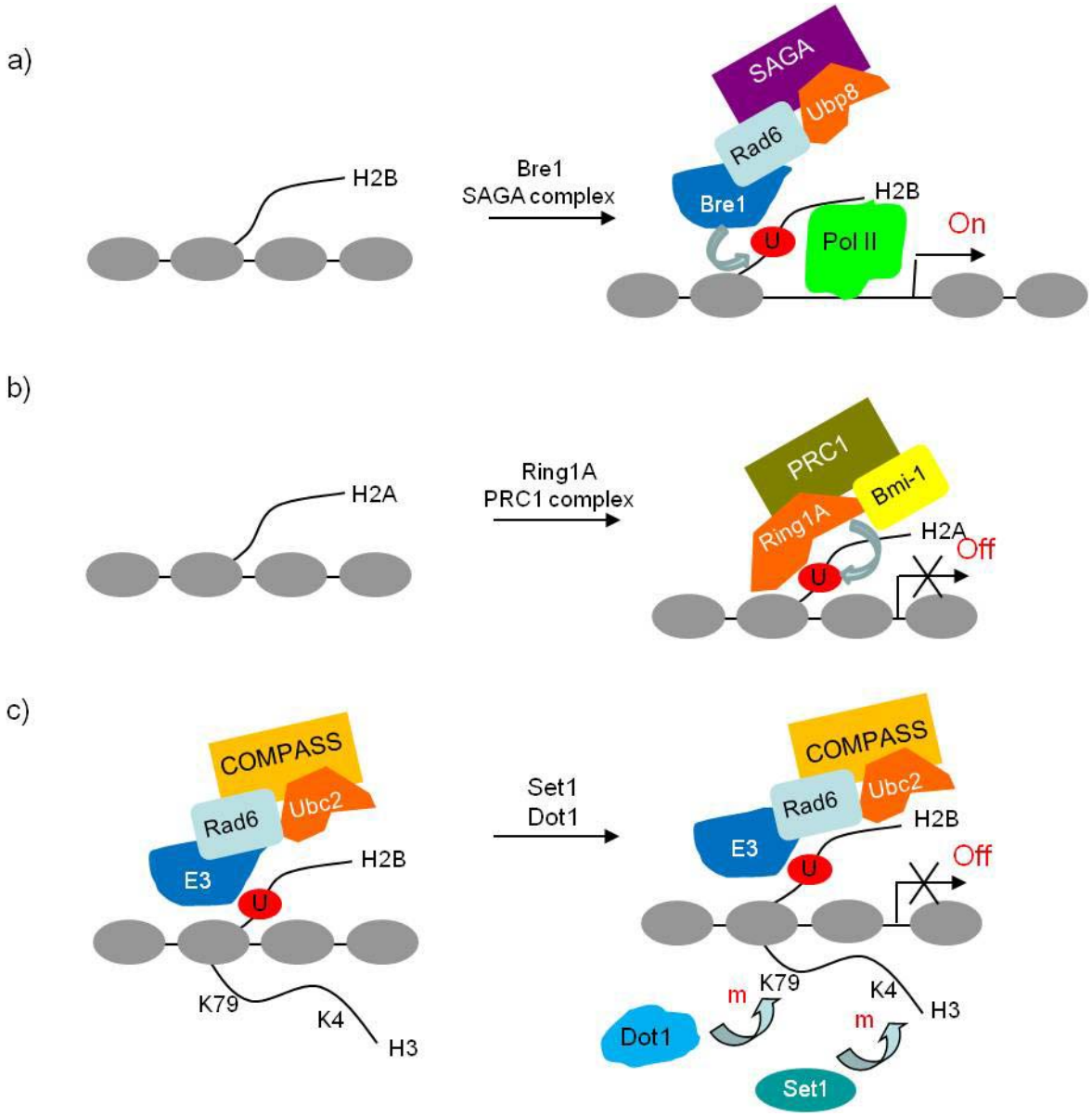

Figure 9. Proposed mechanisms of transcriptional regulation by histone ubiquitination
a) Ubiquitination of H2B mediated by a E3 ligase Bre1, a subunit of SAGA complex, might serve a signal for RNA polymerase II binding and activate gene transcription. b) Ring1A, a subunit of polycomb complex 1, catalyzes H2A monoubiquitination and cooperates with repressive polycomb complex 1 to mediate gene silencing. c) Ubiquitination of histone H2B mediated by ubiquitin-conjugation enzyme Rad6 may be a signal for recruiting Set1 and Dot1, the histone H3K4 and H3K79 methyltransferases respectively, to chromatin (Sun and Allis, 2002; Nakanishi et al., 2009). In yeast, the methylation of histone H3K4 might recruit other transcription factors and in turn regulate gene silencing. COMPASS means the Complex Proteins Associated with Set1.

Ring1B null ESCs reveal a global reduction of the ubiquitinated H2A. Additionally, Ring1B is thought to recruit PRC1 proteins to the X chromosome and mediate its silencing in female mammals (Fig. 9b; Fang et al., 2004). Ring1A associates with another component of PRC1 Bmi-1 to positively regulate the ubiquitination of H2A (Cao et al., 2005). A significant reduction of H2A ubiquitination and up-regulation of the *Hox13* gene were observed by deletion of Bmi-1. These results suggest that the ubiquitination of H2A participates in PcG-

dependent gene silencing pathways.

Concerning the mechanism how histone ubiquitination and deubiquitination affect transcription, a possible explanation is that the ubiquitination of histone influences higher order chromatin structure, and in turn affect gene transcription. However, since ubiquitin is about half of the size of core histones, it likely sticks out of the nucleosome core particle. Thus, a reasonable speculation is that the ubiquitination state of histones may serve as a binding site for other factors and in turn modulate gene activation or repression (Sridhar et al., 2007). A connection between the ubiquitination of histone H2B and the methylation of histone H3K4 and H3K79 was reported (Fig. 9c; Sun and Allis, 2002; Nakanishi et al., 2009), that implicates a regulatory pathway wherein Rad6-mediated H2B ubiquitination regulates Set1-catalyzed H3K4 methylation and Dot1-mediated H3K79 methylation and subsequently regulates gene silencing. In this pathway, the ubiquitination of H2B (Lys123) is prerequisite for the methylation of H3K4, as the H3K4 methylation is abolished when lysine 123 is mutated to arginine, whereas the ubiquitination of H2B is not affected when lysine 4 of H3 is mutated to arginine. However, an opposite evidence was shown that methylation of H3K4 and H3K79 is not strictly dependent on the ubiquitination of histone H2B (Lys123; Foster et al., 2009). In addition to the cross-talk between the ubiquitination of H2B and the methylation of histone H3K4, H2A deubiquitination mediated by deubiquitinase Ubp-M, is critically involved in the cell cycle progression and gene expression through communication with phosphorylation of histone H3S10 (Joo et al., 2007)

1.4. Aims of the work

Dnmt1 has been extensively studied over the past two decades. However, it is still not fully understood how Dnmt1 is regulated by intra- and intermolecular interactions. Therefore, the main objective of this PhD thesis was to identify and study the regulation of Dnmt1 by novel interactions. For this purpose, an ES cell line stably expressing GFP-Dnmt1 was generated and used to identify potential interacting proteins of Dnmt1. Additionally, the essential cofactor Uhrf1 was supposed to recruit Dnmt1 to DNA target sites. Basing on this observation, I was interested in understanding the mechanism of Dnmt1 recruitment by Uhrf1. To clarify this question, several mutants of Uhrf1 were generated and used to functionally characterize the domains involved in recruiting Dnmt1 to hemimethylated CpG sites.

Besides the intermolecular interactions, we were also interested in characterizing the regulation of Dnmt1 by intra-molecular interactions. In particular, we aimed to understand the role of CXXC domain in the regulation of Dnmt1. This highly conserved CXXC zinc finger domain is also present in the histone methyltransferase MLL and the methyl-CpG binding protein MBD1. Since the CXXC zinc finger of MLL and MBD1 was shown to preferentially bind unmethylated DNA. Thus, it is likely that the CXXC domain of Dnmt1 has a function in the discrimination of hemimethylated CpG sites generated after DNA replication.

2. Results

2.1. USP7 AND UHRF1 CONTROL UBIQUITINATION AND STABILITY OF THE MAINTENANCE DNA METHYLTRANSFERASE DNMT1

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Usp7 and Uhrf1 Control Ubiquitination and Stability of the Maintenance DNA Methyltransferase Dnmt1

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ABSTRACT

In mammals Dnmt1 is the DNA methyltransferase chiefly responsible for maintaining genomic methylation patterns through DNA replication cycles, but how its maintenance activity is controlled is still not well understood. Interestingly, Uhrf1, a crucial cofactor for maintenance of DNA methylation by Dnmt1, is endowed with E3 ubiquitin ligase activity. Here, we show that both Dnmt1 and Uhrf1 coprecipitate with ubiquitin specific peptidase 7 (Usp7), a de-ubiquitinating enzyme. Overexpression of Uhrf1 and Usp7 resulted in opposite changes in the ubiquitination status and stability of Dnmt1. Our findings suggest that, by balancing Dnmt1 ubiquitination, Usp7 and Uhrf1 fine tune Dnmt1 stability. J. Cell. Biochem. 112: 439–444, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: HAUSP; NP95; PROTEIN STABILITY; UBIQUITINATION; DNA METHYLATION

By affecting transcriptional activity DNA methylation plays key roles in development and differentiation, genomic imprinting, X chromosome inactivation and genome stability [Bird, 2002]. In higher eukaryotes only the C5 position of cytosine is enzymatically methylated and this is mostly, but not exclusively, in the context of CpG dinucleotides. A family of mammalian cytosine-C5 DNA methyltransferases (Dnmts) establishes and maintains genomic patterns of cytosine methylation [Goll and Bestor, 2005; Spada et al., 2006]. The best characterized roles for these enzymes are establishment of DNA methylation patterns during gametogenesis and development by Dnmt3a and 3b and maintenance of genomic methylation after replication by Dnmt1 [Leonhardt et al., 1992; Li et al., 1992; Lei et al., 1996; Okano et al., 1999].

Dnmt1 is a relatively large protein with an N-terminal regulatory region spanning two thirds of the molecule and a C-terminal catalytic domain connected by seven lysyl–glycyl dipeptide repeats referred to as $(KG)_7$ linker [Margot et al., 2000]. The N-terminal region comprises a proliferating cell nuclear antigen (PCNA) binding domain (PBD), a heterochromatin targeting sequence (TS), a CXXC-type zinc finger domain and two bromo-adjacent homology domains (BAH1 and 2). PCNA targets Dnmt1 to replication and DNA repair sites in vivo to restore DNA methylation during the respective processes [Leonhardt et al., 1992; Chuang et al., 1997; Easwaran et al., 2004; Mortusewicz et al., 2005]. The methylation efficiency is enhanced by the association of Dnmt1 with the replication machinery, but this association is not strictly necessary to maintain genomic methylation [Schermelleh et al., 2007; Spada et al., 2007]. The TS domain has been shown to mediate recruitment of Dnmt1 to pericentric heterochromatin from the ensuing of its replication during mid S phase through G2 phase [Easwaran et al., 2004]. The N-terminal region of Dnmt1 is subject to various types of post-translational modification (PTM), several of which were involved in the control of Dnmt1 stability. It has been proposed that the phosphatidylinositol 3-kinase/protein kinase B pathway targets Dnmt1 and increases its stability [Sun et al., 2007]. The methylation state of several lysine residues of Dnmt1 was reported to be controlled by histone methyltransferase SET7 and histone demethylase LSD1, the methylated state being prone to proteosomal degradation [Esteve et al., 2009; Wang et al., 2009]. Finally, Dnmt1 protein stability was shown to be controlled also by ubiquitin mediated proteosomal degradation, although the enzymes controlling the ubiquitination state of Dnmt1 have not been reported [Agoston et al., 2005].

Usp7 (also known as Hausp) belongs to the ubiquitin specific peptidase class of deubiquitinating enzymes (DUBs). Genetic

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ablation of Usp7 in mice results in arrest of embryonic development shortly after implantation (E6.5–7.5) [Kon et al., 2010]. The best characterized function of Usp7 is the modulation of the p53-Mdm2 pathway as Usp7 deubiquitinates and stabilizes both p53 and Mdm2 [Li et al., 2002, 2004; Meulmeester et al., 2005]. Usp7 was also found to affect transcriptional activity by removing monoubiquitin from both the transcription factor FoxO4 and histone H2B. Deubiquitination of FoxO4 by Usp7 reduced its activity as a transcription factor, while deubiquitination of H2B by a Usp7-GMP synthetase complex enhanced both Polycomb-mediated silencing and transcriptional activation by EBNA1 [van der et al., 2005; van der Horst et al., 2006; Sarkari et al., 2009].

In the present study we identified Usp7 as an interacting partner of Dnmt1 and Uhrf1. We show that, while Uhrf1 promotes ubiquitination of Dnmt1 and decreases its stability, Usp7 mediates deubiquitination of both Uhrf1 and Dnmt1 and increases Dnmt1 stability.

MATERIALS AND METHODS

EXPRESSION CONSTRUCTS

Expression constructs for GFP-Dnmt1 (wt, full length), GFP-Dnmt1$^{\Delta 1\text{-}171}$, GFP-Dnmt1$^{1\text{-}309}$, GFP-Dnmt1$^{1\text{-}1111}$, GFP-Dnmt1$^{310\text{-}629}$, GFP-Dnmt1$^{630\text{-}1111}$, GFP-Dnmt1$^{1124\text{-}1620}$ and HA-ubiquitin were described previously [Easwaran et al., 2004; Pohl and Jentsch, 2008; Fellinger et al., 2009]. Expression constructs for GFP-Dnmt1$^{\Delta 459\text{-}501}$ and GFP-Dnmt1$^{\Delta 651\text{-}698}$ were derived from GFP-Dnmt1 by overlap extension PCR. To generate the Ch-Usp7 construct the Usp7 coding sequence was amplified using cDNA from mouse E14 ESCs as template and subcloned into the pCAG-Cherry-IB vector [Meilinger et al., 2009]. Expression constructs for Cherry fusions of the various Usp7 fragments were cloned into pCAG-Cherry-IB vector by PCR amplification. The Ch-Usp7^{C224S} construct was derived from Ch-Usp7 by overlap extension PCR. The GFP-Uhrf1 construct was described previously [Meilinger et al., 2009]. The Uhrf1-Ch construct was derived from the GFP-Uhrf1 construct by standard subcloning procedures. All constructs were verified by DNA sequencing.

CELL CULTURE AND TRANSFECTION

HEK293T, BHK, and ESCs were cultured and transfected as described [Meilinger et al., 2009; Szwagierczak et al., 2010]. The *dnmt1*$^{-/-}$ ESCs used in this study are homozygous for the c null allele [Lei et al., 1996] For stable complementation with GFP-Dnmt1 transfected *dnmt1*$^{-/-}$ ESCs were selected with 10 μg/ml of blasticidin (PAA) and individual clones were picked manually and expanded. For the in vivo ubiquitination assay, transfected HEK293T cells were incubated with medium supplemented with 2 mM N-ethylmaleimide (NEM; Sigma) for 30 min before harvesting. For cycloheximide treatment transfected cells were incubated in medium containing 10 μg/ml cycloheximide (Sigma) and harvested at the indicated time points.

MASS SPECTROMETRY

In-gel digests were performed according to standard protocols. Briefly, after washing the excised gel slices proteins were reduced by adding 10 mM DTT prior to alkylation with 55 mM iodoacetamide. After washing and shrinking of the gel pieces with 100% acetonitrile, trypsin (Sequencing Grade Modified, Promega) was added and proteins were digested overnight in 40 mM ammoniumbicarbonate at 37°C. For protein identification 10 μl of each sample were first purified and concentrated on a C18 reversed phase pipette tip (ZipTip, Millipore). Peptides were eluted with 1 μl of α-cyano-4-hydroxycinnamic acid (Sigma) and directly spotted on a MALDI sample plate (Applied Biosystems). MALDI-TOF measurements were then performed on a Voyager-DE STR mass spectrometer (Applied Biosystems). The resulting spectra where analyzed with the Mascot™ Software (Matrix Science) using the NCBInr Protein Database.

COIMMUNOPRECIPITATION AND F2H ASSAY

GFP fusion pulldowns with the GFP-trap and the F2H assay were performed as described [Meilinger et al., 2009]. For detection of ubiquitinated proteins by immunoprecipitation, cells were lysed in buffer containing 150 mM KCl, 50 mM Tris–HCl (pH7.4), 5 mM $MgCl_2$, 1% Triton X-100, 5% Glycerol, 2 mM phenylmethyl sulphonyl fluoride and 2 mM Mercaptoethanol and 5 mM NEM. After brief sonication cell lysates were cleared by centrifugation at 4°C for 10 min and supernatants were incubated with GFP-trap beads (Chromotek) for 2 h at 4°C with gentle rotation. The beads were then washed three times with lysis buffer and resuspended in SDS–PAGE sample buffer. The anti-HA mouse monoclonal antibody 12CA5 was used for detection of ubiquitinated proteins. Ch-Usp7 was detected with either an affinity purified polyconal antibody specific for human USP7 (a gift from Grigory Dianov, University of Oxford) or anti-red monoclonal antibody (Chromotek) [Rottach et al., 2008]. Rabbit antisera used for detection of Dnmt1 and Uhrf1 have been described [Li et al., 1992; Citterio et al., 2004]. The goat anti-Lamin B1 antibody was from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibodies (Sigma) and ECL Plus reagent (GE Healthcare) were used for chemiluminescent detection.

RESULTS

Usp7 INTERACTS WITH Dnmt1 AND Uhrf1

To identify Dnmt1 interaction partners we established *dnmt1*$^{-/-}$ embryonic stem cell (ESC) lines stably expressing GFP-tagged Dnmt1 (GFP-Dnmt1). A clone expressing steady state levels of GFP-Dnmt1 similar to those of the endogenous Dnmt1 in wild-type ESCs was selected for affinity purification of GFP-Dnmt1 using the GFP-trap [Rothbauer et al., 2008]. Bound proteins were separated by SDS–PAGE and Usp7 was identified by MALDI-TOF mass spectrometry (Fig. 1A). This was confirmed by immunoprecipition of GFP-Dnmt1 from the same clone of stably complemented *dnmt1*$^{-/-}$ ESCs and probing of the blot with an anti-USP7 antibody (Fig. 1B). To test for unspecific interaction of Usp7 with the GFP tag we transiently co-expressed GFP or GFP-Dnmt1 with Cherry-tagged Usp7 (Ch-Usp7) in HEK293T cells and immunoprecipitated protein complexes with the GFP-trap. Ch-Usp7 coimmunoprecipitated with GFP-Dnmt1 but not with GFP (Fig. 1C). In addition, we could not detect interaction of Ch-Usp7 with GFP fusions of the de novo methyltransferases Dnmt3a and Dnmt3b, further supporting the specificity of the interaction between Usp7 and Dnmt1 (Suppl. Fig. 1).

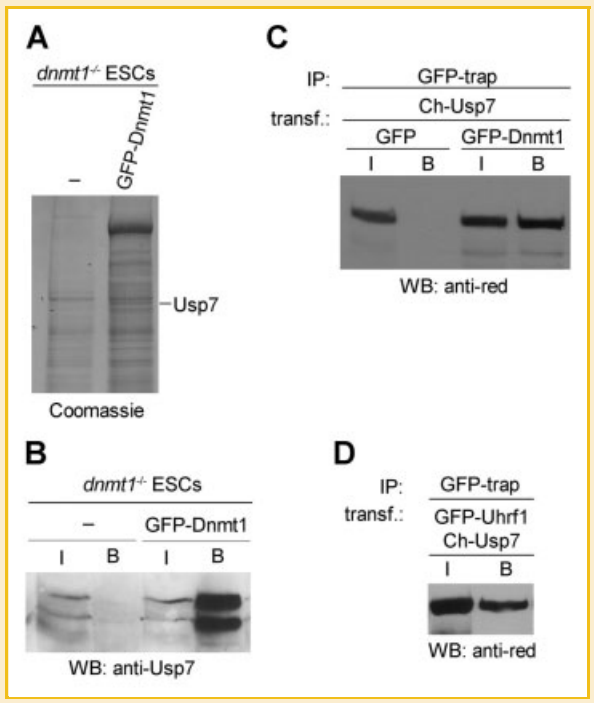

Fig. 1. Usp7 interacts with Dnmt1 and Uhrf1. A: GFP-trap pulldowns from *dnmt1*$^{-/-}$ ESCs and a clone of the same cells stably expressing GFP-Dnmt1. A colloidal Coomassie blue stained SDS–PAGE of bound fractions is shown and the position of Usp7 as identified by mass spectrometry is shown. B: GFP-trap pulldowns as in A were probed with an affinity purified anti-USP7 antibody. C: GFP-trap pulldowns from HEK293T cells coexpressing Ch-Usp7 and either GFP or GFP-Dnmt1. D: GFP-trap pulldowns from HEK293T cells coexpressing Ch-Usp7 and GFP-Uhrf1. The blots in C and D were probed with the anti-red monoclonal antibody that recognizes several red fluorescent proteins including Cherry. I, input; B, bound fractions.

Uhrf1 (also known as Np95 in the mouse and ICBP90 in human) interacts with Dnmt1 and is a crucial cofactor for maintaining genomic methylation [Bostick et al., 2007; Sharif et al., 2007]. To investigate whether Usp7 interacts also with Uhrf1 we immunoprecipitated a Uhrf1-GFP fusion from lysates of cells coexpressing Ch-Usp7. The latter clearly coprecipitated with Uhrf1-GFP, indicating that Usp7 forms complexes with both Dnmt1 and Uhrf1 (Fig. 1D).

MAPPING OF THE INTERACTION BETWEEN Dnmt1 AND Usp7

To define the domains that are responsible for the interaction between Dnmt1 and Usp7, we generated several domain and deletion constructs for both GFP-Dnmt1 and Ch-Usp7 and tested their interaction by co-immunoprecipitation from transfected cells and fluorescent two hybrid assay (F2H). In the latter assay a GFP-tagged bait protein is anchored to a *lac* operator array inserted in the genome of BHK cells, so that the array is visible as a spot of enriched GFP fluorescence in the nucleus. Accumulation of Cherry-tagged prey proteins at this spot reflects the interaction between prey and bait [Fellinger et al., 2009; Meilinger et al., 2009]. Both methods showed that only the C-terminal region (amino acids 561–1,103) of Usp7, containing four ubiquitin like domains, strongly interacted with GFP-Dnmt1 and that deletion of the same region abolished the interaction (Suppl. Fig. 2). In contrast, individual deletion of several domains in Dnmt1 did not overtly disrupt the interaction with Usp7, only the deletion of the highly conserved central part of the TS domain [Fellinger et al., 2009] resulting in a substantially weaker interaction (Suppl. Fig. 3). However, the strong interaction detected with the full length Dnmt1 construct was not preserved with any of several Dnmt1 fragments, including the entire N-terminal region and C-terminal catalytic domain (Suppl. Fig. 4). These major parts of Dnmt1 are known to engage in intramolecular interaction required for catalytic activity [Margot et al., 2000; Fatemi et al., 2001; Pradhan and Esteve, 2003]. Therefore, we tested whether mutation of the $(KG)_7$ linker between the N-terminal region and the C-terminal catalytic domain of Dnmt1 affects the interaction with Usp7. Substitution of all lysines in the $(KG)_7$ linker with glutamine residues generating a neutrally charged $(QG)_7$ linker completely abrogated the interaction with Usp7 (Suppl. Fig. 3). These data suggest that the $(KG)_7$ linker contributes to the interaction with Usp7 either directly or indirectly, by mediating a specific conformation of Dnmt1.

Usp7 REGULATES THE UBIQUITINATION STATUS OF Dnmt1 AND Uhrf1

DNMT1 was shown to be ubiquitinated in human cell lines [Agoston et al., 2005]. To determine whether Usp7 affects the ubiquitination levels of Dnmt1, GFP-Dnmt1 was coexpressed with either HA-tagged ubiquitin or both HA-ubiquitin and Ch-Usp7 in HEK293T cells. GFP-Dnmt1 was then immunoprecipitated with the GFP-trap and its ubiquitination was probed with anti-HA antibody. In the absence of Ch-Usp7 the ubiquitinated GFP-Dnmt1 appeared as a smear reflecting relatively broad size heterogeneity (Fig. 2A). Overexpression of Ch-Usp7 resulted in both reduced signal strength and altered migration of ubiquitinated GFP-Dnmt1, which appeared as a sharp band comigrating with the lowest part of the smear obtained in the absence of Ch-Usp7 (Fig. 2A). In contrast, overexpression of Ch-Usp7^{C224S}, a catalytically inactive point mutant [Li et al., 2002], led to an apparent increase in the ubiquitination levels of GFP-Dnmt1. Using the same assay and mutation analysis we could map the ubiquitination sites of Dnmt1 within amino acids 524–629, corresponding to the C-terminal part of the TS domain (Suppl. Fig. 5).

Uhrf1 contains a Ring domain endowed with E3 ubiquitin ligase activity and has been shown to ubiquitinate itself as well as core histones both in vitro and upon overexpression in HEK293T cells [Citterio et al., 2004; Karagianni et al., 2008]. As we showed that Usp7 also interacts with Uhrf1 we used the same immunoprecipitation assay as described above to determine whether Usp7 affects the ubiquitination levels of Uhrf1. The levels of ubiquitinated GFP-Uhrf1 were clearly reduced by coexpression of Ch-USP7 (Fig. 2B). Thus, our results suggest that ubiquitination status of both Dnmt1 and Uhrf1 is regulated by Usp7.

Uhrf1 ENHANCES UBIQUITINATION OF Dnmt1 IN VIVO

The E3 ubiquitin ligases responsible for ubiquitination of Dnmt1 are unknown. As the Ring domain of Uhrf1 has E3 ubiquitin ligase activity, we explored the possibility that Uhrf1 ubiquitinates Dnmt1.

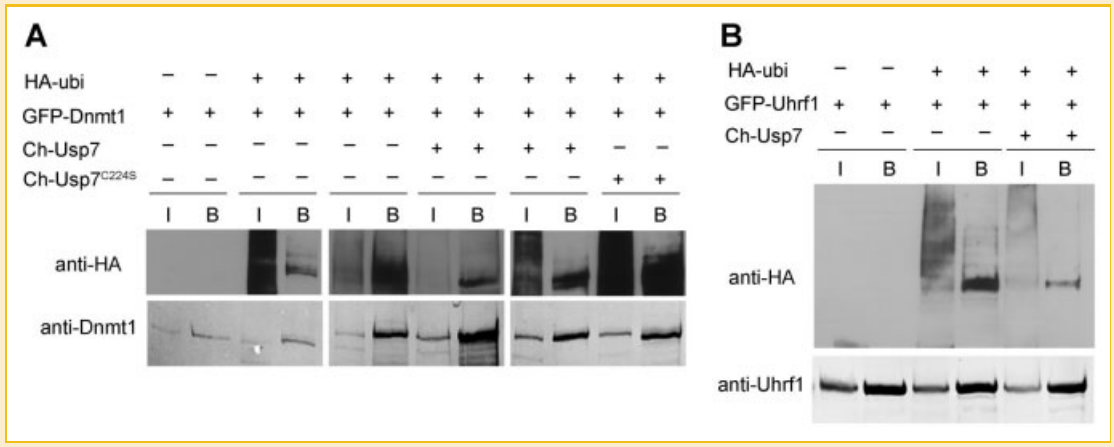

Fig. 2. Usp7 deubiquitinates both Dnmt1 and Uhrf1. GFP-trap pulldowns from HEK293T cells expressing the indicated combinations of HA-ubiquitin, Ch-Usp7, Ch-Usp7^{C224S}, and either GFP-Dnmt1 (A) or GFP-Uhrf1 were probed with an anti-HA antibody to detect ubiquitinated proteins and either anti-Dnmt1 (A) or anti-Uhrf1 (B) antibodies as loading controls.

GFP-Dnmt1 was coexpressed with either HA-ubiquitin or both HA-ubiquitin and Uhrf1-Cherry in HEK293T cells (Fig. 3). The latter condition generated a clear increase in size of ubiquitinated GFP-Dnmt1, indicating that Uhrf1 is able to ubiquitinate Dnmt1 in vivo.

USP7 CONTROLS Dnmt1 STABILITY

As Usp7 dependent deubiquitination is known to stabilize both p53 and Mdm2 we asked whether Usp7 affects Dnmt1 protein stability. To this aim we over-expressed either Cherry or Ch-Usp7 in HEK293T cells and treated them with cycloheximide to block protein synthesis. With prolonged cycloheximide treatment endogenous Dnmt1 levels steadily decreased in cells overexpressing Cherry, showing a half-life of about 5 h, while they remained unaltered in cells overexpressing Ch-Usp7 (Fig. 4). This result clearly supports the idea that deubiquitination by Usp7 increases Dnmt1 stability.

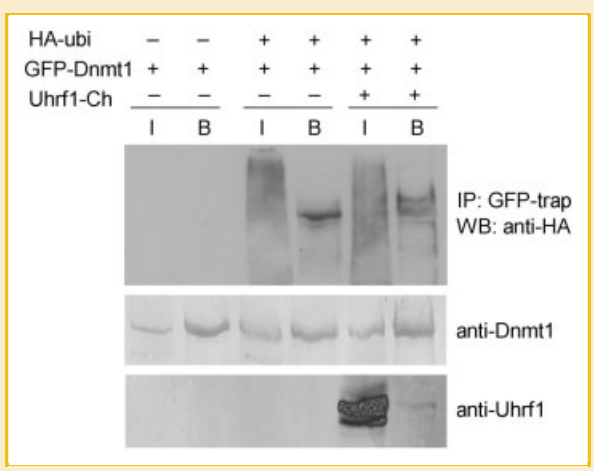

Fig. 3. Uhrf1 ubiquitinates Dnmt1. GFP-trap pulldowns from HEK293T cells expressing the indicated combinations of HA-ubiquitin, GFP-Dnmt1 and Uhrf1-Ch were probed with anti-HA and anti-Uhrf1 antibodies as in Figure 2B.

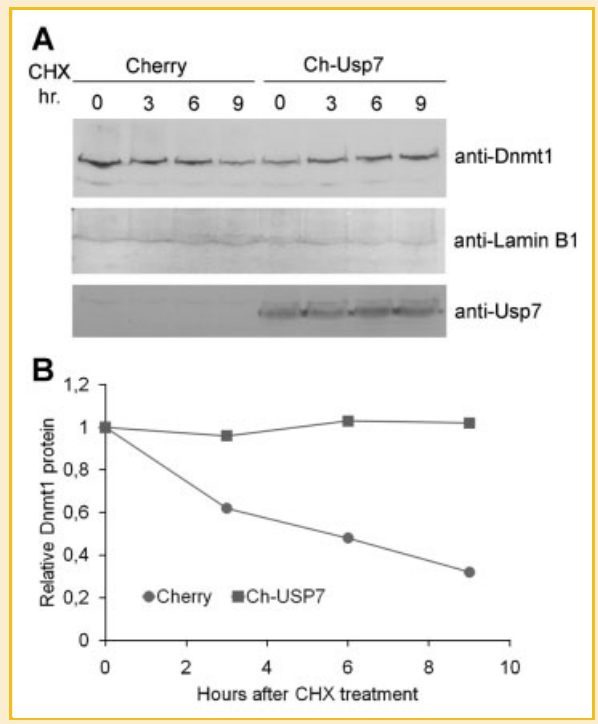

Fig. 4. USP7 stabilizes Dnmt1. HEK293T cells expressing either Cherry or Ch-Usp7 were treated with cycloheximide (CHX) for the indicated time periods before harvesting. A: Endogenous Dnmt1 levels were detected with an anti-Dnmt1 antibody, while anti-Lamin B1 and anti-Usp7 blots served as loading controls. B: shows quantification of the blots in (A).

DISCUSSION

Here we show that the DUB Usp7 interacts with both Dnmt1 and Uhrf1, two factors crucial for maintenance of genomic methylation patterns. We provide evidence that ubiquitination of Dnmt1 is controlled by the Uhrf1 and Usp7 and that deubiquitination by the latter stabilizes Dnmt1. In addition several Ring domain E3 ubiquitin

ligases were shown to regulate their own stability by autoubiquitination. Here we confirm that Uhrf1 ubiquitinates itself and show that in turn it is deubiquitinated by Usp7. Our data are consistent with a very recent report [Du et al., 2010] in supporting a network of ubiquitination mediated feedback loops that ultimately fine tune the levels of two central effectors of DNA methylation maintenance. Interestingly, two other types of PTM have been proposed to modulate Dnmt1 stability, namely phosphorylation and methylation [Sun et al., 2007; Esteve et al., 2009; Wang et al., 2009]. This observation raises the question as to whether these PTMs are part of distinct mechanisms that mediate the control of Dnmt1 stability independently or belong to a common pathway. In any case, Dnmt1 stability seems to be under control of a complex system of mechanisms that likely reflects the necessity for tight regulation of Dnmt1 levels, as altered Dnmt1 expression has been associated to several pathologic states ranging from cancer [Gaudet et al., 2003] to major psychoses [Costa et al., 2007].

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Usp7 and Uhrf1 control ubiquitination and stability of the maintenance DNA methyltransferase Dnmt1

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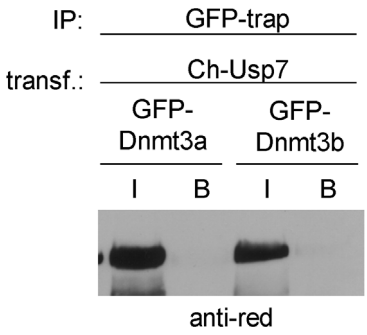
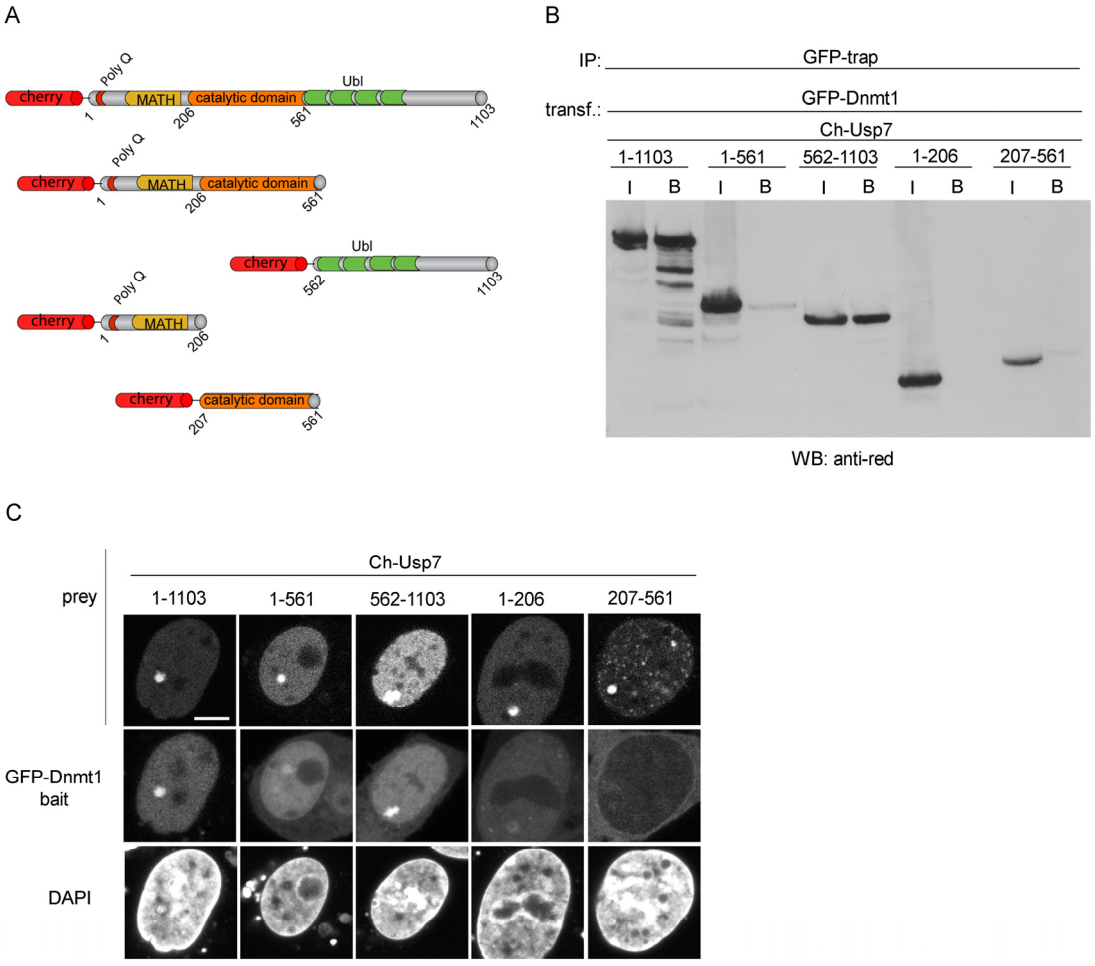
SUPPLEMENTARY MATERIAL

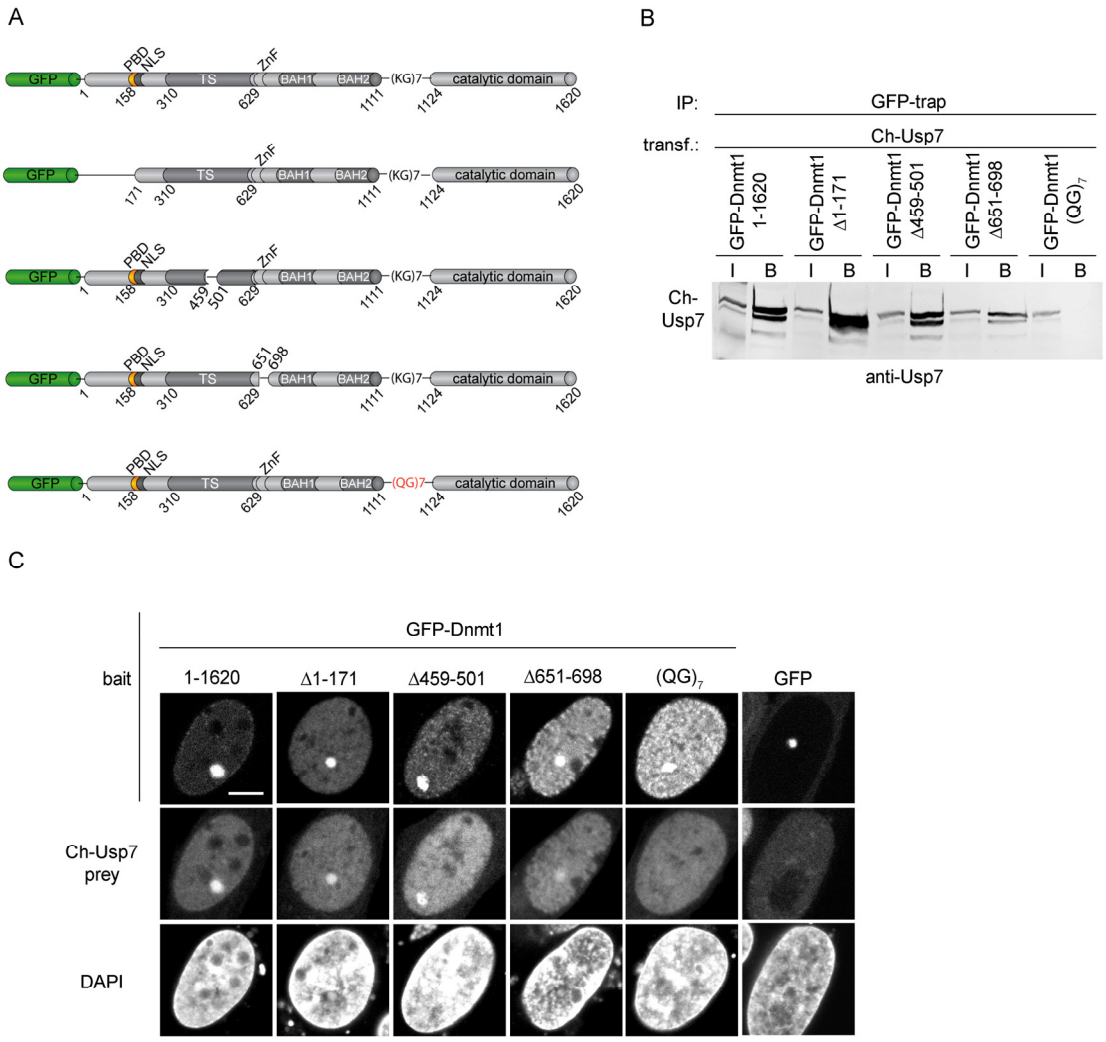
Supplementary Figures 1-5



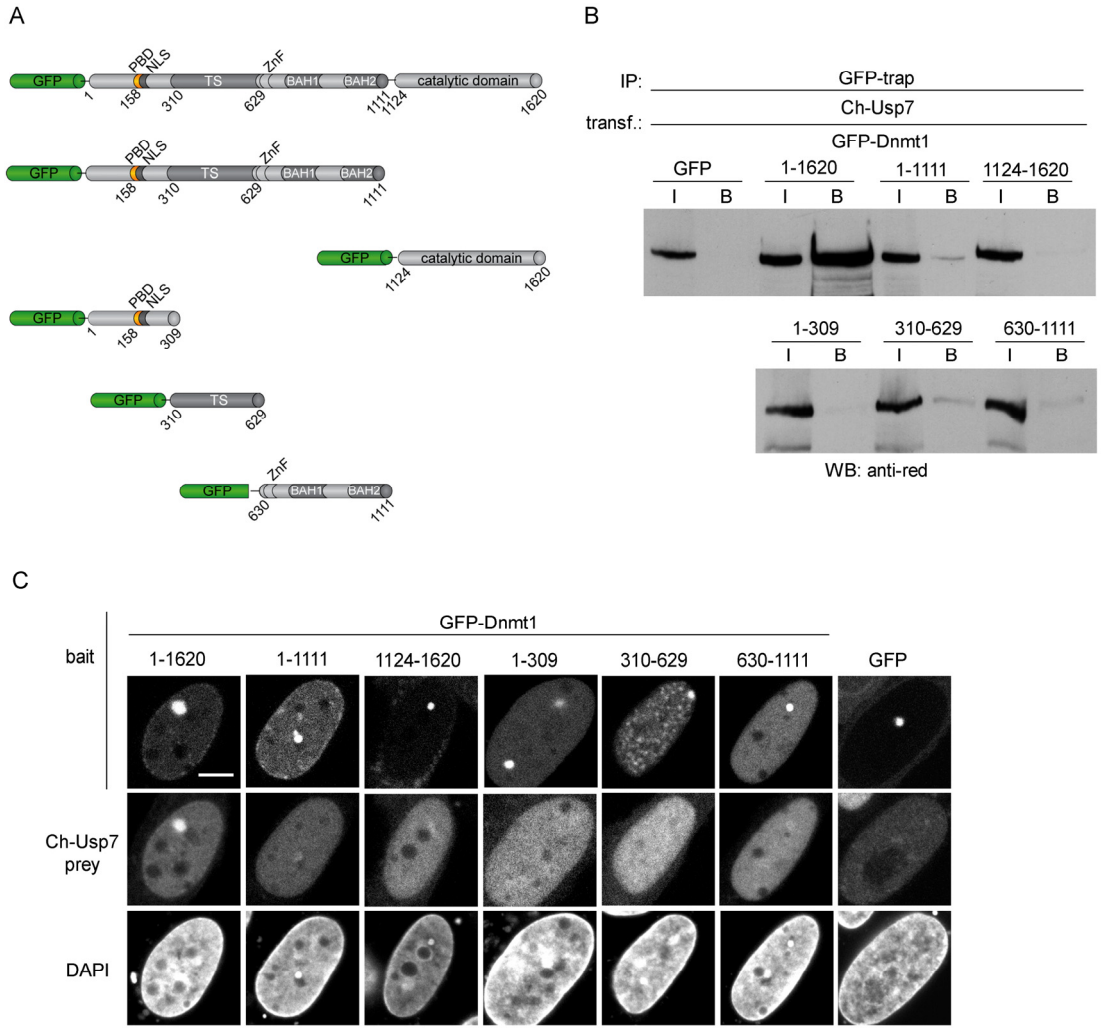
Supplementary Figure 1. Usp7 does not interact with de novo DNA methyltransferases Dnmt3a and 3b. GFP-trap pulldowns from HEK293T cells transiently coexpressing Ch-Usp7 and either GFP-Dnmt3a or GFP-Dnmt3b were probed with the anti-red monoclonal antibody that recognizes several red fluorescent proteins including Cherry. I=input and B=bound fractions.



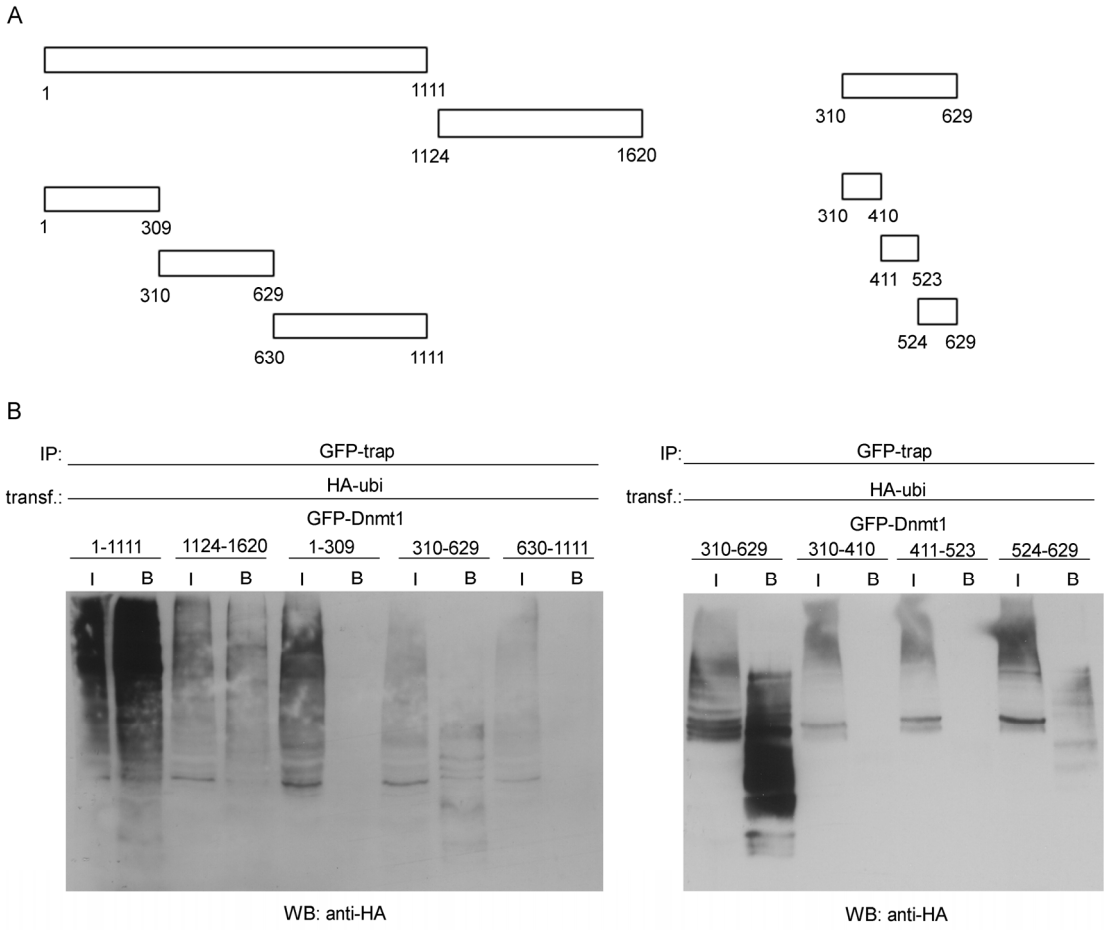
Supplementary Figure 2. Mapping of the Usp7 domain responsible for the interaction with Dnmt1. (A) Schematic representation of Ch-Usp7 constructs used for the mapping. (B) GFP-trap pulldowns from HEK293T cells transiently coexpressing GFP-Dnmt1 and the indicated Ch-Usp7 constructs as shown in A. The blot was probed with probed with the anti-red monoclonal antibody. I=input and B=bound fractions. (C) F2H assays (Zolghadr et al., 2008, Mol Cell Proteomics 7:2279) using GFP-Dnmt1 as bait and the Ch-Usp7 constructs shown in A as preys.



Supplementary Figure 3. Mapping of the Dnmt1 domain responsible for the interaction with Usp7. (A) Schematic representation of GFP-Dnmt1 constructs used for the mapping. (B) GFP-trap pulldowns from HEK293T cells transiently coexpressing Ch-Usp7 and the indicated GFP-Dnmt1 constructs as shown in A. the blot was probed with probed with an anti-Usp7 antibody. I=input and B=bound fractions. (C) F2H assays (Zolghadr et al., 2008, Mol Cell Proteomics 7:2279) using GFP-Dnmt1 constructs shown in A as baits and Ch-Usp7 as prey.



Supplementary Figure 4. Further mapping of the Dnmt1 domain responsible for the interaction with Usp7. (A) Schematic representation of GFP-Dnmt1 constructs used for the mapping. (B) GFP-trap pulldowns from HEK293T cells transiently coexpressing Ch-Usp7 and the indicated of GFP-Dnmt1 constructs as shown in A. The blot was probed with probed with the anti-red monoclonal antibody. I=input and B=bound fractions. (C) F2H assays (Zolghadr et al., 2008, Mol Cell Proteomics 7:2279) using GFP-Dnmt1 constructs shown in A as baits and Ch-Usp7 as prey.



Supplementary Figure 5. Mapping of the ubiquitinated domain in Dnmt1. (A) Schematic representation of the GFP-Dnmt1 fragment constructs used for the mapping. (B) GFP-trap pulldowns from HEK293T cells transiently coexpressing HA-ubiquitin and the indicated GFP-Dnmt1 constructs as shown in A. The blot was probed with probed with an anti-HA antibody to detect ubiquitinated proteins. I=input and B=bound fractions. Amino acids 524-629 corresponding to the C-terminal part of the TS domain harbor the ubiquitinated sites. Some additional ubiquitinated sites may be present in the C-terminal catalytic domain.

2.2. DIFFERENT BINDING PROPERTIES AND FUNCTION OF CXXC ZINC FINGER DOMAINS IN DNMT1 AND TET1

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Different Binding Properties and Function of CXXC Zinc Finger Domains in Dnmt1 and Tet1

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Abstract

Several mammalian proteins involved in chromatin and DNA modification contain CXXC zinc finger domains. We compared the structure and function of the CXXC domains in the DNA methyltransferase Dnmt1 and the methylcytosine dioxygenase Tet1. Sequence alignment showed that both CXXC domains have a very similar framework but differ in the central tip region. Based on the known structure of a similar MLL1 domain we developed homology models and designed expression constructs for the isolated CXXC domains of Dnmt1 and Tet1 accordingly. We show that the CXXC domain of Tet1 has no DNA binding activity and is dispensable for catalytic activity *in vivo*. In contrast, the CXXC domain of Dnmt1 selectively binds DNA substrates containing unmethylated CpG sites. Surprisingly, a Dnmt1 mutant construct lacking the CXXC domain formed covalent complexes with cytosine bases both *in vitro* and *in vivo* and rescued DNA methylation patterns in *dnmt1*$^{-/-}$ embryonic stem cells (ESCs) just as efficiently as wild type Dnmt1. Interestingly, neither wild type nor ΔCXXC Dnmt1 re-methylated imprinted CpG sites of the *H19a* promoter in *dnmt1*$^{-/-}$ ESCs, arguing against a role of the CXXC domain in restraining Dnmt1 methyltransferase activity on unmethylated CpG sites.

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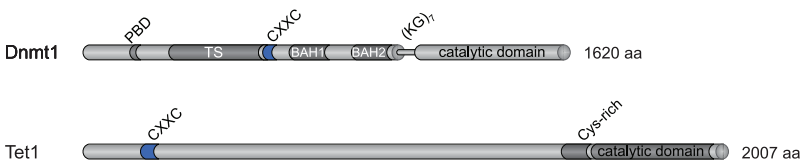
Introduction

In mammals DNA methylation is restricted to cytosine residues and mainly involves CpG dinucleotides. CpG methylation is widespread across mammalian genomes, including gene bodies regardless of their transcriptional activity [1–4]. However, highly CpG-rich regions (CpG islands) are refractory to methylation and mostly coincide with promoters of constitutively active genes. The methylation state of other regulatory sequences with moderate to low CpG density, including promoters and enhancers, shows developmental and/or tissue-specific variations and positively correlates with a transcriptionally silent state [1,3–8]. Dense methylation of repetitive sequences is also thought to maintain these elements in a silent state and thus contribute to genome stability [9–11]. In mammals cytosine methylation is catalyzed by a family of DNA methyltransferases (Dnmts) [12]. Dnmt3a and Dnmt3b establish methylation patterns during embryonic development of somatic as well as germ cell lineages and, consistently, show developmental stage and tissue specific expression patterns. In contrast, Dnmt1 is ubiquitous and generally the most abundant DNA methyltransferase in mammalian tissues, where it associates with the replication machinery and restores symmetrical methylation at hemimethylated CpG sites generated by the semi-conservative DNA replication process [13]. Thus, Dnmt1 maintains methylation patterns with high fidelity and is essential for embryonic development and genome integrity [9,14,15].

Dnmt1 is a large enzyme with a complex domain structure that likely evolved by fusion of at least three genes [16]. It comprises a regulatory N-terminal region and a C-terminal catalytic domain connected by a linker of seven glycine-lysine repeats (Figure 1A)[17]. The N-terminal part contains a PCNA binding domain (PBD), a heterochromatin targeting sequence (TS), a CXXC-type zinc finger domain and two Bromo-Adjacent Homology domains (BAH1 and BAH2). The C-terminal domains of mammalian Dnmts contain all ten catalytic motifs identified in bacterial DNA (cytosine-5) methyltransferases [12]. Thus, prokaryotic and mammalian cytosine methyltransferases are thought to adopt the same catalytic mechanism. However, the C-terminal domain of Dnmt1 is the only DNA methyltransferase domain in Dnmts that is not catalytically active when expressed separately. Indeed, interaction with the N-terminal part is required for allosteric activation of the enzyme [18]. Remarkably, the first 580 amino acids (aa) of human DNMT1 are dispensable for both enzymatic activity and substrate recognition, whereas deletion of the first 672 aa results in an inactive enzyme [19]. Interestingly, this truncation eliminates part of the CXXC domain, suggesting an involvement of this domain in allosteric activation. However, addition

A


Dnmt1
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TS
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BAH1
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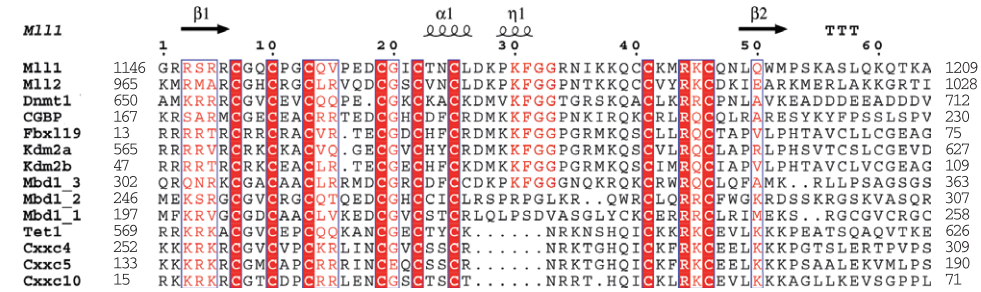
catalytic domain
1620 aa
Tet1
CXXC
Cys-rich
catalytic domain
2007 aa

B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CGBP 167 KRSARMCGECEACRRTEDCGHCDFCRDMKKFGGPNKIRQKCRLRQCQLRARESYKYFPSSLSPV 230
Fbxl19 13 RRRRTRCRRCRACVR.TECGDCHFCRDMKKFGGPGRMKQSCLLRQCTAPVLPHTAVCLLCGEAG 75
Kdm2a 565 RRRRVRCRKCKACVQ.GECGVCHYCRDMKKFGGPGRMKQSCVLRQCLAPRLPHSVTCSLCGEVD 627
Kdm2b 47 RRRRTRCRKCEACLR.TECGECHFCKDMKKFGGPGRMKQSCIMRQCIAPVLPHTAVCLVCGEAG 109
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Mbd1_2 246 MEKSRGCGVCRGCQTQEDCGHCCICLRSPRPGLKR..QWRCLQRRCFWGKRDSSKRGSKVASQR 307
Mbd1_1 197 MFKRVGCGDCAACLVKEDCGVCSTCRLQLPSDVASGLYCKCERRRCLRIMEKS..RGCGVCRGC 258
Tet1 569 RRKRKACGVCEPCQQKANCGECTYCK.....NRKNSHQICKKRKCEVLKKKPEATSQAQVTKE 626
Cxxc4 252 KKKRKRCGVCVPCKRLINCGVCSSCR.....NRKTGHQICKFRKCEELKKKPGTSLERTPVPS 309
Cxxc5 133 KKKRKRCGMCAPCRRRINCEQCSSCR.....NRKTGHQICKFRKCEELKKKPSAALEKVMLPS 190
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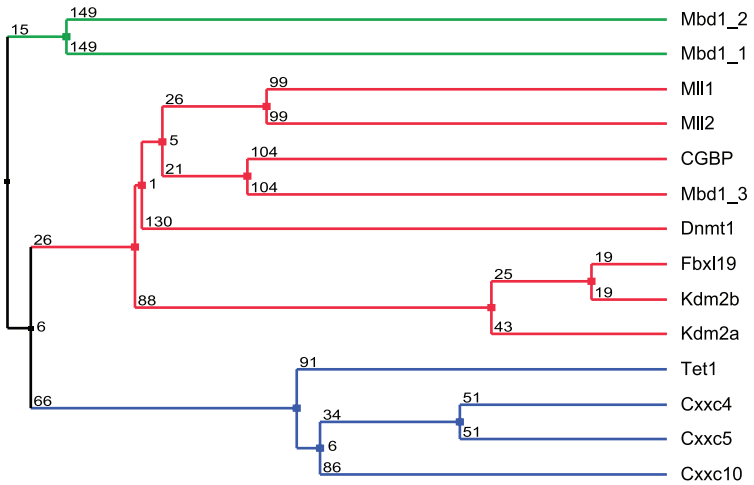
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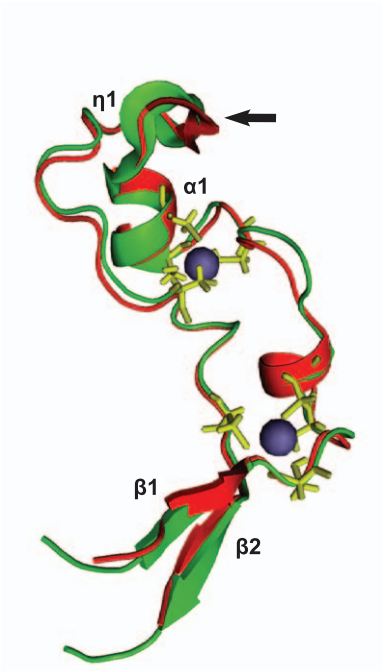
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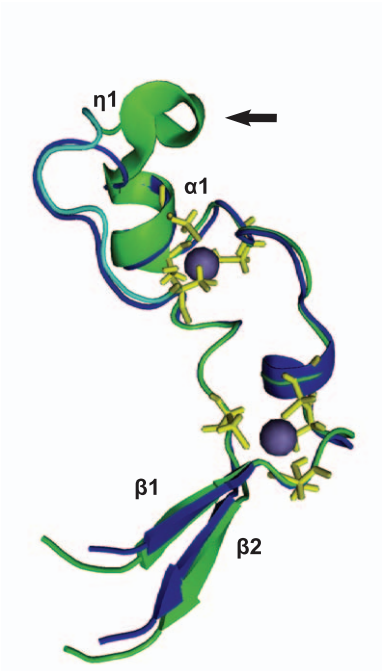
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Figure 1. Sequence and predicted structural homology of CXXC domains. (**A**) Schematic representation of the domain structure in Dnmt1 and Tet1. The catalytic domain and the N-terminal region of Dnmt1 are connected by seven lysine-glycine repeats [$(KG)_7$]. PBD: PCNA binding domain; TS: targeting sequence; CXXC: CXXC-type zinc finger domain; BAH1 and 2: bromo-adjacent homology domain; NLS: nuclear localization signal; Cys-rich: cysteine rich region. (**B**) Alignment of mammalian CXXC domains. Numbers on the right side indicate the position of the last amino acid in the corresponding protein. The Mbd1a isoform contains three CXXC motifs (Mbd1_1-3). Absolutely conserved residues, including the eight cysteines involved in zinc ion coordination are highlighted in red and the conserved KFGG motif is in red bold face. Positions with residues in red face share 70% similarity as calculated with the Risler algorithm [66]. At the top residues of MLL1 involved in β sheets β1 and β2 (black arrows), α helices α1 and α2 and strict α turns (TTT) are indicated. All sequences are from *M. musculus*. Accession numbers (for GenBank unless otherwise stated): Dnmt1, NP_034196; Mll1, NP_001074518; Mll4, O08550 (SwissProt); CGBP, NP_083144; Kdm2a, NP_001001984; Kdm2b, NP_001003953; Fbxl19, NP_766336; Mbd1, NP_038622; CXXC4/Idax, NP_001004367; CXXC5, NP_598448; CXXC10 (see Materials and Methods). (**C**) A homology tree was generated from the alignment in (B). The three subgroups of CXXC domains identified are in different colors. Average distances between the sequences are indicated. (**D–E**) Homology models of the mouse Dnmt1 (D; red) and Tet1 (E; blue) CXXC domains superimposed to the CXXC domain of MLL1 (green; [35]). MLL1 residues that were described to contact DNA according to chemical shift measurements [35] are cyan in (E), while cysteines involved in coordination of the two zinc ions are yellow. Arrows point to the KFGG motif in MLL1 and Dnmt1. The locations of α helices and β sheets are indicated as in (B).
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of an N-terminal fragment containing the isolated CXXC domain to the catalytic domain was not sufficient for catalytic activation [20].

CXXC-type zinc finger domains are found in several other proteins with functions related to DNA or chromatin modification, including the histone H3 lysine 4 (H3K4) methyltransferases mixed-lineage leukaemia (MLL) proteins 1 and 4, the CpG-binding protein (CGBP, also known as Cfp1 or CXXC1), the methyl-CpG binding domain protein 1 (MBD1), the H3 lysine 36 (H3K36) demethylases KDM2A and B (also known as JHD1A/FBXL11 and JHD1B/FBXL10) and the MLL1 fusion partner TET1 (Figure 1A) [21–28]. The CXXC domains of some of these proteins were shown to mediate specific binding to double stranded DNA templates containing unmethylated CpG sites [21,22,29,30]. A region of Dnmt1 which mainly includes the CXXC domain (aa 628–753) was also shown to bind Zn ions and DNA [20,31,32]. However, available data on the selectivity of this DNA binding activity are conflicting. Whereas a fragment including aa 613–748 of mouse Dnmt1 was shown to bind DNA with a slight preference for hemimethylated CpG sites [20], aa 645–737 of human DNMT1 were shown to selectively bind unmethylated DNA [32]. As these studies used different constructs and species, the selectivity of DNA binding by the CXXC domain of Dnmt1 with regard to CpG methylation state and the role of the CXXC domain in allosteric activation and substrate discrimination remain to be firmly established.

Notably, not all CXXC domains show DNA binding activity, as exemplified by the fact that only one out of three CXXC domains in MBD1 binds DNA [29]. Interestingly, TET1 was recently shown to be a 2 oxoglutarate- and Fe(II)-dependent dioxygenase responsible for converting genomic 5-methylcytosine (mC) to 5-hydroxymethylcytosine (hmC) [33,34]. However, it is not known whether the CXXC domain of TET1 is involved in recognition of methylated DNA substrates.

Here we report a functional study and characterization of the DNA binding activity for the CXXC domains of mouse Dnmt1 and Tet1 proteins. We generated isolated CXXC domain and deletion constructs based on structural homology models to minimize structural alterations. We show that the CXXC domain of Dnmt1 preferentially binds DNA substrates containing unmethylated CpG sites, but does not contribute significantly to the DNA binding properties of the full length enzyme and is dispensable for its catalytic activity *in vitro* and *in vivo*. In addition, we found that the CXXC domain of Tet1 does not bind DNA *in vitro* and is also dispensable for catalytic activity of Tet1 *in vivo*.

Results

Sequence homology and structural modeling identify distinct CXXC domain subtypes

Dnmt1 contains a zinc finger domain of the CXXC type, which is present in several mammalian proteins including MLL1 (Figure 1A–C) and is highly conserved among Dnmt1 sequences from various animal species (Figure S1 in File S1). The primary structure of CXXC domains spans two clusters of 6 and 2 cysteine residues separated by a stretch of variable sequence and length. Sequence alignment and homology tree construction identified three distinct groups of CXXC domains (Figure 1B and C). The sequence between the two cysteine clusters in the CXXC domains of Dnmt1, CGBP/Cfp1, Fbxl19, Mll1, Mll2 and Kdm2 proteins and CXXC domain 3 of Mbd1 is highly conserved and contains a KFGG motif. The two other homology groups, including the CXXC domains 1 and 2 of Mbd1 on one side and those of Tet1, Cxxc4/Idax, Cxxc5/RINF and Cxxc10 on the other side, lack the KFGG motif and diverge from the first group and from each other in the sequence between the cysteine clusters. We generated structural homology models for the CXXC domains of mouse Dnmt1 and Tet1 using the NMR structure of the MLL1 CXXC domain as a template (Figure 1D and E)[35]. The CXXC domains of these proteins adopt an extended crescent-like structure that incorporates two Zn^{2+} ions each coordinated by four cysteine residues. The peptide of the MLL1 CXXC domain predicted to insert into the major groove of the DNA double helix (cyan in Fig. 1E) is located on one face of the structure and is contiguous to the KFGG motif [35]. The predicted structure of the Tet1 CXXC domain lacks the short 3_{10} helix (η1 in Figure 1E) formed by residues PKF and partially overlapping the KFGG motif, but is similar to the MLL1 CXXC domain in the region of the DNA-contacting peptide. However, each of the two predicted β-strands in Tet1 carries three positive charges, whereas there is only one or no charged residue in the C-terminal strands of the CXXC domains in MLL1 and Dnmt1. Depending on the orientation of the positively charged side chains, it cannot be excluded that the charge density prevents strand pairing in the Tet1 CXXC domain.

The Dnmt1 CXXC domain binds unmethylated DNA

To investigate the binding properties of the Dnmt1 CXXC domain, we generated a GFP fusion construct including aa 652–699 (GFP-$CXXC^{Dnmt1}$). According to our homology model the ends of this fragment form an antiparallel β-sheet that structurally delimits the domain as in MLL1. We first compared the localization and mobility of GFP-$CXXC^{Dnmt1}$ and GFP in mouse C2C12 myoblasts. While GFP was diffusely distributed in both nucleus and cytoplasm, GFP-$CXXC^{Dnmt1}$ was exclusively nuclear with a punctuated pattern throughout the nucleoplasm and was enriched in nucleoli, a pattern independent of cell cycle stage (Figure 2A and Figure S2 in File S1). Enrichment in the nucleus and nucleoli is frequently observed with constructs containing stretches with high density of basic residues. After photobleaching half of the nuclear volume we observed a slower fluorescence recovery rate for GFP-$CXXC^{Dnmt1}$ than for GFP (Figure 2B). To rule out a contribution of nucleolar interactions to the slower kinetics of GFP-$CXXC^{Dnmt1}$,

we separately bleached nucleoplasmic and nucleolar regions and found that GFP-CXXC^{Dnmt1} has even faster kinetics within the nucleolus (Figure S3 in File S1). These results are consistent with a binding activity of GFP-CXXC^{Dnmt1} in the nucleus and very transient, unspecific binding in the nucleolus. To investigate whether the CXXC domain of Dnmt1 binds DNA and its possible selectivity with respect to CpG methylation we used a recently developed fluorescent DNA binding assay [36,37]. GFP-CXXC^{Dnmt1} was transiently expressed in HEK293T cells, immunopurified with the GFP-trap (Figure S4 in File S1) and incubated with fluorescent DNA substrates containing either no CpG site or one central un-, hemi- or fully methylated CpG site in direct competition. As shown in Figure 2C, GFP-CXXC^{Dnmt1} displayed a significant preference for the substrate containing one unmethylated CpG site, which increased substantially with a five-fold higher concentration of the DNA substrates (Figure S5 in File S1). These results are consistent with the reported binding preference of the CXXC domains in human DNMT1 and other factors belonging to the same CXXC homology group [21,22,29,32]. Notably, the CXXC domains 1 and 2 of Mbd1 lack the KFGG motif and do not bind DNA, while mutation of this motif prevented DNA binding by the CXXC domain of MLL1 [29,38]. Therefore, we generated a GFP-CXXC^{Dnmt1} construct where the KFGG motif was mutated to AAGG (GFP-CXXC$^{Dnmt1KF/AA}$, Figure S4 in File S1) to test the requirement of the KFGG motif for binding by the CXXC domain of Dnmt1. The mutant domain showed significantly decreased binding to all DNA substrates and complete loss of preferential binding to the unmethylated substrate *in vitro* (Figure 2B). In addition, GFP-CXXC$^{Dnmt1KF/AA}$ showed faster recovery after photobleaching (FRAP) *in vivo* compared to the corresponding wild type construct (Figure 2C). These results further support the importance of the KFGG motif for DNA binding by CXXC domains.

The CXXC domain of Tet1 shows no specific DNA binding activity and is dispensable for enzymatic activity *in vivo*

It was recently shown that Tet1 oxidizes genomic mC to hmC. However, the mechanism by which Tet1 is targeted to genomic mC is not known. Our model for the structure of the Tet1 CXXC domain diverged from the structure of the MLL1 CXXC domain with respect to the KFGG motif but not to the DNA-contacting peptide, suggesting that the Tet1 CXXC domain may still bind DNA. To test this we generated a GFP-tagged Tet1 CXXC

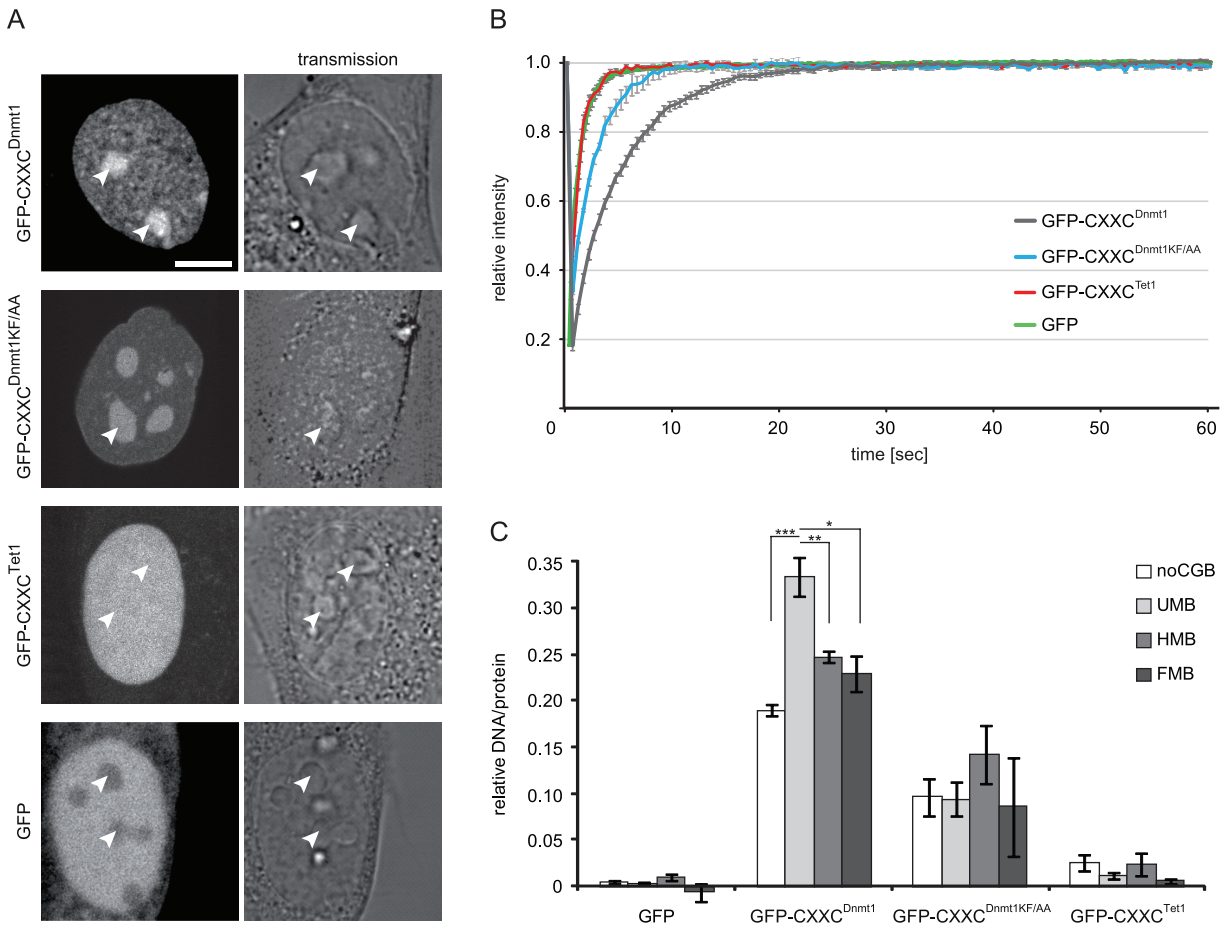

Figure 2. Properties of isolated Dnmt1 and Tet1 CXXC domains. (**A–B**) Subcellular localization (A) and binding kinetics (B) of GFP-CXXC^{Dnmt1}, GFP-CXXC$^{Dnmt1KF/AA}$, GFP-CXXC^{Tet1} and GFP in mouse C2C12 myoblasts. Localization and binding kinetics were independent from the cell cycle stage (Figures S2 and S5 in File S1). Arrowheads in (A) point to nucleoli. Scale bar: 5 μm. Binding kinetics were analyzed by FRAP. (**C**) DNA binding specificity of the Dnmt1 and Tet1 CXXC domains. GFP, GFP-CXXC^{Dnmt1}, GFP-CXXC$^{Dnmt1KF/AA}$ and GFP-CXXC^{Tet1} were pulled down from extracts of transiently transfected HEK293T cells and incubated with fluorescent DNA substrates containing no CpG site or one central un-, hemi- or fully methylated CpG site in direct competition (noCGB, UMB, HMB, FMB, respectively). Shown are the mean DNA/protein ratios and corresponding standard errors from 5 (GFP), 4 (GFP-CXXC^{Dnmt1} and GFP-CXXC$^{Dnmt1KF/AA}$) and 2 (GFP-CXXC^{Tet1}) independent experiments. * $P = 0.01$; ** $P = 0.007$; *** $P = 0.001$.
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construct (GFP-CXXC^{Tet1}) following the same criteria as for GFP-CXXC^{Dnmt1} and investigated its cellular localization, *in vivo* binding kinetics and *in vitro* DNA binding activity. GFP-CXXC^{Tet1} was prevalently nuclear with a homogeneous distribution including nucleoli that was independent of cell cycle stage (Figure 2A and Figure S6 in File S1). After photobleaching GFP-CXXC^{Tet1} showed very fast recovery kinetics similar to GFP (Figure 2B) and its DNA binding activity *in vitro* was also similar to the background levels of the GFP control (Figure 2C). We conclude that the isolated CXXC domain of Tet1 has no specific DNA binding activity. Together with the observation that the CXXC domains 1 and 2 of Mbd1 also lack the KFGG motif and do not bind DNA [29] and that mutation of this motif reduced DNA binding by the CXXC domains of both Dnmt1 (Figure 2C) and MLL1 [38], this result indicates that the KFGG motif is a major determinant for DNA binding by CXXC domains.

To assess whether the CXXC domain is required for catalytic activity of Tet1 we generated a GFP-Tet1 fusion construct and a corresponding mutant lacking the CXXC domain (GFP-Tet1$^{\Delta CXXC}$). In C2C12 myoblasts GFP-Tet1 and GFP-Tet1$^{\Delta CXXC}$ showed punctuated nuclear patterns that did not depend on the cell cycle stage (Figure 3A and data not shown). The same constructs were transfected in HEK293T cells and global levels of genomic hmC were measured using a recently described hmC glucosylation assay [39]. Overexpression of GFP-Tet1 and GFP-Tet1$^{\Delta CXXC}$ determined a similar 5-fold increase of genomic hmC levels relative to control samples overexpressing GFP (Figure 3B), indicating that the CXXC domain is not required for enzymatic activity of Tet1 *in vivo*.

Deletion of the CXXC domain does not affect the activity of Dnmt1 *in vitro*

To explore the role of the CXXC domain in Dnmt1 function we generated GFP-Dnmt1 fusion constructs where the CXXC domain, as defined by our homology model, was deleted. We reasoned that precise deletion of the entire structure delimited by the antiparallel β-sheet (Figure 1D) would have the highest chances to preserve native folding of the rest of the protein. We introduced this deletion in GFP fusion constructs encoding either the full length Dnmt1 or the isolated N-terminal region (GFP-Dnmt1$^{\Delta CXXC}$ and GFP-NTR$^{\Delta CXXC}$, respectively; Figure 4A and Figure S4 in File S1). We then compared DNA binding properties, catalytic activity and interaction between N-terminal region and C-terminal catalytic domain of ΔCXXC and corresponding wild type constructs. Competitive DNA binding assays with the same set of substrates as used for the experiments with GFP-CXXC^{Dnmt1} and GFP-CXXC^{Tet1} reported above (Figure 2C) showed that both GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ bind DNA independently of the presence and methylation state of a CpG site (Figure 4B). As the isolated CXXC domain preferentially bound the substrate containing an unmethylated CpG site, the result with GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ indicates that the CXXC domain contributes negligibly to the DNA binding specificity of the full-length enzyme.

Several groups reported that interaction between the N-terminal region and the C-terminal catalytic domain of Dnmt1 leads to allosteric activation of Dnmt1 [16,18–20,40]. To test whether the CXXC domain is involved in this intramolecular interaction, we co-expressed either GFP-tagged N-terminal region (GFP-NTR) or GFP-NTR$^{\Delta CXXC}$ constructs with a Cherry- and His-tagged C-terminal domain (Ch-CTD-His) in HEK293T cells and performed co-immunoprecipitation experiments. Ch-CTD-His co-precipitated both GFP-NTR and GFP-NTR$^{\Delta CXXC}$, indicating that the CXXC domain is dispensable for the interaction between the N-terminal region and the C-terminal domain of Dnmt1 (Figure 4C).

To investigate whether the CXXC domain is needed for enzymatic activity or substrate recognition, we tested formation of the covalent complex with cytosine and transfer of the methyl group for GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$. We first employed an assay to monitor covalent complex formation that exploits the formation of an irreversible covalent bond between the enzyme and the mechanism-based inhibitor 5-aza-2-deoxycytosine (5-aza-dC). This results in permanent trapping of the enzyme by DNA substrates containing 5-aza-dC, as opposed to the reversible complex formed with substrates containing the natural substrate 2-deoxycytosine (dC) [36]. GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ were incubated with fluorescent DNA substrates containing either dC (binding) or 5-aza-dC (trapping) at a single CpG site in direct competition. DNA-protein complexes were then isolated by GFP pulldown and molar DNA/protein ratios were calculated from fluorescence measurements (Figure 4D). Covalent complex

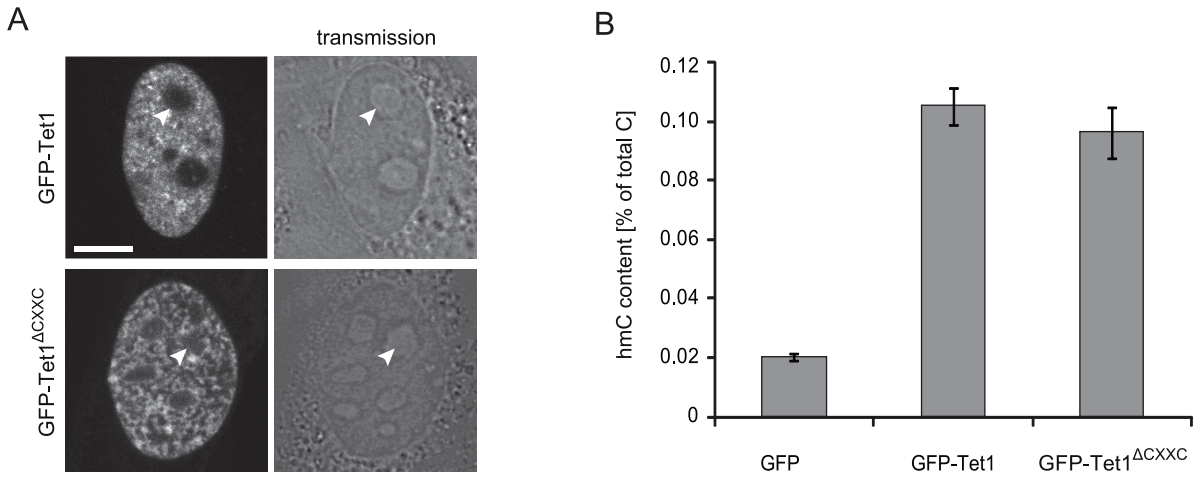

Figure 3. Cellular localization and *in vivo* catalytic activity of GFP-Tet1 and GFP-Tet1$^{\Delta CXXC}$. (A) Live images of C2C12 myoblasts expressing GFP-Tet1. Scale bar: 5 μm. (B) Genomic hmC content in HEK293T cells overexpressing GFP, GFP-Tet1 and GFP-Tet1$^{\Delta CXXC}$. Shown are mean values and standard deviation of hmC percentage over total cytosine for three measurements from one transfection.
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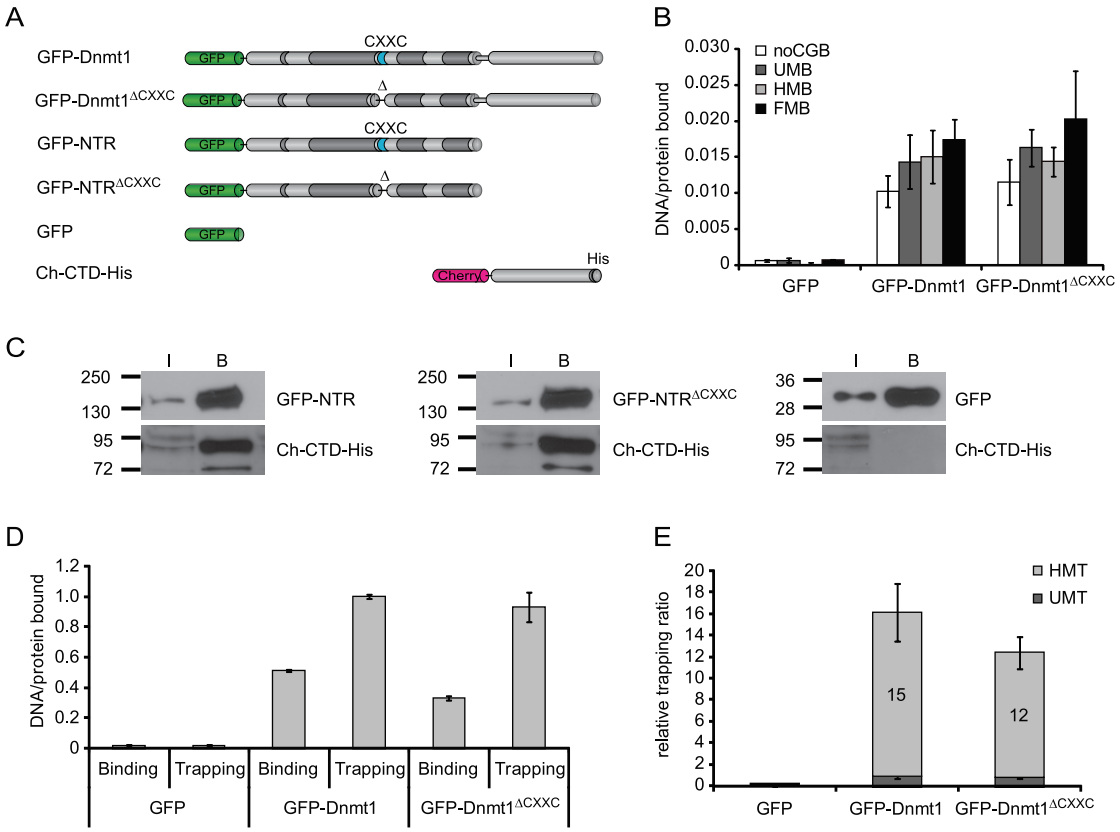

Figure 4. DNA binding specificity, intramolecular interaction and trapping of wild-type Dnmt1 and CXXC deletion constructs *in vitro*. (**A**) Schematic representation of Dnmt1 expression constructs. (**B**) DNA binding specificity of GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ were assayed as described in Figure 2C. (**C**) Co-immunoprecipitation of the C-terminal domain of Dnmt1 (Ch-CTD-His) and the N-terminal region with and without deletion of the CXXC domain (GFP-NTR and GFP-NTR$^{\Delta CXXC}$, respectively). GFP fusions were detected using an anti-GFP antibody, while the C-terminal domain construct was detected using an anti-His antibody. GFP was used as negative control. I = input, B = bound. (**D**) Comparison of binding and trapping activities for GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ to monitor irreversible covalent complex formation with hemimethylated substrates. (**E**) Relative covalent complex formation rate of GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ on substrates containing one un- (UMT) or hemi-methylated CpG site (HMT) in direct competition. The trapping ratio for GFP-Dnmt1 on unmethylated substrate was set to 1. In (D) and (E) the means and corresponding standard deviations of triplicate samples from three independent experiments are shown. GFP was used as negative control.
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formation was then estimated by comparing trapping and binding activities. GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ showed comparable covalent complex formation rates (trapping/binding ratios), which were about 15- and 12-fold higher for hemi- than unmethylated substrates, respectively (Figure 4E). Together with the data from binding experiments (Fig. 4B), this result indicates that the preference of Dnmt1 for hemimethylated substrates is determined at the covalent complex formation step rather than upon DNA binding. Furthermore, the CXXC domain clearly does not play a major role in determining either the efficiency or the methylation state-specificity of covalent complex formation.

Next, we tested whether deletion of the CXXC domain affects the ability of Dnmt1 to transfer [^{3}H]methyl groups from the donor S-adenosylmethionine (SAM) to a poly(dI·dC)-poly(dI·dC) substrate, a standard DNA methyltransferase activity assay. This showed that *in vitro* GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ are equally active methyltransferases (Figure S7 in File S1). This result is in contrast with a previous report showing that deletion of aa 647–690 in human DNMT1 encompassing the CXXC domain resulted in a drastic loss of catalytic activity [32]. However, according to our homology model the deletion by Pradhan *et al.* would eliminate the predicted N-terminal β-strand (β1 in Figure 1) preventing the formation of the antiparallel β-sheet and potentially distort the folding of the rest of the protein. This is in contrast with our GFP-Dnmt1$^{\Delta CXXC}$ mutant that was designed to retain the β-sheet structure. To test whether this may account for the observed discrepancy, we generated GFP fusion constructs of wild type human DNMT1 and the same deletion as reported by Pradhan *et al.* and tested covalent complex formation with 5-aza-dC containing DNA substrates as described above. While the human wild type construct showed the same preference for hemimethylated over unmethylated trapping substrates as the mouse constructs, this preference was clearly reduced for the human CXXC deletion mutant (Figure S8 in File S1). This result is consistent with the loss of enzymatic activity shown by Pradhan *et al.* for this mutant and together with the retention of trapping and catalytic activity by the different deletion in GFP-Dnmt1$^{\Delta CXXC}$ suggests that disruption of the antiparallel β-sheet delimiting the CXXC domain results in further distortion and loss of activity of the enzyme.

In conclusion, we showed that, *in vitro*, deletion of the CXXC domain does not affect the interaction between N-terminal region and C-terminal domain, DNA binding, the preference for hemimethylated substrates upon covalent complex formation and the methyltransferase activity of Dnmt1. Together, these data strongly argue against an involvement of the CXXC domain in allosteric activation of Dnmt1.

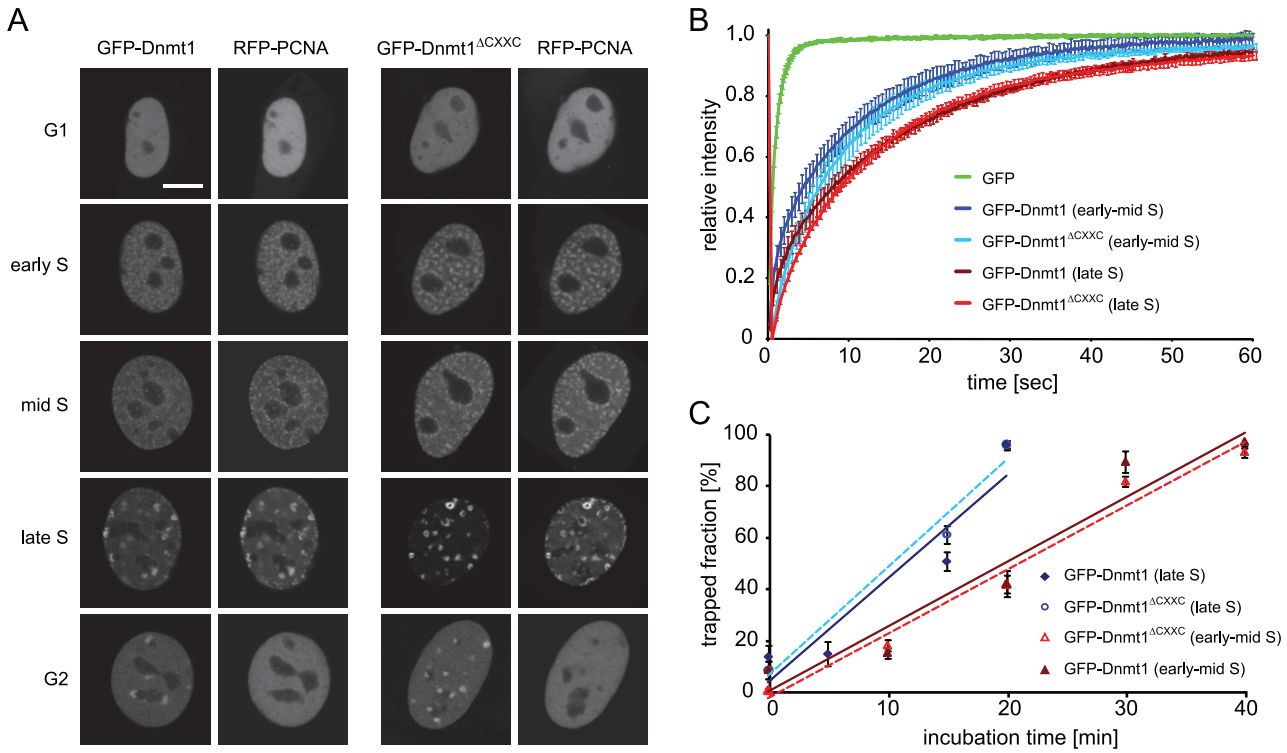

Figure 5. Cell cycle dependant cellular localization, protein mobility and trapping of wild-type Dnmt1 and CXXC deletion constructs in mouse C2C12 myoblasts. (**A**) Cell cycle dependent localization of GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ constructs. Scale bar: 5 μm. (**B**) Analysis of binding kinetics of GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ in early and late S-phase cells by FRAP. The recovery curve for GFP is shown for comparison. (**C**) *In vivo* trapping by FRAP analysis in cells treated with 5-aza-dC. The trapped enzyme fraction is plotted over time for early and late S-phase cells. For each construct three to six cells in early-mid and late S phase were analysed per time point. Shown are mean values ± SEM. In (A–C) RFP-PCNA was cotransfected to identify cell cycle stages in living cells.
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Deletion of the CXXC domain does not affect Dnmt1 activity *in vivo*

We then undertook a functional characterization of the GFP-Dnmt1$^{\Delta CXXC}$ construct *in vivo*. We first compared localization and binding kinetics of GFP-Dnmt1 or GFP-Dnmt1$^{\Delta CXXC}$ in mouse C2C12 myoblasts co-transfected with RFP-PCNA, which served as S-phase marker [41]. GFP-Dnmt1$^{\Delta CXXC}$ showed the same cell-cycle dependent nuclear localization pattern as previously shown for GFP-Dnmt1 and endogenous Dnmt1 (Figure 5A)[42,43]. Interaction with PCNA via the PBD directs Dnmt1 to replication foci throughout S-phase. In addition, in late S-phase and G2 Dnmt1 is enriched at chromocenters, clusters of pericentric heterochromatin (PH) that are observed as discrete domains densely stained by DNA dyes in mouse interphase cells. Association of Dnmt1 with PH at these stages is mediated by the TS domain [42]. Thus, the CXXC domain clearly does not contribute to the subnuclear localization of Dnmt1 at this level of resolution.

We also compared the mobility of GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ in living C2C12 myoblasts by FRAP analysis (Figure 5B). These experiments revealed that the kinetics of Dnmt1 is not significantly affected by deletion of the CXXC domain in early-mid as well as late S-phase.

To test covalent complex formation in living cells, we used a previously established trapping assay [44]. Mouse C2C12 myoblasts were co-transfected with RFP-PCNA and either GFP-Dnmt1 or GFP-Dnmt1$^{\Delta CXXC}$ and treated with 5-aza-dC. Immobilization of the Dnmt1 constructs at the site of action was then measured by FRAP analysis (Figure 5C). GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ showed very similar trapping kinetics, the immobile enzyme fraction reaching nearly 100% after 20 and 40 minutes in early-mid and late S-phase, respectively. This result clearly shows that the CXXC domain is dispensable for covalent complex formation also *in vivo*.

Finally, we compared the ability of GFP-Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$ to restore DNA methylation patterns in mouse *dnmt1*$^{-/-}$ ESCs. Cells transiently expressing either GFP-Dnmt1 or GFP-Dnmt1$^{\Delta CXXC}$ were FACS sorted 48 h after transfection. Isolated genomic DNA was then bisulfite treated and fragments corresponding to major satellite repeats, intracisternal type A particle (IAP) interspersed repeats, *skeletal α-actin* and *H19a* promoters were amplified and subjected to pyrosequencing (Figure 6). As shown previously [43], under these conditions GFP-Dnmt1 partially restored methylation of major satellite and IAP repeats and the *skeletal α-actin* promoter, but not of the imprinted *H19a* promoter, as establishment of the methylation imprint requires passage through the germ line [45]. Methylation patterns of all these sequences in cells expressing GFP-Dnmt1$^{\Delta CXXC}$ were very similar to those in GFP-Dnmt1 expressing cells, including the lack of (re-) methylation at the *H19a* promoter. These results suggest that the CXXC domain is not required for maintenance of DNA methylation patterns by Dnmt1 and does not restrain the DNA methyltransferase activity of Dnmt1 on unmethylated CpG sites. Thus, the CXXC domain does not play a major role in subcellular localization, it does not contribute to the global binding kinetics of Dnmt1 and, consistent with the *in vitro* data reported above, is dispensable for maintaining DNA methylation patterns in living cells.

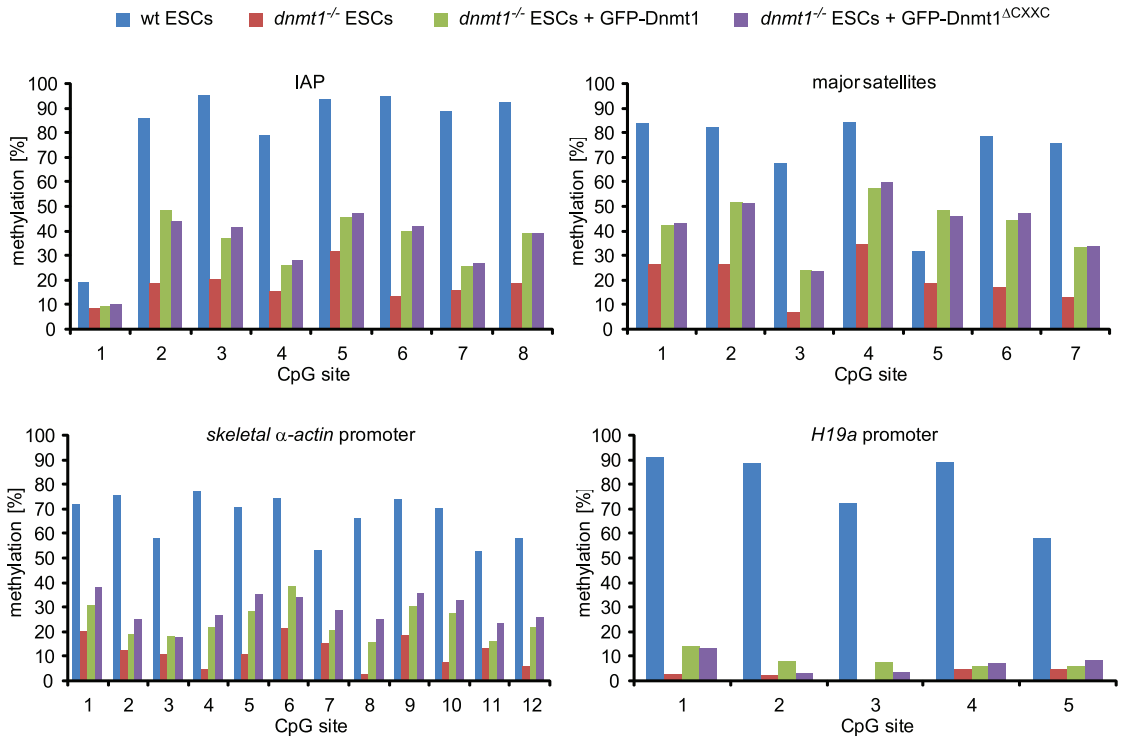

Figure 6. The CXXC deletion construct of Dnmt1 restores methylation in *dnmt1* null cells. Mouse *dnmt1*$^{-/-}$ ESCs transiently expressing GFP-Dnmt1 or GFP-Dnmt1$^{\Delta CXXC}$ were isolated by FACS-sorting 48 h after transfection and CpG methylation levels within the indicated sequences were analyzed by bisulfite treatment, PCR amplification and direct pyrosequencing. Methylation levels of untransfected wild type and *dnmt1*$^{-/-}$ ESCs are shown for comparison.
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Discussion

We generated homology models based on the reported structure of the MLL1 CXXC domain to design isolated CXXC domain constructs and CXXC domain deletion mutants for Dnmt1 and Tet1 with minimal probability of structural alteration. According to these models CXXC domains are delimited by an antiparallel β-sheet, a discrete structural element. Our data show that the CXXC domain of mouse Dnmt1 preferentially binds DNA substrates containing unmethylated CpG sites as previously shown for CXXC domains of human DNMT1 and other mammalian proteins. We note that sequences C-terminal to the corresponding peptide in CGBP/Cfp1 were reported to be required for DNA binding *in vitro* [22] and that only a significantly larger peptide spanning the CXXC-3 domain of Mbd1a was tested for DNA binding. However, sequences C-terminal to CXXC domains are not conserved (Figure 1B) and our data show that they are not required for DNA binding by the CXXC domain of Dnmt1. Nevertheless, all the CXXC domains reported to selectively bind unmethylated CpG sites cluster in a distinct homology group and contain the KFGG motif. The latter was shown to be crucial for DNA binding by the CXXC domain of MLL1 [38] and here we extend this observation to the CXXC domain of Dnmt1. Sequence alignment reveals two distinct CXXC domain homology groups that lack the KFGG motif (Figure 1A). Consistent with a role of this motif in DNA binding, members of these groups such as CXXC-1/2 of Mbd1 [29] and the CXXC domain of Tet1 (this study) show no DNA binding activity. While no specific function is known for CXXC-1/2 of Mbd1, the CXXC domain of Tet1 is closely related to those in CXXC4/Idax and CXXC5/RINF that were shown to mediate protein-protein interactions [46–48]. This suggests that the CXXC domain of Tet1, rather than mediating DNA binding, may function as a protein-protein interaction domain. However, our data do not rule out the possibility that the DNA binding properties of the CXXC domain within the context of full length Tet1 may be different from those of the isolated domain. Nevertheless, we show that the CXXC domain is not required for enzymatic activity of Tet1 *in vivo*.

Although we observed a clear DNA binding activity by the isolated CXXC domain of Dnmt1, we found that, within the context of the full length enzyme, this domain is dispensable for overall DNA binding properties, preference for hemimethylated substrates upon covalent complex formation, methyltransferase activity and allosteric activation as well as for the ability to restore methylation of representative sequences in *dnmt1* null ESCs. Consistent with our data, a recent report showed a preference of the CXXC domain of human DNMT1 for substrates containing unmethylated CpG sites [32]. However, the same report showed that deletion of the CXXC domain from the human enzyme results in a significant decrease in methyltransferase activity on hemimethylated substrates *in vitro* and 25% lower methylation at rDNA repeats upon overexpression in HEK293 cells, suggesting a dominant negative effect of the deletion construct. These discrepancies may be due to the fact that the fragment deleted by Pradhan *et al.* includes the N-terminal strand of the predicted antiparallel β-sheet, potentially leading to disruption of native folding, to species-specific differences and/or to the analysis of non-physiological expression levels in HEK293 cells. In our trapping assay the same human deletion mutant showed reduced covalent complex formation, consistent with loss of enzymatic activity. The report from Pradhan *et al.* also showed that mutation of cysteine 667 to glycine within the CXXC domain of human

DNMT1 disrupts DNA binding and enzymatic activity. However, as this point mutation involves one of the zinc coordinating residues it is not unlikely to alter peptide folding with negative consequences potentially extending beyond the CXXC domain and including reduced enzymatic activity. In this respect the dominant negative effect observed upon overexpression of this mutant may be explained by the prevalent occurrence of Dnmt1 as a dimer [49]. These observations, together with preserved ability for covalent complex formation and catalytic activity of our CXXC domain deletion, support the validity of our homology model-driven approach for functional characterization of the CXXC domain. In addition, our genetic complementation approach constitutes a rather physiologic functional assay. However, due to the transient approach and the analysis of genomic methylation at only a few representative sequences, subtle or highly sequence specific effects of deletion of the CXXC domain cannot be excluded.

It was recently shown that binding of Cfp1/CGBP and KDM2A to CpG islands through their CXXC domains leads to local enrichment and depletion of H3K4 and H3K36 methylation, respectively [26,30]. Analogously, Dnmt1 may bind CpG islands through its CXXC domain. However, this interaction would not lead to a straightforward functional interpretation as CpG islands with high CpG density are generally refractive to DNA methylation and a function of Dnmt1 as a *de novo* DNA methyltransferase is not well established. It could be envisaged that binding to unmethylated CpG sites/islands by the CXXC domain may have a negative effect on the enzymatic activity of Dnmt1 and restrain its function as a *de novo* DNA methyltransferase. However, we show that in *dnmt1* null ESCs methylation of the imprinted *H19a* promoter is not restored upon expression of either wild type or ΔCXXC Dnmt1 constructs, arguing against a negative regulatory function of the CXXC domain.

Notably, binding of unmethylated CpG sites by KFGG motif-containing CXXC domains does not exclude a role in protein-protein interaction as the CXXC domain of MLL1 was reported to interact with both DNA and Polycomb Repressive Complex 1 components HPC2/CBX4 and BMI-1 [21,50]. Therefore, it is possible that the CXXC domain of Dnmt1 has regulatory functions in specific cell types or developmental stages that may involve DNA binding and/or interaction with other proteins. The generation of dedicated animal models may be instrumental for testing these possibilities.

Materials and Methods

Bioinformatic methods

Alignments were performed using the ClustalW2 software [51]. The CXXC domain homology tree (Figure 1C) was generated from the alignment in Figure 1B with Jalview 2.4 by unweighted pair group method with arithmetic mean (UPGMA). The neighbor-joining method gave the same result. Average distances between the sequences were calculated using the BLOSSUM62 matrix. The human CXXC10 coding sequence [52] was determined by assembling ESTs AI438961, BX114363, BX492895, BU633058.1, AW207644.1 and the genomic sequence AC073046.7. The putative translational start site is located 16308 bp upstream of the annotated transcriptional start site of *TET3*. A partial coding sequence of murine Cxxc10 containing the CXXC domain was identified by aligning the human CXXC10 protein sequence to the ORFs present in NT_039353.7 upstream of the *tet3* gene from position 35663306 to 35808487). A very high match was found 13266 nt upstream of *tet3* at positions 35676374-35676572 of NT_039353.7. To build homology models for the CXXC domains of Dnmt1 (aa 645–696) and Tet1 (aa 561–614), we submitted the respective sequences to the HHpred server [53]. The best template was the CXXC domain of MLL1 (PDB-ID: 2J2S). The 49 residues of the CXXC domain in Dnmt1 can be aligned to this domain with 45% sequence identity and only a single amino acid gap after residue 661 (Figure 1B). 3D models were calculated with the homology modeling software MODELLER [54] (version 9.5) using this alignment. Distance restraints were given to MODELLER to enforce a distance of 2.3±0.1 Å between the eight sulphurs in the Zn-coordinating cysteines and the Zn^{2+} ions. TM-align [55] was used to superpose the model structure with the template domain. Images were generated using the PyMol Molecular Graphics System (Version 1.3, Schrödinger, LLC). The quality of the models and the underlying alignments were checked with DOPE [56] and Verify3D [57] and results for both models were found to be comparable to the MLL1 template structure (2J2S).

Expression constructs

Fusion constructs were generated using enhanced green fluorescent protein, monomeric red fluorescent protein or monomeric cherry and are here referred to as GFP, RFP and Cherry fusions, respectively. Mammalian expression constructs for GFP, mouse GFP-Dnmt1, GFP-NTR and human RFP-PCNA were described previously [42,44,49,58]. The deletion construct GFP-Dnmt1$^{\Delta CXXC}$ was obtained by replacing the sequence coding for aa 655–696 with three alanine codons in the GFP-Dnmt1 construct as described [59]. The GFP-DNMT1$^{\Delta CXXC}$ construct was generated by subcloning the sequence coding for human DNMT1$^{\Delta CXXC}$ from the homonymous construct by Pradhan *et al.* [32] in the pEGFP-C2 vector (Clonetech). To generate GFP-Tet1 three partially overlapping fragments spanning the Tet1 coding sequence were amplified using E14 ESCs cDNA as template. The fragments were then joined by overlap extension PCR and inserted into the pCAG-GFP-IB vector [43]. To generate GFP-Tet1$^{\Delta CXXC}$ aa 569-621 of murine Tet1 were deleted from GFP-Tet1 using a type IIs restriction endonuclease approach as described [60]. To generate GFP-CXXC^{Dnmt1} and GFP-CXXC^{Tet1} sequences coding for the respective CXXC domains (aa 643-700 for Dnmt1 and 561-614 for Tet1) were amplified by PCR using the GFP-Dnmt1 expression construct and cDNA from E14 ESCs as templates, respectively. PCR fragments were then inserted into the pCAG-GFP-IB vector. GFP-NTR$^{\Delta CXXC}$ was obtained by replacing the BglII-XhoI fragment of GFP-NTR with the same fragment of GFP-Dnmt1$^{\Delta CXXC}$. Ch-CTD-His was generated by replacing the GFP coding sequence in a GFP-CTD construct [49] with the Cherry coding sequence. All constructs were confirmed by sequencing.

Cell culture, transfection and cell sorting

HEK293T cells [61] and mouse C2C12 myoblasts [62] were cultured in DMEM supplemented with 50 μg/ml gentamicin and 10% and 20% fetal calf serum, respectively. For expression of fusion proteins HEK293T cells were transfected with polyethylenimine (Sigma). For live cell imaging, C2C12 cells were grown to 40% confluence on Lab-Tek chambers (Nunc) or μ-slides (Ibidi) and transfected with TransFectin transfection reagent (BioRad) according to the manufacturer's instructions. Mouse ESCs were cultured as described [63] and transfected with FuGENE HD (Roche) according to the manufacturer's instructions. ESCs were sorted with a FACS Aria II instrument (Becton Dickinson). The *dnmt1*$^{-/-}$ J1 ESCs used in this study are homozygous for the c allele [14].

In vitro DNA binding and trapping assays

In vitro DNA binding and trapping assays were performed as described previously [36,37] with the following modifications. DNA substrates labeled with four different ATTO fluorophores (Tables S1 and S2 in File S1) were used at a final concentration of 125 nM each in the pull-down assay with immobilized GFP fusions. After removal of unbound substrate, the amounts of protein and DNA were determined by fluorescence intensity measurements with a Tecan Infinite M1000 plate reader using calibration curves from purified GFP or DNA coupled ATTO fluorophores, respectively. The following excitation/emission ± detection bandwidth settings were used: 490/511±10 nm for GFP, 550/580±15 nm for ATTO550, 600/630±15 nm for ATTO590, 650/670±10 nm for ATTO647N and 700/720±10 nm for ATTO700. Cross detection of GFP and different ATTO dyes was negligible with these settings. Binding and trapping ratios were calculated dividing the concentration of bound DNA substrate by the concentration of GFP fusion on the beads.

In vivo mC hydroxylation assay

Genomic DNA was isolated from HEK293T cells 24 h after transfection with the GFP-Tet1 and GFP-Tet1$^{\Delta CXXC}$ constructs and global hmC levels were measured using the *in vitro* glucosylation assay as previously described [63], except that 100 nM β-glucosyltransferase and only UDP-[^{3}H]glucose donor (0.43 μM) were used.

Co-immunoprecipitation

Co-immunoprecipitation was performed as described previously [49,64]. Shortly, HEK293T cells were transiently co-transfected with expression plasmids for GFP fusions and the Ch-CTD-His construct, harvested and lysed. GFP fusions were pulled down using the GFP-Trap [65] (Chromotek) and subjected to western blotting using anti-GFP (Roche or Chromotek) and anti-His (Invitrogen) monoclonal antibodies.

Live cell microscopy, FRAP analysis and live cell trapping assay

Live cell imaging and FRAP experiments were performed as described previously [43]. For each construct 6-15 nuclei were averaged and the mean values as well as the standard errors were calculated. For presentation, we used linear contrast enhancement on entire images. The DNA methyltransferase trapping assay was described previously [44]. Briefly, transfected cells were incubated with 30 μM 5-aza-dC (Sigma) for the indicated periods of time before photobleaching experiments. FRAP analysis was performed with a confocal laser scanning microscope (TCS SP5, Leica) equipped with a 63×/1.4 NA Plan-Apochromat oil immersion objective. Microscope settings were as described except that a smaller region of interest (3 μm×3 μm) was selected for photobleaching. Mean fluorescence intensities of the bleached region were corrected for background and for total loss of nuclear fluorescence over the time course, and normalized by the mean of the last 10 prebleach values.

DNA Methylation Analysis

Genomic DNA was isolated with the QIAmp DNA Mini Kit (Qiagen) and 1.5 μg were bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo research) according to the manufacturer's instructions. Primer sets and PCR conditions for IAP-LTR, *skeletal α-actin* and *H19* promoters were as described [43]. Primer sequences for major satellites were AAAATGAGAAA-CATCCACTTG (forward primer) and CCATGATTTT-CAGTTTTCTT (reverse primer). For amplification we used Qiagen Hot Start Polymerase in 1x Qiagen Hot Start Polymerase buffer supplemented with 0.2 mM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer, 1.3 mM betaine (Sigma) and 60 mM tetramethylammonium-chloride (TMAC, Sigma). Promoter regions and IAP-LTR were amplified with two subsequent (nested) PCR reactions and major satellite repeats were amplified with a single amplification reaction. Pyrosequencing reactions were carried out by Varionostic GmbH (Ulm, Germany). Pyrosequencing primers are listed in Table S3 in File S1.

Supporting Information

File S1 Tables S1–S3, Figures S1–S8 and Supplemental methods.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: FS HL. Performed the experiments: CF AR SB DM KF SH MW WQ. Analyzed the data: CF AR SB DM KF SH JS. Contributed reagents/materials/analysis tools: JS. Wrote the paper: FS HL. Generated homology models: JS.

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Different binding properties and function of CXXC zinc finger domains in Dnmt1 and Tet1

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SUPPLEMENTAL INFORMATION FILE

Tables S1-3

Figures S1-8

Supplemental Methods

Table S1. Sequences of DNA oligonucleotides used for preparation of double stranded DNA substrates. M: 5-methylcytosine.

Name	Sequence
CG-up	5'- CTCAACAACTAACTACCATCCGGACCAGAAGAGTCATCATGG -3'
MG-up	5'- CTCAACAACTAACTACCATCMGGACCAGAAGAGTCATCATGG -3'
noCG-up	5'- CTCAACAACTAACTACCATCTGGACCAGAAGAGTCATCATGG -3'
Fill-In-550	5'- ATTO550-CCATGATGACTCTTCTGGTC -3'
Fill-In-590	5'- ATTO590-CCATGATGACTCTTCTGGTC -3'
Fill-In-647N	5'- ATTO647N-CCATGATGACTCTTCTGGTC -3'
Fill-In-700	5'- ATTO700-CCATGATGACTCTTCTGGTC -3'

Table S2. DNA substrates used for the *in vitro* DNA binding and trapping assays.

Name	CpG site	Label	Oligo I	Oligo II	dCTP reaction	Purpose
noCGB 700	no CpG site	700	noCG-up	Fill-In-700	dCTP	Binding
UMB 550	unmethylated	550	CG-up	Fill-In-550	dCTP	Binding
UMB 590		590		Fill-In-590		
UMB 647N		647N		Fill-In-647N		
UMB 700		700		Fill-In-700		
UMT 550		550		Fill-In-550	5-aza-dCTP	Trapping
HMB 590	hemimethylated	590	MG-up	Fill-In-590	dCTP	Binding
HMB 647N		647N		Fill-In-647N		
HMT 550		550		Fill-In-550	5-aza-dCTP	Trapping
HMT 647N		647N		Fill-In-647N		
FMB 647N	fully methylated	647N	MG-up	Fill-In-647N	5methyl dCTP	Binding

Table S3. Primers used for pyrosequencing. Each primer is biotinylated at the 5' end.

Name	Sequence
skeletal α-actin-1	5'- AGTTGGGGATATTTTTTATA -3'
skeletal α-actin-1b	5'- TTTTGGTTAGTGTAGGAGAT -3'
skeletal α-actin-2	5'- TGGGAAGGGTAGTAATATTT -3'
H19-1	5'- ATAGTTATTGTTTATAGTTT -3'
H19-2	5'- AGGAATATGTTATATTTAT -3'
IAP LTR-1	5'- CCCTAATTAACTACAACCCA -3'
IAP LTR-2	5'- TGTAGTTAATTAGGGAGTGA -3'
Major Satellite-1	5'- AAAATGAGAAATATTTATTTG -3'
Major Satellite-2	5'- GAGAAATATATACTTTAGGA -3'

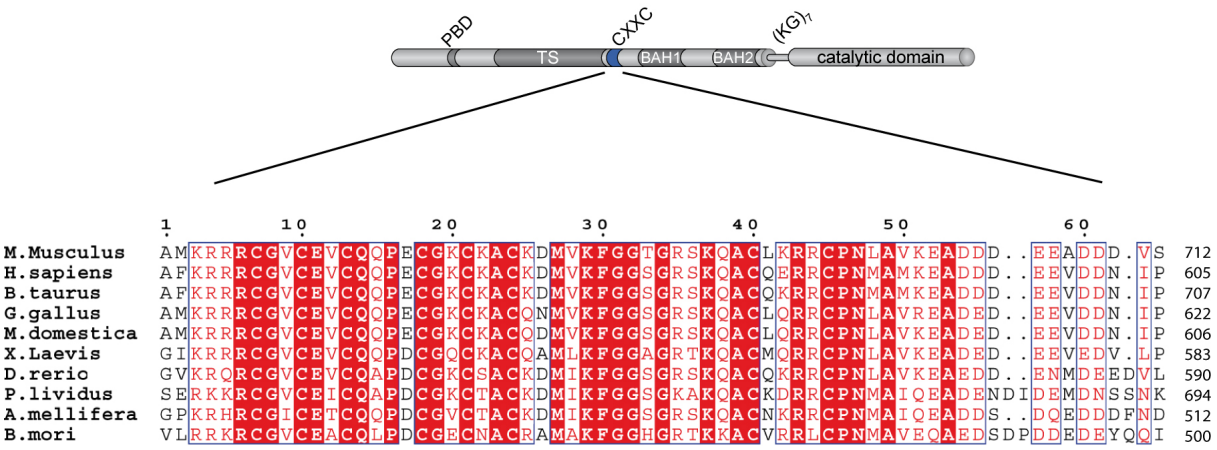

Figure S1. Dnmt1 domain structure and alignment of Dnmt1 CXXC domains from different species. Numbers on the right side indicate the position of the last amino acid in each sequence. PBD: PCNA binding domain; TS: targeting sequence; CXXC: CXXC-type zinc finger domain; BAH1 and 2: bromo-adjacent homology domain; (KG)7: seven lysine-glycine repeats. Absolutely conserved residues are highlighted in red. Positions with residues in red face share 70% similarity as calculated with the Risler algorithm {Mohseni-Zadeh, 2004 #133}. The alignment was generated with ClustalW2 and displayed with ESPript 2.2. GenBank accession numbers are: *Mus musculus*: NP_034196; *Homo sapiens*: NP_001124295; *Bos taurus*: NP_872592; *Monodelphis domestica*: NP_001028141; *Gallus gallus*: NP_996835*;* *Xenopus laevis*: NP_001084021; *Danio rerio*: NP_571264; *Paracentrotus lividus*: Q27746 (Swiss Prot); *Apis mellifera*: NP_001164522 (Dnmt1a); *Bombyx mori*: NP_001036980.

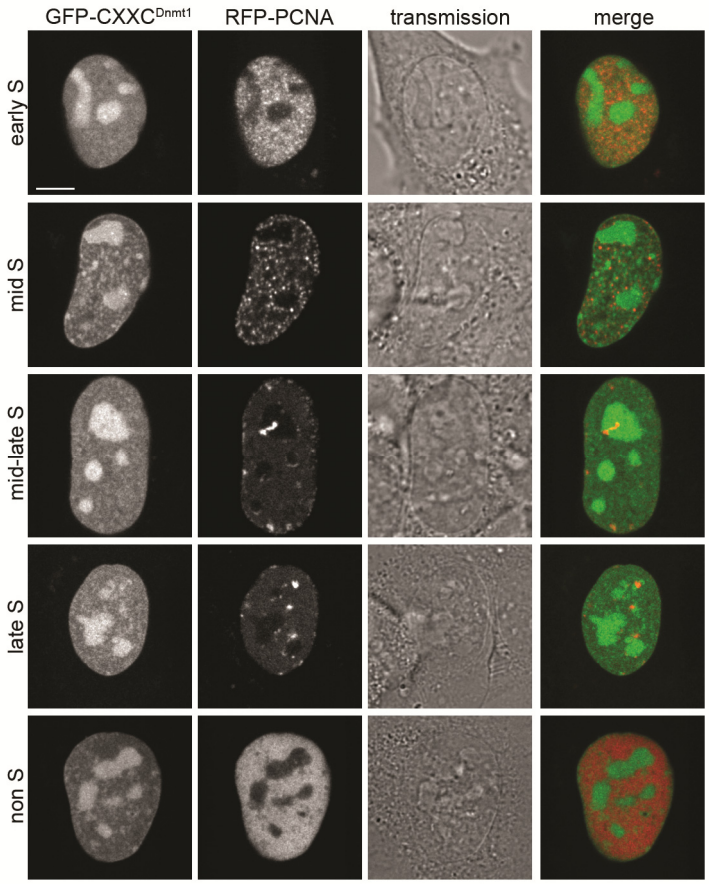

Figure S2. The cellular localization of GFP-CXXC^{Dnmt1} is independent of cell cycle stage. Live images of C2C12 mouse myoblasts cotransfected with expression constructs for GFP-CXXC^{Dnmt1} and RFP PCNA. The latter served for identification of the cell cycle stage. Scale bar: 5 µm.

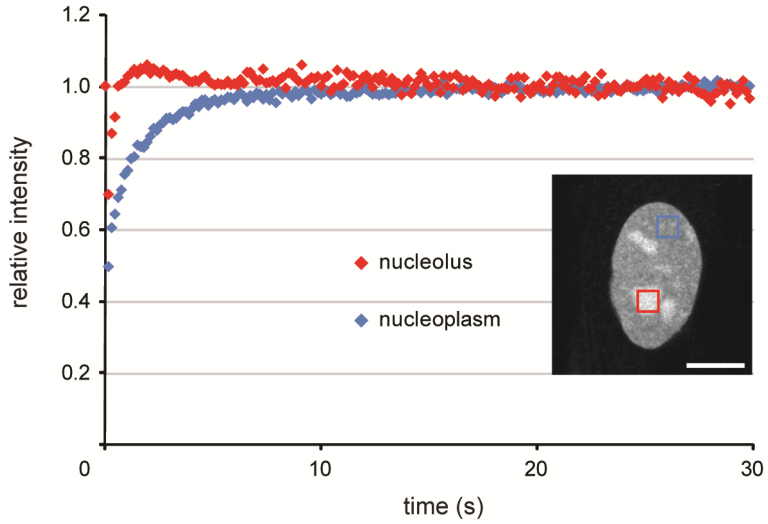

Figure S3. Differential mobility of GFP-CXXC^{Dnmt1} in nucleoli and nucleoplasm of mouse C2C12 myoblasts measured by FRAP analysis. Identical regions of interest over the nucleoplasm or nucleoli (as exemplified in the inset) were bleached and recovery curves were recorded over 30 seconds. GFP-CXXC^{Dnmt1} kinetics is faster in nucleoli than in the nucleus, which indicates more transient (possibly unspecific) binding in the former than in the latter. Scale bar: 5 µm.

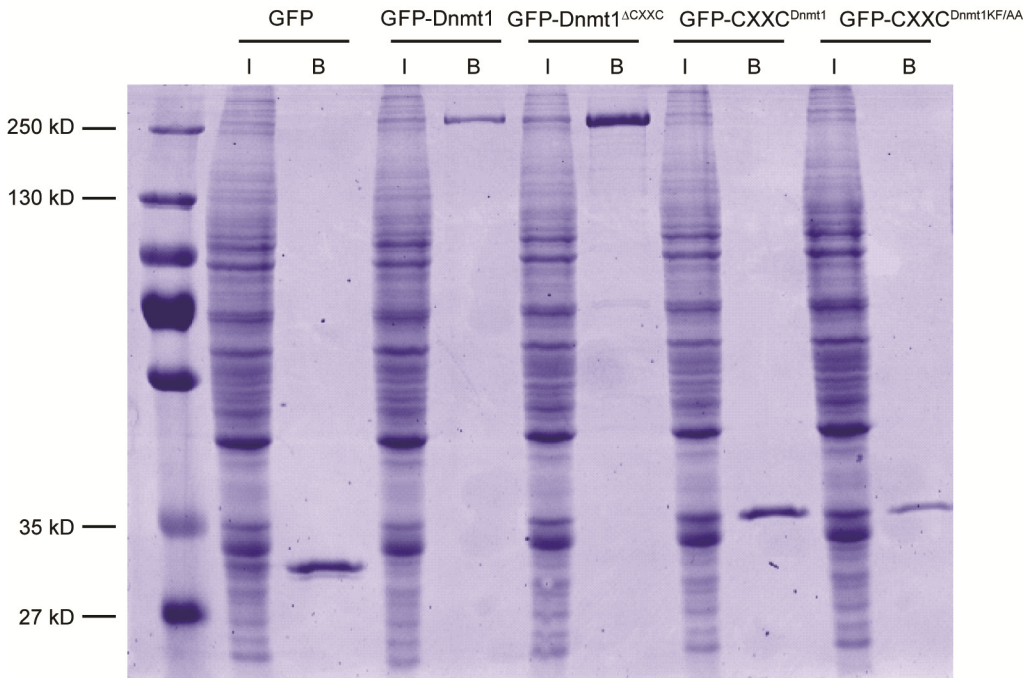

Figure S4. GFP fusion pulldowns from transiently transfected HEK293T cells using the GFP-trap. Shown is a SDS polyacrylamide gel stained with coomassie blue. I = input (1%); B = bound (10%).

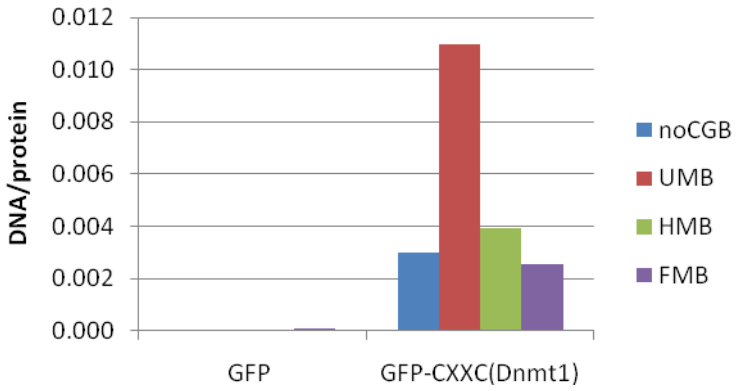

Figure S5. The CXXC domain of Dnmt1 preferentially binds unmethylated CpG sites. GFP and GFP-CXXC^{Dnmt1} purified from transiently transfected HEK293T cells with the GFP trap were challenged with fluorescent DNA substrates containing no CpG site or one central un-, hemi- or fully methylated CpG site in direct competition (noCGB, UMB, HMB and FMB, respectively) as in Figure 2C, except that a five-fold higher concentration (625 nM) of each substrate was used.

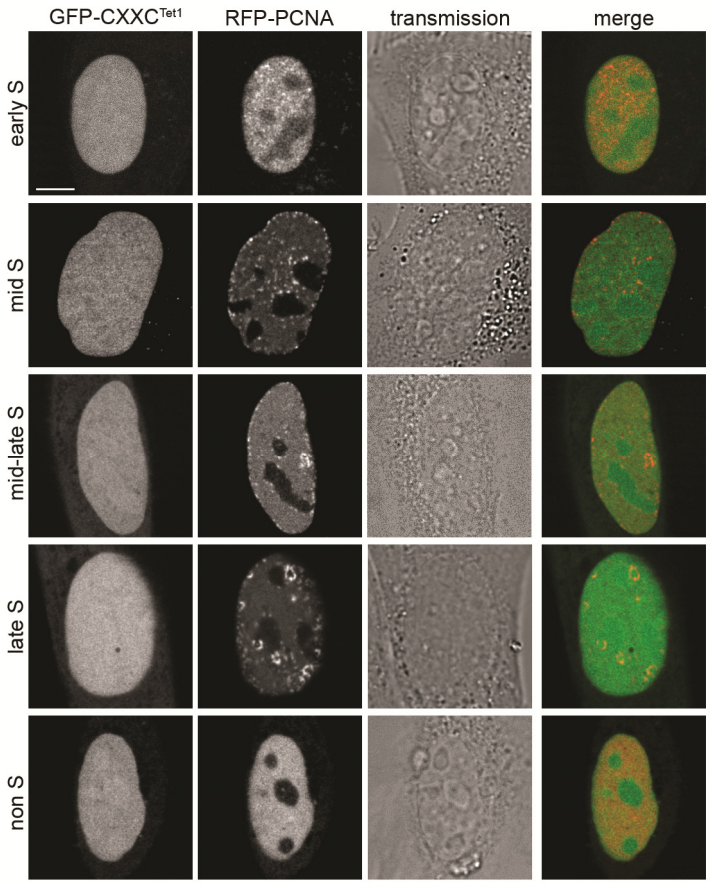

Figure S6. The cellular localization of GFP-CXXC^{Tet1} is independent of cell cycle stage. Live images of C2C12 mouse myoblasts cotransfected with expression constructs for GFP-CXXC^{Tet1} and RFP PCNA. The latter served for identification of the cell cycle stage. Scale bar: 5 µm.

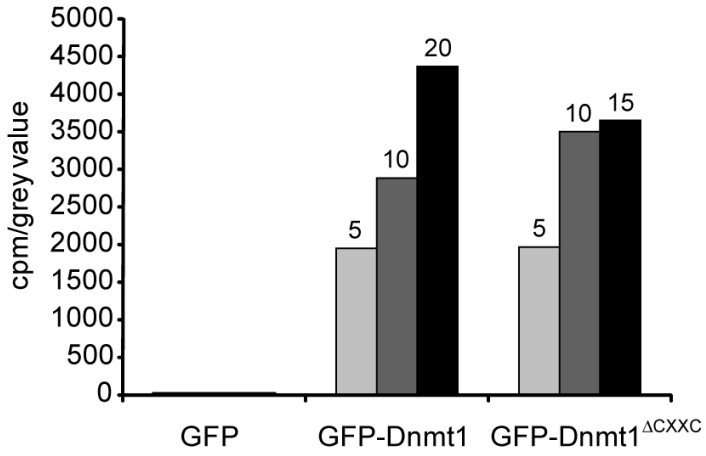

Figure S7. Radioactive methyltransferase activity assay for GFP Dnmt1 and GFP-Dnmt1$^{\Delta CXXC}$. The transfer of [3H]-methyl groups to poly(dI•dC)-poly(dI•dC) substrate was measured for increasing volumes of GFP fusion proteins immunopurified from transiently transfected HEK293T cells. Counts per minute (cpm) were normalized to the relative protein concentration as determined by SDS-PAGE analysis. GFP was used as negative control. Numbers above the bars indicate the volume (µl) of protein solution added.

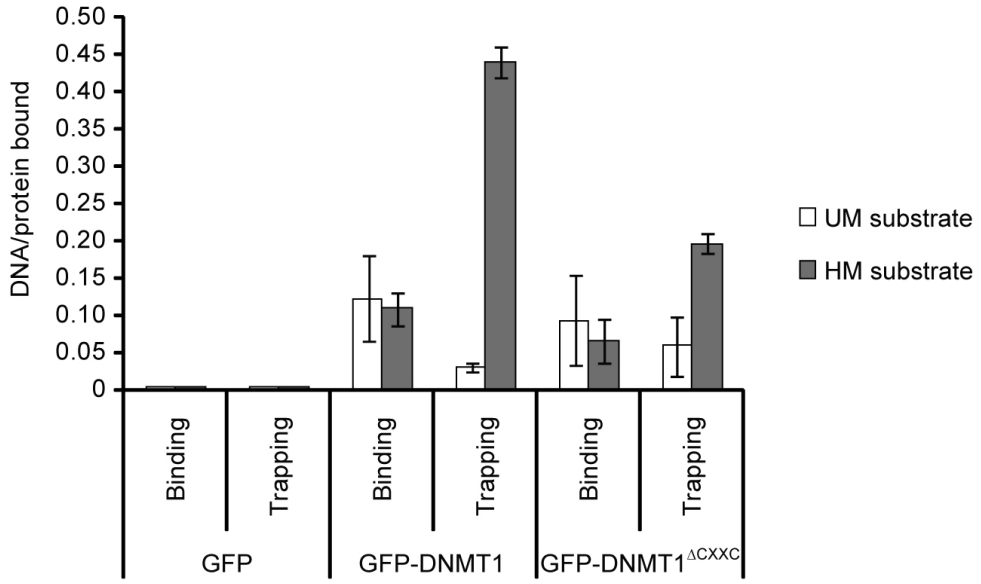

Figure S8. Competitive DNA binding and trapping assays for human GFP-DNMT1 and GFP-DNMT1$^{\Delta CXXC}$. GFP, GFP-DNMT1 and GFP-DNMT1$^{\Delta CXXC}$ were purified from transfected HEK293T cells using the GFP-trap and incubated with fluorescent DNA substrates containing one central unmethylated (UM) or hemimethylated (HM) CpG site in direct competition. Both substrates contained either dC (binding) or 5 aza dC (trapping) on the strand opposite to the differentially methylated one. The comparison of binding and trapping ratios reflects irreversible covalent complex formation. Note the reduction in trapping of GFP-DNMT1$^{\Delta CXXC}$ relative to GFP-DNMT1 by the hemimethylated substrate. Shown are mean values and standard deviation of DNA/protein ratios from two independent experiments.

SUPPLEMENTARY METHODS

In vitro methyltransferase activity assay

Eight milligrams of His-tagged GFP-binding protein (GBP; Chromotek) were coupled to 1ml Ni-NTA agarose beads (Qiagen) by incubating for 2 h at 4°C in PBS and unbound protein was washed out twice with PBS. Extracts of HEK293T cells expressing GFP or a GFP fusions were prepared in 200 µl lysis buffer II (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.5 % Tween-20, 2 mM $MgCl_2$, 1 mg/ml DNaseI, 2 mM PMSF, 1X mammalian protease inhibitor mix). After centrifugation, supernatants were diluted to 500 µl with immunoprecipitation buffer II (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.05 % Tween-20) and precleared by incubation with 25 µl of equilibrated Ni-NTA agarose beads for 30 min at 4°C followed by centrifugation. Precleared extracts were then incubated with 40 µg of His-tagged GFP-trap coupled to Ni-NTA beads for 2 h at 4°C with constant mixing. GFP or GFP fusions were pulled down by centrifugation at 540 *g*. After washing twice with wash buffer II (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.05 % Tween-20), complexes were eluted with 60 µl of elution buffer (10 mM Tris pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 250 mM imidazole) for 10 min at 25°C with constant mixing. 10 µl aliquots of all eluates were subjected to western blot analysis using mouse or rat monoclonal antibodies to GFP (Roche and Chromotek, respectively) and quantified by densitometry. Indicated volumes of eluate were incubated with 1 µg of poly(dI·dC)-poly(dI·dC) substrate (Sigma), 0.5 µg/µl of BSA and 1 µCi of S-adenosyl-[^{3}H-methyl]-methionine in 50 µl of trapping buffer (10 mM Tris pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM DTT) for 60 min at 37°C. 15 µl of each sample were spotted onto blotting paper and the DNA was precipitated with ice cold 5 % TCA. After washing twice with 5% TCA and once with cold 70 % ethanol, paper filters were air dried and analyzed by scintillation in 4 ml scintillation cocktail (Rotiszint® eco plus, Roth) for 5 min.

2.3. UHRF1 PLAYS MULTIPLE ROLES IN THE REGULATION OF DNA METHYLTRANSFERASE 1

Uhrf1 plays multiple roles in the regulation of DNA methyltransferase 1

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Key words: Uhrf1; ubiquitination; DNA methylation; Dnmt1

Abstract

In mammals Dnmt1 is the major DNA methyltransferase responsible for maintaining genomic methylation patterns after DNA replication. Uhrf1, a crucial cofactor of Dnmt1, was suggested to recruit Dnmt1 to hemimethylated CpG sites. In this study, we show that the interaction of Dnmt1 with Uhrf1 is essential but not sufficient for maintenance of DNA methylation and that the PHD and Ring domains of Uhrf1 are involved in Dnmt1 recruitment. A deletion mutant of Dnmt1 lacking amino acids (aa 459-501) essential for the interaction with Uhrf1 is enzymatically active *in vitro*. Interestingly, this mutant is unable to methylate naked hemimethylated DNA when cell extracts were added to the methylation reaction, suggesting that activation and inhibition of Dnmt1 occur *in vivo*. Uhrf1 did inhibit maintenance of DNA methylation by competitively binding hemimethylated CpG sites *in vitro*. These results suggest that Uhrf1 plays multiple roles in the maintenance of DNA methylation. We propose that *in vivo* maintenance of DNA methylation by Dnmt1 might involve three steps: recruitment, access and enzymatic activation.

Introduction

Vertebrate genomes are modified by DNA methylation at the C5 position of cytosine residues, mostly within cytosine-guanine dinucleotides (CpG). DNA methylation is involved with imprinting, X-chromosome inactivation, stable transcriptional repression, genome stability and tumorigenesis (Bird, 2002). Establishment of DNA methylation patterns is mediated by *de novo* methyltransferases Dnmt3a and Dnmt3b during gametogenesis and early development, and is maintained by maintenance methyltransferase Dnmt1 after DNA replication.

Dnmt1 is a relatively large protein comprising a N-terminal regulatory region, which covers two thirds of the molecule, and a C-terminal catalytic domain which contains all essential motifs of active C5 DNA methyltransferases. The N-terminal domain mediates the subcellular distribution of Dnmt1 during the cell cycle and controls enzymatic activity. Dnmt1 associates with proliferating cell nuclear antigen (PCNA) at replication sites via PCNA binding domain (PBD; Chuang et al., 1997; Leonhardt et al., 1992). The association of Dnmt1 with the replication machinery enhances methylation efficiency, but is not strictly required for postreplicative maintenance of DNA methylation (Schermelleh et al., 2007; Spada et al., 2007). A targeting sequence (TS domain) mediates Dnmt1 dimerization (Fellinger et al., 2009) and targets Dnmt1 to pericentric heterochromatin (Easwaran et al., 2004). A recent crystal structure shows that the TS domain inserts into the DNA binding pocket of the catalytic domain, suggesting a role in autoinhibition in the regulation of Dnmt1 activity (Syeda et al., 2011; Takeshita et al., 2011). The CXXC-type zinc finger domain of Dnmt1 preferentially binds to unmethylated DNA (Frauer et al., 2011). Another recent crystal structure of Dnmt1 incomplete with unmethylated DNA suggests that the linker between CXXC and the bromo-adjacent homology 1 (BAH1) domain inhibits *de novo* activity of Dnmt1 by blocking the catalytic center (Song et al., 2011).

Uhrf1, also known as Np95 (mouse) or ICBP90 (human), is involved in maintenance of DNA methylation by Dnmt1. Mice lacking *uhrf1* show a similar phenotype to that observed in *dnmt1* null mice, which is genomic hypomethylation and developmental arrest at embryonic day 9.5 (Bostick et al., 2007; Sharif et al., 2007). Uhrf1 shows a preferential binding to hemimethylated DNA and histone H3 tail containing trimethylated lysine 9 (Rottach et al., 2010). In addition, Uhrf1 harbors a Ring domain and endows ubiquitin E3 ligase activity *in vitro*, which is required for the growth regulation of tumor cells (Jenkinis et al., 2005; Citterio

et al., 2004). Recently we reported that the ubiquitination state and stability of Dnmt1 controlled by Uhrf1 and the ubiquitin specific protease Usp7 (Qin et al., 2011).

Here we aimed at elucidating the role of Uhrf1 in controlling maintenance of DNA methylation by Dnmt1. The interaction of Dnmt1 with Uhrf1 was mapped to the TS domain. Moreover, a Dnmt1 mutant lacking part of the TS domain, Dnmt1$^{\Delta TS}$, failed to restore DNA methylation patterns in *dnmt1*$^{-/-}$ embryonic stem cells (ESCs). Similarly, two Uhrf1 mutants with single amino acid substitution in the PHD and Ring domains, H346G and H730A, respectively, could not rescue DNA methylation patterns in *uhrf1*$^{-/-}$ ESCs. These data suggest that the interaction between Dnmt1 and Uhrf1 is necessary but not sufficient for propagation of DNA methylation. Interestingly, we showed that Dnmt1$^{\Delta TS}$ is enzymatically inactive when cell extracts were added to methylation reaction, implicating that the effect on DNA methylation is not only due to a failure in recruiting Dnmt1, but also a lack of enzymatic activation. *In vitro* Uhrf1 inhibits DNA methylation by competitively binding hemimethylated CpG sites. These results suggest that Uhrf1 plays multiple roles in maintenance of DNA methylation by Dnmt1. Thus, we propose that *in vivo* maintenance of DNA methylation might involve three steps: recruitment, access and enzymatic activation.

Result

TS domain of Dnmt1 is required for the interaction with Uhrf1

Uhrf1 was suggested to target Dnmt1 to hemimethylated CpG sites. To investigate the mechanism of recruitment, we first mapped the domains involved in the interaction of Dnmt1 with Uhrf1 by co-immunoprecipitation (Fig. 1B and D). Our data showed that the N-terminal part, but not the C-terminal catalytic domain of Dnmt1, interacts with Uhrf1. Furthermore, the association with Uhrf1 was narrowed down to the conserved TS domain (Fig. 1B and 2B). Consistently, the Dnmt1 deletion construct lacking conserved region (aa 459-501, Dnmt1$^{\Delta TS}$) did not interact with Uhrf1 (data not shown). Notably, an *in vitro* radioactive methyltransferase assay showed that Dnmt1$^{\Delta TS}$ is able to transfer the methyl group from donor of S-Adenosyl-L-Methionine (AdoMet) to poly(dI•dC)-poly(dI•dC) substrate (Fig. 2C). This data suggest that the catalytic activity of Dnmt1 is not affected by deletion of amino acids 459 to 501 *in vitro*. Similarly, the Ubl domain of Uhrf1 was found to mediate the interaction with Dnmt1 (Fig. 1D).

Dnmt1$^{\Delta TS}$ cannot reestablish DNA methylation patterns in dnmt1$^{-/-}$ ESCs

To test if the interaction of Dnmt1 with Uhrf1 is indeed required for maintenance of DNA methylation, embryonic stem cells (ESCs) where expression of endogenous Dnmt1 is abolished by genetic deletion (*dnmt1$^{-/-}$,* C/C) were stably transfected with constructs for the expression of GFP fusion with either wild type Dnmt1 (Dnmt1^{wt}) or Dnmt1$^{\Delta TS}$. The resulting ESC lines express GFP-Dnmt1^{wt} and GFP-Dnmt1$^{\Delta TS}$ as the only Dnmt1 protein. In contrast to Dnmt1^{wt}, Dnmt1$^{\Delta TS}$ did not colocalize with Uhrf1 and showed a dispersed distribution in the nucleus (Fig. 3A), suggesting that the interaction with Uhrf1 is essential for cellular localization of Dnmt1. The activity of Dnmt1^{wt} and Dnmt1$^{\Delta TS}$ in living cells further was also tested by using an *in vivo* trapping assay developed by Schermelleh (Schermelleh et al., 2005). In this assay, cytosine analogue 5-aza-2'-deoxycytidine (5-aza-dC) is used. 5-aza-dC can form a covalent complex with the C6 position of the cytosine residue, if incorporated into DNA, which is in contrast to cytosine irreversible. Dnmt1 is therefore trapped at DNA replication foci. Trapped Dnmt1 fraction increases overtime and reflects enzymatic activity of Dnmt1. In ESCs stably expressing either GFP-Dnmt1^{wt} or mutant GFP-Dnmt1$^{\Delta TS}$, foci of immobilized GFP-Dnmt1^{wt} emerged within 20 min (Fig. 3B). GFP-Dnmt1$^{\Delta TS}$ did not accumulate at replication foci even after 2 hours, suggesting that GFP-Dnmt1$^{\Delta TS}$ is unable to methylate DNA in the context of living cells. We next analyzed both global DNA

methylation levels and methylation patterns of specific sequence. Our data showed that GFP-Dnmt1$^{\Delta TS}$ cannot reestablish the DNA methylation patterns in contrast to Dnmt1^{wt}. Consistently the rescue experiment was also carried out in *p53 and dnmt1* knockout somatic cells. After expression of GFP-Dnmt1$^{\Delta TS}$ the global DNA methylation level is similar to that observed in *dnmt1*$^{-/-}$ ESCs (Fig. S2). Moreover the re-methylation of specific genes sequences, like major satellites and skeletal α-actin promoters, did not occur (Fig. 3C and S1). In summary, GFP-Dnmt1$^{\Delta TS}$ does not interact with Uhrf1 and is able to methylate DNA substrate *in vitro*. However, GFP-Dnmt1$^{\Delta TS}$ cannot restore the DNA methylation patterns in *dnmt1*$^{-/-}$ ESCs, suggesting that the association of Dnmt1 with Uhrf1 is required to maintain DNA methylation in living cells.

The PHD and Ring domains of Uhrf1 are functionally required for maintenance methylation

To elucidate the functional mechanism of Dnmt1 recruitment by Uhrf1, we introduced point mutations in the PHD and Ring domain, (Uhrf1^{H346G} and Uhrf1^{H730A}, respectively). These mutations are expected to prevent coordination of zinc ions by zinc finger motifs. The preferential binding of Uhrf1 to hemimethylated DNA and trimethylated H3K9 was not affected by these mutations (Fig. S3 and S4). Also we tested whether the point mutations introduced in the PHD and Ring domains influence the interaction with Dnmt1. By co-immunoprecipitation, Dnmt1 complex was precipitated by using RFP binder (Chromotek, Germany) from the cell lysate which transiently expressed RFP tagged Dnmt1 in combination with GFP-tagged wild type Uhrf1, or Uhrf1^{H346G} or Uhrf1^{H730A}. Co-precipitated Uhrf1 was visualized with a GFP antibody in all bound fractions, showing that both mutants still interact with Dnmt1 (Fig. 4A). Consistently the interactions were confirmed by a modified method of a recently developed fluorescent two-hybrid assay (F2H; Zolghadr et al., 2008). Uhrf1-GFP fusion constructs were used as baits by tethering them to a *lac* operator array present in BHK cells, so that the array was visible as a distinct nuclear spot of enriched GFP fluorescence. The RFP-Dnmt1 fusion (prey) accumulated at this spot when Uhrf1^{wt}, Uhrf1^{H346G} or Uhrf1^{H730A} were used (Fig. 4B). Additionally, the data showed that the ubiquitin ligase activity of Uhrf1 was abolished by substitution of histidine 730 to alanine (Fig. S5).

In order to clarify the role of the PHD and Ring domains in maintenance of DNA methylation, we stably expressed GFP tagged Uhrf1^{wt}, or Uhrf1^{H346G} or Uhrf1^{H730A} in *uhrf1*$^{-/-}$ ESCs, respectively. In these ESCs, Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP showed a normal cellular localization and accumulate at heterochromatin comparable to Uhrf1^{wt}-GFP (Fig. 4C). Thus,

the mutations do not affect localization of Uhrf1. Interestingly, Dnmt1 did not colocalize with Uhrf1^{H346G} or Uhrf1^{H730A} in contrast to Uhrf1^{wt} and showed a dispersed distribution in nucleus. This observation is consistent with the result done by visualization of the colocalization between the mutants and the endogenous Dnmt1 (Fig. 4D). Similarly, we also analyzed DNA methylation level at specific loci. Indeed, our data showed that both Uhrf1^{H346G} and Uhrf1^{H730A} cannot rescue DNA methylation patterns in *uhrf1*$^{-/-}$ ESCs in contrast to Uhrf1^{wt} (Fig. 4E).

In summary, the interaction of Dnmt1 with Uhrf1 is essential but not sufficient for maintenance of DNA methylation, suggesting that the PHD and Ring domains are involved in recruiting Dnmt1 to hemimethylated CpG sites.

Uhrf1 inhibits methylation of naked DNA and nucleosomal substrate by Dnmt1

Since the PHD domain of Uhrf1 is involved in large-scale chromocenter reorganization, it can be speculated that Uhrf1 might modulate chromatin accessibility for Dnmt1. To test this hypothesis, an assay was established to test Dnmt1 activity on nucleosomal substrates in the presence or absence of Uhrf1. To generate the nucleosomal substrates, 200 base pairs of nucleosome positioning sequence (601 sequence; Fig. S6A; Lowary et al., 1998), that was un- or hemimethylated at CpG sites, was reconstituted with recombinant core histones by salt dialysis (Fig. S6B). These nucleosomal substrates and the corresponding naked DNA substrates were incubated with ESCs extracts stably expressing GFP-Dnmt1^{wt} or GFP-Dnmt1$^{\Delta TS}$ and methylation of the substrates was analyzed. In hemimethylated nucleosomal substrates only the linker DNA was methylated by extracts containing GFP-Dnmt1^{wt} (Fig. 5A and 5B), suggesting that an enzymatic activation of Dnmt1 *in vivo* is required. To clarify the role of Uhrf1 at local chromatin sites, transiently expressed GFP fusion proteins were immunoprecipitated from HEK293T cell lysates and incubated with the same DNA and nucleosomal substrates as above. Methylation analysis showed that GFP-Dnmt1^{wt} methylated the naked hemimethylated DNA (Fig. 6A). As the same with cell lysates, GFP-Dnmt1^{wt} preferentially methylated the hemimethylated CpG in linker DNA, but not in the sequence wrapped with the histone octamer. Interestingly, a reduction of DNA methylation in the presence of Uhrf1 was observed (Fig. 6B). This effect is likely due to competitive binding of Uhrf1 to hemimethylated CpG sites, as deletion of the SRA domain abolished this influence on DNA methylation (Fig. 6C). Consistently, a time course experiment confirmed the inhibitory effect of Uhrf1 on DNA methylation by Dnmt1 (Fig. 6D).

Usp7 has an activating effect on the enzymatic activity of Dnmt1

As the results above suggested that an activation of Dnmt1 is required *in vivo*, Uhrf1 however has an inhibitory function in maintenance of DNA methylation *in vitro*. Therefore, we next aimed to identify the proteins involved in the activation of Dnmt1. Since Usp7 was reported as a novel interacting protein with Dnmt1 in our previous study, the role of Usp7 was investigated by *in vitro* trapping assay (Frauer et al., 2009). In this approach, transiently expressed GFP fusion proteins were immunoprecipitated from HEK293T cell lysates and incubated with fluorescently labeled DNA substrates. Dnmt1 activity could be reflected by fluorescence intensity ratios of bound probe/bound GFP fusion. Notably, Dnmt1 activity on hemimethylated substrates was increased two-fold in the presence of GFP-Usp7 (Fig. 7), suggesting that Usp7 is an activator of Dnmt1.

Discussion

Here we showed that the interaction of Dnmt1 with Uhrf1 is required for enrichment of Dnmt1 at DNA target sites *in vivo*. The PHD and Ring domains of Uhrf1 are involved in Dnmt1 recruitment. Importantly, these domains are not involved in the interaction of Uhrf1 with either hemimethylated CpG sites (SRA domain) or Dnmt1 (Ubl domain). To clarify the mechanism of Dnmt1 recruitment, we tested Dnmt1 activity on nucleosomal substrates with or without Uhrf1. We showed that in hemimethylated nucleosomal substrates only the linker DNA was methylated and that Uhrf1 acts as a competitive inhibitor of Dnmt1 on naked DNA and mononucleosomal substrates. The mononucleosomal substrates in this study likely do not reflect the complexity of chromatin *in vitro*. We could not gain deeper insights into the mechanism by which Uhrf1 allows Dnmt1 to maintain DNA methylation. However, altogether our results strongly indicate that the role of Uhrf1 is far more complex than recruitment of Dnmt1 to hemimethylated CpG sites. The PHD domains have been reported to bind histone modifications and it might mediate Uhrf1 binding to another histone modifications in addition to trimethylated H3K9 (tandem Tudor domain). Recognition of "bivalent histone modification marks" by Uhrf1 might be required for Dnmt1 targeting to chromatin substrate *in vivo*. Therefore, histone modifications should be considered when making the chromatin substrates and the polynucleosomal substrates should be used. Additionally, in this study we observed that the Ring domain of Uhrf1 was also involved in maintenance of DNA methylation. In previous studies, Uhrf1 was shown to ubiquitinate core histones *in vitro* (Citterio et al., 2004). Ubiquitination of core histones by Uhrf1 might also modulate the accessibility of chromatin for the recruitment of Dnmt1. Mapping the ubiquitination sites of histones catalyzed by Uhrf1 might likely allow us to understand the mechanism of Dnmt1 targeting *in vivo*.

A recent crystal structure shows that the TS domain plays an autoinhibition role by insertion into the catalytic DNA binding pocket of Dnmt1 (Takeshita et al., 2011), suggesting that an activation of Dnmt1 might be required. Here, we showed an opposite role of Uhrf1 and Usp7 in the regulation of Dnmt1 activity. In our previous study we reported that the interaction between Dnmt1 and Usp7 was abolished by substitution of all lysine in the GK linker, suggesting a function of Usp7 in modulating the folding of Dnmt1, or in controlling the Dnmt1 activity by affecting the intra-molecular interaction of Dnmt1. Additionally, Uhrf1 and Usp7 coordinately control the ubiquitination state of Dnmt1. Therefore, it is interesting to

investigate whether the inhibition and activation of Dnmt1 by Uhrf1 and Usp7, respectively, are ubiquitination dependent or independent.

Materials and Methods

Expression constructs

Expression construct for GFP-Dnmt1^{wt} was described previously (Easwaran et al., 2004; Schermelleh et al., 2007). Different fragments of Dnmt1, named as GFP-Dnmt1$^{1-1111}$, GFP-Dnmt1$^{1124-1620}$, GFP-Dnmt1$^{1-309}$, GFP-Dnmt1$^{310-629}$ and GFP-Dnmt1$^{630-1111}$ were cloned into pcDNA3-GFP by PCR amplification. Uhrf1^{wt}-GFP, Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP constructs for expression were cloned into pCAG-GFP-IRES-IP vector. The expression construct for HA-tagged ubiquitin was kindly provided by Stefan Jentsch (MPI Biochemistry, Germany). All constructs were verified by DNA sequencing.

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells and baby hamster kidney (BHK) cells were cultured in DMEM supplemented with 10% fetal calf serum and 50 µg/ml gentamycine (PAA, Germany). The embryonic stem cells (ESCs) here used, including wild type J1, *dnmt1*$^{-/-}$, E14 and *uhrf1*$^{-/-}$ cultured without feeder cells in gelatinized flasks as described (Schermelleh et al., 2005). The *dnmt1*$^{-/-}$ ESCs used in this study are homozygous for the c allele (Lei et al., 1996). Mouse ESCs were transfected with FuGENE HD (Roche) according to the manufacturer's instructions and sorted with a FACS Aria II instrument (Becton Dikinson). HEK293T cells and BHK cells were transfected using polyethylenimine as transfection reagent (Sigma, Germany) according to the manufacturer's instructions. Cell fixation and microscopy were carried out as described by Zolghadr *et al.* (2008).

Co-immunoprecipitation and Western blotting

Extracts from HEK293T cells were prepared in lysis buffer (20 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 1 µg/µl DNase I, 2 mM $MgCl_2$, 2 mM phenylmethyl sulphonyl fluoride and 0.5% NP40) containing 150 mM NaCl and diluted with lysis buffer without NP40. GFP trap (Rothbauer et al, 2008) was used for immunoprecipitation of GFP fusion proteins. GFP trap was washed twice with dilution buffer containing 300 mM NaCl and re-suspended in SDS–PAGE sample buffer.

Rabbit antisera were used for detection of Dnmt1 and Uhrf1 (Grohmann et al, 2005; Citterio et al., 2004). Horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit secondary antibodies (Sigma) and ECL Plus reagent (GE Healthcare, Munich, Germany) were used for detection.

DNA methylation assay

Genomic DNA was isolated with the QIAmp DNA Mini Kit (Qiagen) and was bisulfate converted using the EZ DNA Methylation-Gold Kit (Zymo research) according to the manufacturer's instructions.

For *in vitro* methylation, GFP-Dnmt1 was purified by immunoprecipitation from HEK293T cells over-expressed GFP-Dnmt1. The concentration of GFP-Dnmt1 in bound fraction is measured by TECAN reader. Incubate 3 μg of GFP-Dnmt1 with 88 ng of DNA template in the methylation buffer containing 160 μM of SAM, 100 ng/μl of BSA at 37°C for 3 hours. After inactivation of reaction at 65°C for 30 min, the DNA was isolated with Nucleospin PCR cleaning kit (Macherey-Nagel) and bisulfate treated with EZ DNA Methylation-Gold Kit (Zymo research). Primer sequences for 601 DNA were TGTATGTATTGAATAG (forward primer) and TACACAAAATATATATATC (reverse primer). For amplification we used Qiagen Hot Start Polymerase in 1x Qiagen Hot Start Polymerase buffer supplemented with 0.2 mM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer, 1.3 mM betaine (Sigma) and 60 mM tetramethylammonium-chloride (TMAC, Sigma). Pyrosequencing reactions were carried out by Varionostic GmbH (Ulm, Germany).

To measurement of total DNA methylation level, a slot blot assay was used. In this assay, a series amount of genomic DNA, including 150, 300 and 600 ng, were loaded into nylon membrane. 5mC antibody is used to detect the methylation level and anti single strand DNA was used to normalization (IBL, Japan).

Preparation of reconstituted mononucleosomes

601 DNA was kindly provided by Peter Becker's group. In order to get enough unmodified DNA templates, the 601 DNA sequences were amplified with primers: TGCATGTATTGAACAG (forward) and TGCACAGGATGTATATATC (reverse).

To prepare the hemimethylated DNA, an efficient method for preparation of long heteroduplex DNA was used as described (Thomas et al., 2002). In this strategy, one pairs of modified PCR primers were synthesized, which are labeled with phosphate at 5'-end, like 5'-phosphorylated-TGCATGTATTGAACAG-3' and 5'-phosphorylated-TGCACAGGATGTATATATC-3'. To get single and up-strand DNA, the DNA was amplified with the reverse primer labeled with phosphate at 5'-end, following a lambda-nuclease digestion (NEB). The same procedure is required for making low-strand DNA. However, in order to prepare the methylated low-strand DNA, one more step, *in vitro*

methylation by bacterial methyltransferase M.SssI (NEB), is required before treatment with lambda-nuclease. In the end, the equal amount of up and low strand DNA were mixed and incubated at 95°C for 5 min, followed annealing.

To get rid of contamination from double strand DNA after lambda-nuclease treatment, the hydroxyapatite chromatography was carried out. Hydroxyapatite column was packaged according to the manufacturer's instructions (Sigma) and the single strand DNA was eluted by elution buffer containing 150 mM sodium phosphate.

Histone ubiquitination assay

HEK293T cells were transfected with HA-ubiquitin alone or in combination with Uhrf1^{wt}-GFP and Uhrf1^{H730A}-GFP. Histone core particles then were isolated as described (Shechter et al., 2007). Ubiquitinated histones were visualized by HA antibody.

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Figure legend

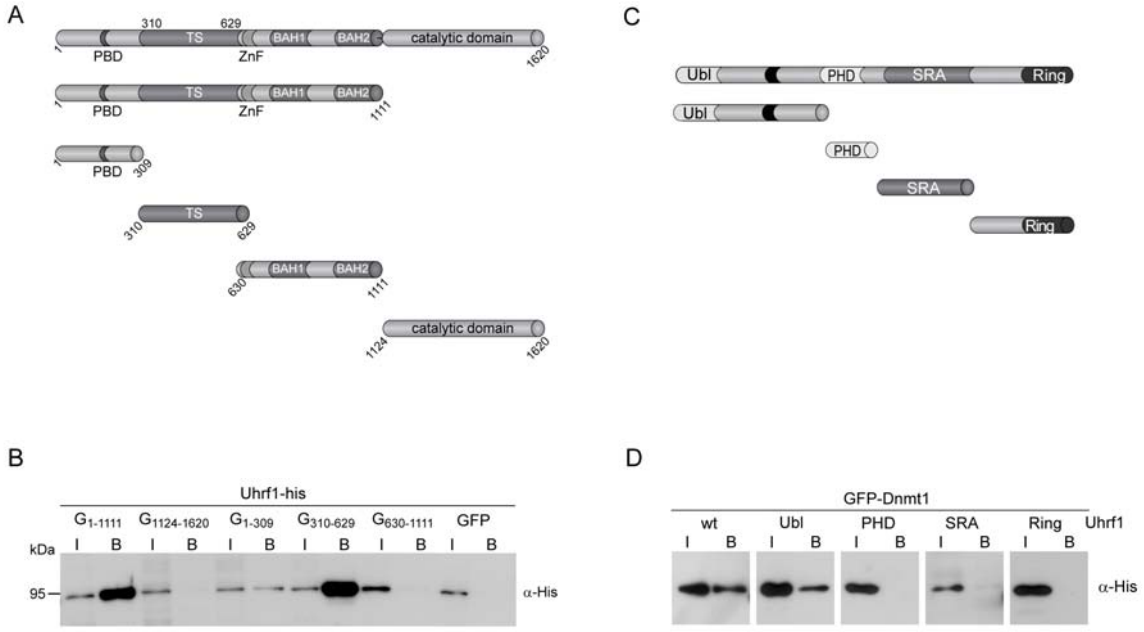

Figure 1. Mapping the interaction of Dnmt1 with Uhrf1

(A) Schematic representation of Dnmt1 and its fragments. Dnmt1 is a relative large protein comprising a N-terminal regulatory domain and a catalytic domain. The N-terminal regulatory domain harbors several functional subdomains: a PCNA binding domain (PBD), a targeting sequence (TS) domain, a zinc finger domain (ZnF) and two bromo-adjacent homology domains (BAH1 and BAH2).

(B) The N-terminal domain, but not the C-terminal catalytic domain of Dnmt1, is responsible for the interaction with Uhrf1. HEK293T cells were transiently transfected with constructs expressing for Uhrf1-his in combination with GFP-Dnmt1^{wt}, or GFP-Dnmt1$^{1-1111}$, or GFP-Dnmt1$^{1-309}$, or GFP-Dnmt1$^{310-629}$, GFP-Dnmt1$^{630-1111}$ or GFP-Dnmt1$^{1124-1620}$. GFP fusion proteins were immunoprecipitated with GFP trap. Bound fraction was separated by SDS-PAGE and detected by anti-his antibody.

(C) Schematic representation of Uhrf1 and its fragments. Uhrf1 harbors several functional domains, an ubiquitin-like domain (Ubl), a plant and homeodomain (PHD), a set and ring associated domain (SRA) and a really interesting new gene (Ring).

(D) Ubl domain is required for the interaction between Dnmt1 and Uhrf1.

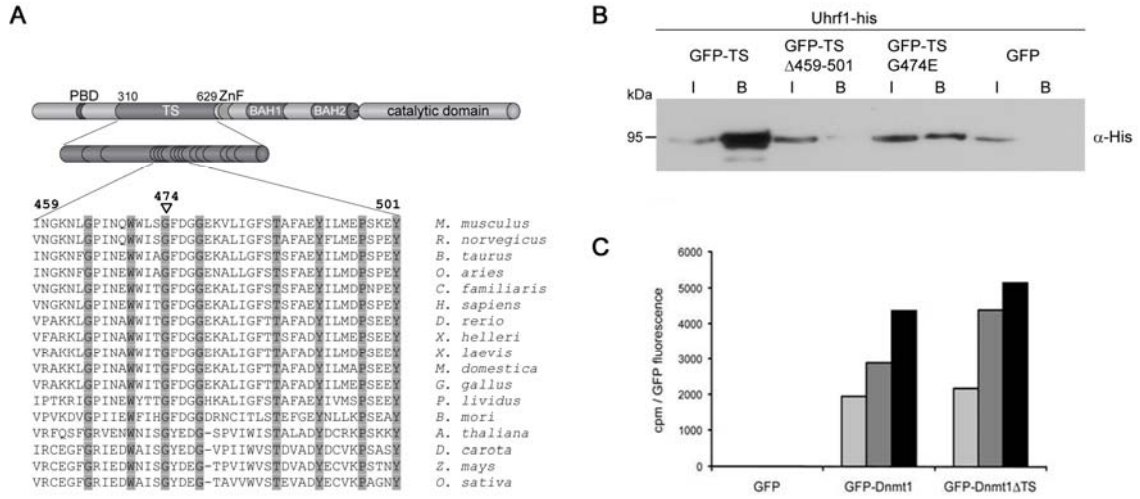

Figure 2. The amino acids of 459 to 501 are essential for the interaction between the TS domain and Uhrf1.

(A) Dnmt1 structure and alignment of the TS domain from amino acids 459 to 501 in various species. Several conserved amino acids in all species were highlighted in gray. NCBI accession numbers of Dnmt1 from different species are as follows: *M. musculus,* NP_034196; *B. Taurus,* NP_872592.2; *R. norvegicus*, NP_001003959.1; *O. sativa,* B1Q3J6.1; *C. familiaris*, XP_533919; *M. domestica*, NP_001028141.1; *H.sapiens*, NP_008823.1; *D. rerio*, NP_571264.1; *X. helleri*, AAF73200; *X. laevis*, NP_001084021.1; *G. gallus,* NP_996835.1; *P. lividus,* CAA90563.1; *B. mori,* NP_001036980.1; *A. thaliana*, NP_199727.1; *Z. mays*, NP_001105186.1; *O. aries,* NP_001009473.1; *D. carota*, AAC39356.1.

(B) The amino acids 459-501 in the TS domain are required for the interaction between the TS domain and Uhrf1. His tagged Uhrf1 was transiently expressed in HEK293T cells with GFP-TS$^{310\text{-}629}$, GFP-TS$^{\Delta 459\text{-}501}$ or GFP-TS^{G474E}. GFP fusion proteins were precipitated by GFP trap and Uhrf1 proteins were detected by anti-his antibody in bound fraction.

(C) GFP-Dnmt1$^{\Delta TS}$ shows enzymatic activity in an *in vitro* radioactive methyltransferase assay. The transfer of [3H]-methyl groups to poly(dI•dC)-poly(dI•dC) substrate was measured for increasing volumes of GFP fusion proteins immunopurified from transiently transfected HEK293T cells. Counts per minute (cpm) were normalized to the relative protein concentration as determined by SDS-PAGE analysis. GFP was used as negative control.

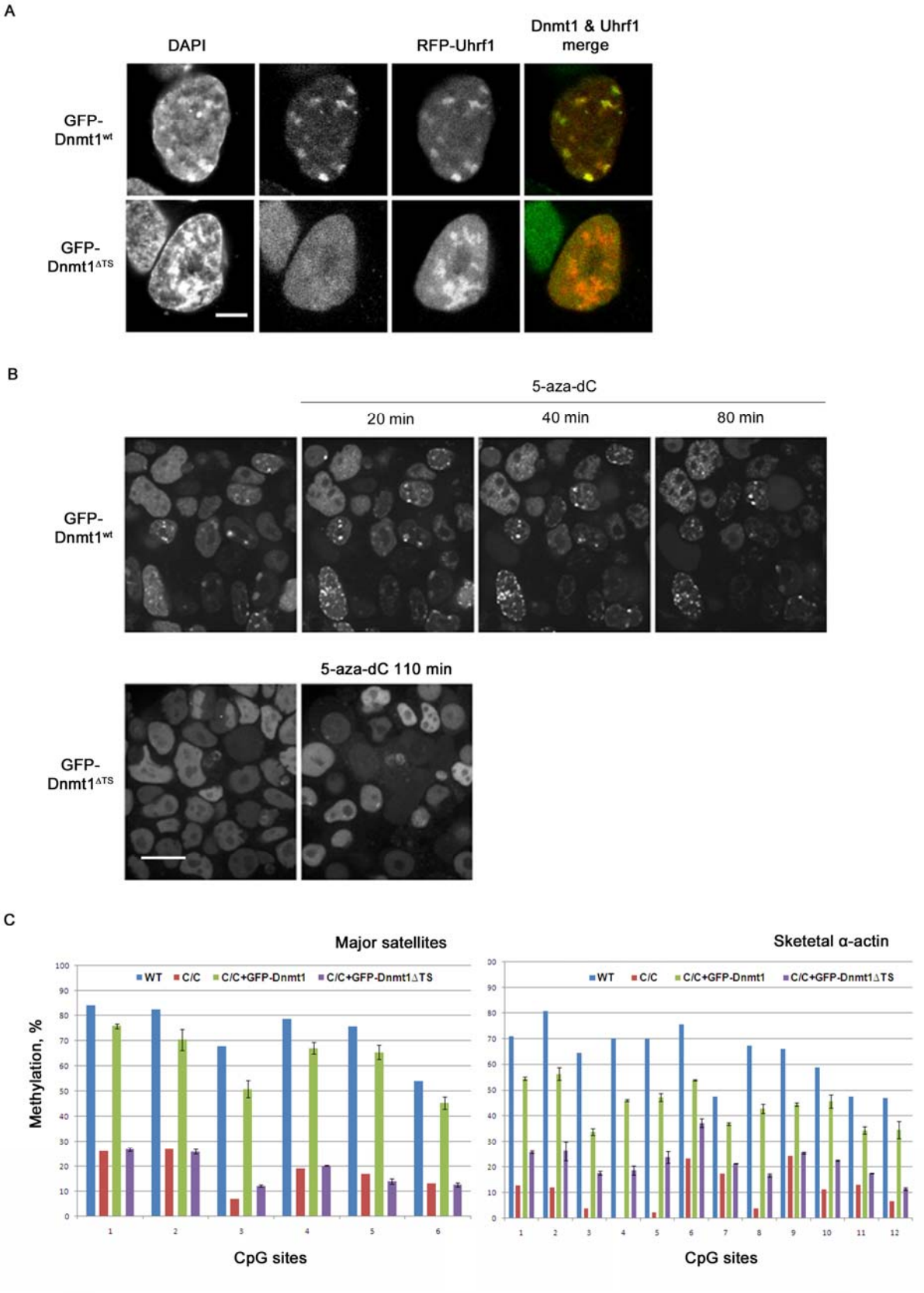

Figure 3. Dnmt1$^{\Delta TS}$ failed to rescue DNA methylation in *dnmt1* knockout ESCs.

(A) RFP-Uhrf1 was transiently overexpressed in *dnmt1* knockout ESCs stably expressing GFP-Dnmt1^{wt} or GFP-Dnmt1$^{\Delta TS}$. GFP-Dnmt1$^{\Delta TS}$ shows a dispersed distribution in the nucleus and not colocalizes with Uhrf1 at heterochromatin in contrast to GFP-Dnmt1^{wt}. The scale bar stands for 5 μm.

(B) Enzymatic activity of GFP-Dnmt1^{wt} and GFP-Dnmt1$^{\Delta TS}$ in living cells was analyzed by *in vivo* trapping assay. *Dnmt1* knockout ESCs stably expressing either GFP-Dnmt1^{wt} or GFP-Dnmt1$^{\Delta TS}$ were incubated with 10 μM of cytosine analogue of 5-aza-deoxycytosine. Trapped GFP-Dnmt1$^{\Delta TS}$ fraction did not emerge at DNA replication foci in contrast to GFP-Dnmt1^{wt}, reflecting GFP-Dnmt1$^{\Delta TS}$ is unable to rescue DNA methylation in *dnmt1* knockout ESCs. The scale bar stands for 20 μm.

(C) Genomic DNA was isolated from *dnmt1* knockout (C/C) ESCs stably expressing GFP-Dnmt1^{wt} or GFP-Dnmt1$^{\Delta TS}$ and was bisulfite-treated. The promoter sequences of major satellite and skeletal α-actin were amplified by PCR and methylation level of these sequences was analyzed by pyrosequencing. C/C ESCs show hypomethylation compared with wild type ESCs (WT). In contrast to GFP-Dnmt1^{wt}, GFP-Dnmt1$^{\Delta TS}$ cannot rescue DNA methylation at promoter regions of major satellites and partially rescue the skeletal α-actin.

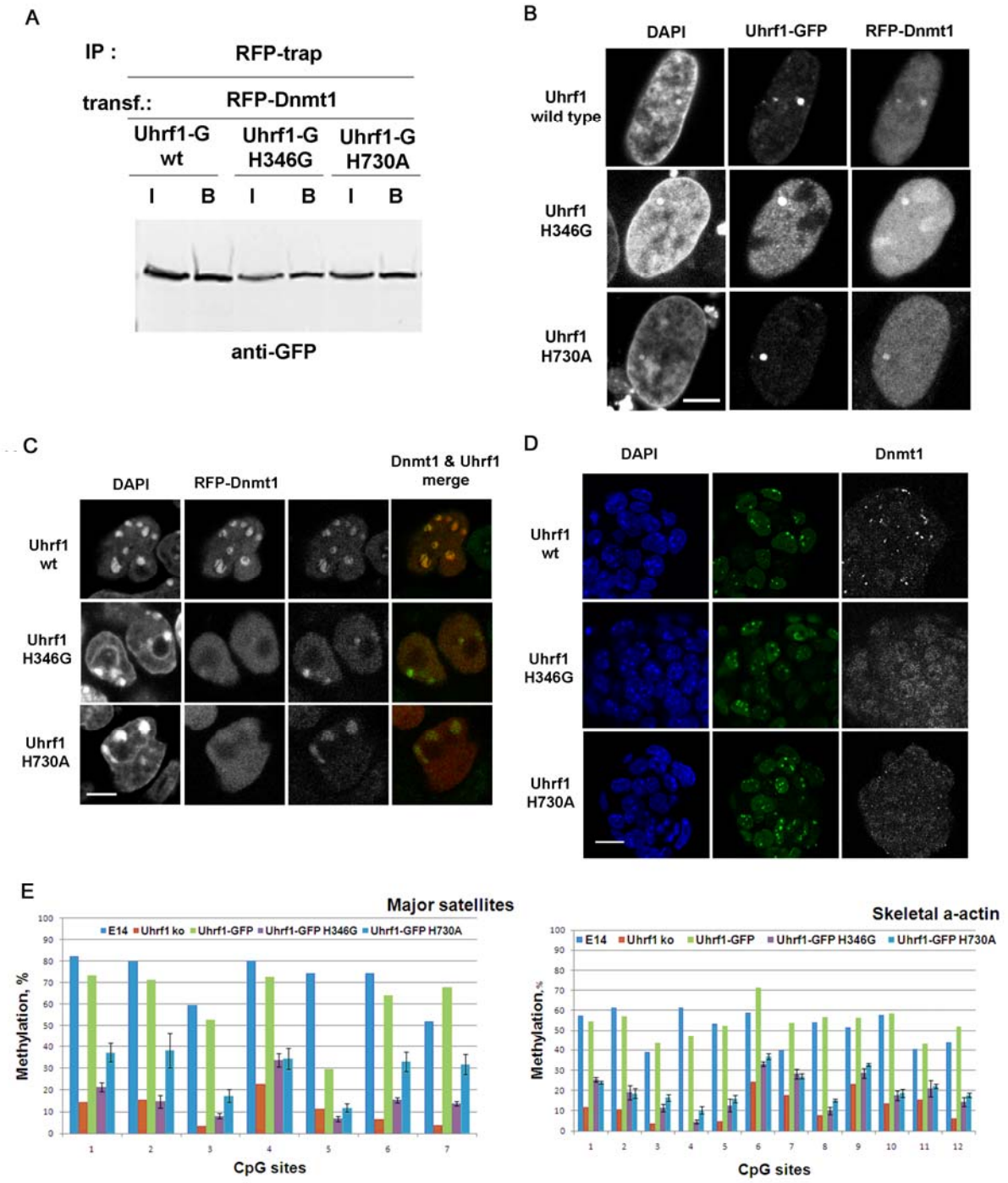

Figure 4. The PHD and Ring domains are involved in maintenance of DNA methylation

(A) The mutants of Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP still interact with Dnmt1 by coimmunoprecipitation. RFP-Dnmt1 was transiently overexpressed in HEK293T cells in combination with Uhrf1^{wt}-GFP, Uhrf1^{H346G}-GFP or Uhrf1^{H730A}-GFP. RFP-Dnmt1 was precipitated by RFP-trap and bound fractions were separated by SDS-PAGE. Uhrf1 proteins in bound fraction were detected by an anti-GFP antibody.

(B) The mutants of Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP still interact with Dnmt1 by a modified F2H assay. Uhrf1-GFP fusion constructs were used as baits by tethering them to a *lac* operator array present in BHK cells, so that the array was visible as a distinct nuclear spot of enriched GFP fluorescence. The RFP-Dnmt1 fusion (prey) accumulated at this spot when Uhrf1^{wt}, Uhrf1^{H346G} or Uhrf1^{H730A} were used.

(C) RFP-Dnmt1 was transiently expressed in *uhrf1*$^{-/-}$ ESCs stably expressing Uhrf1^{wt}-GFP, Uhrf1^{H346G}-GFP or Uhrf1^{H730A}-GFP, respectively. RFP-Dnmt1 accumulates at heterochromatin and overlaps with Uhrf1^{wt}-GFP, whereas it does not colocalize with Uhrf1 mutants, although Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP show a normal cellular distribution. The scale bar stands for 5 μm.

(D) Endogenous Dnmt1 was stained by using a rat antibody against Dnmt1. Dnmt1 colocalizes with Uhrf1^{wt}-GFP at heterochromatin, but not with the mutants, Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP. The scale bar stands for 20 μm.

(E) Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP can partially rescue DNA methylation in *uhrf1*$^{-/-}$ ESCs compared with Uhrf1^{wt}-GFP. With pyrosequencing, the DNA methylation levels were analyzed at promoter regions of major satellite and skeletal α-actin. The *uhrf1*$^{-/-}$ ESCs (Uhrf1 ko) show hypomethylation at promoter regions of both genes compared to wild type E14 ESCs.

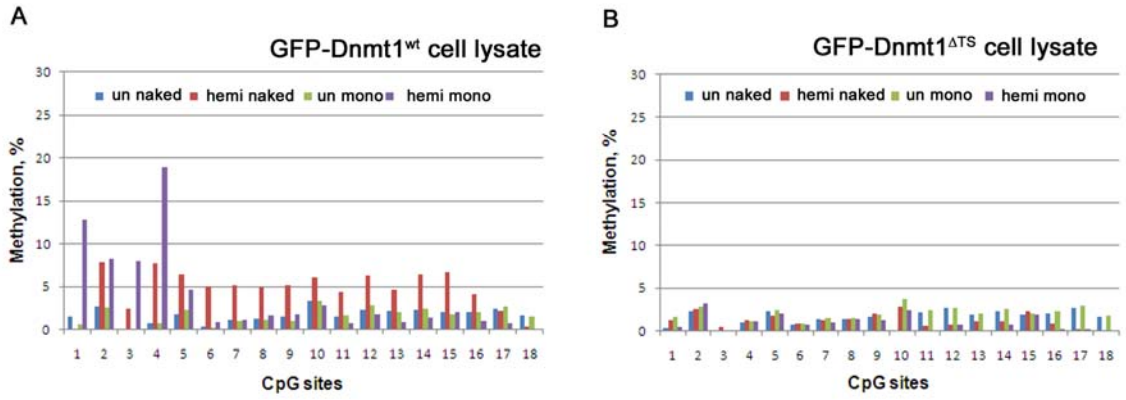

Figure 5. Dnmt1$^{\Delta TS}$ is unable to methylate naked hemimethylated DNA when cell lysate was added to the methylation reaction.

(A) Cell lysates of ESCs stably expressing GFP-Dnmt1^{wt} were incubated with DNA subsates, either un- or hemimethylated mononucleosomal DNA and the corresponding naked DNA substrates. Methylation level of these sequences was measured by pyrosequencing. GFP-Dnmt1^{wt} showed methylation activity on the naked hemimethylated DNA and the linker part of hemimethylated mononucleosomal DNA, but not on the sequence wrapped with core histones.

(B) Cell lysates of ESCs stably expressing GFP-Dnmt1$^{\Delta TS}$ were incubated with DNA subsates, either un- or hemimethylated mononucleosomal DNA and the corresponding naked DNA substrates. The DNA methylation level of these sequences was analyzed by pyrosequencing. No methylation activity of GFP-Dnmt1$^{\Delta TS}$ was observed on all DNA substrates used in this study.

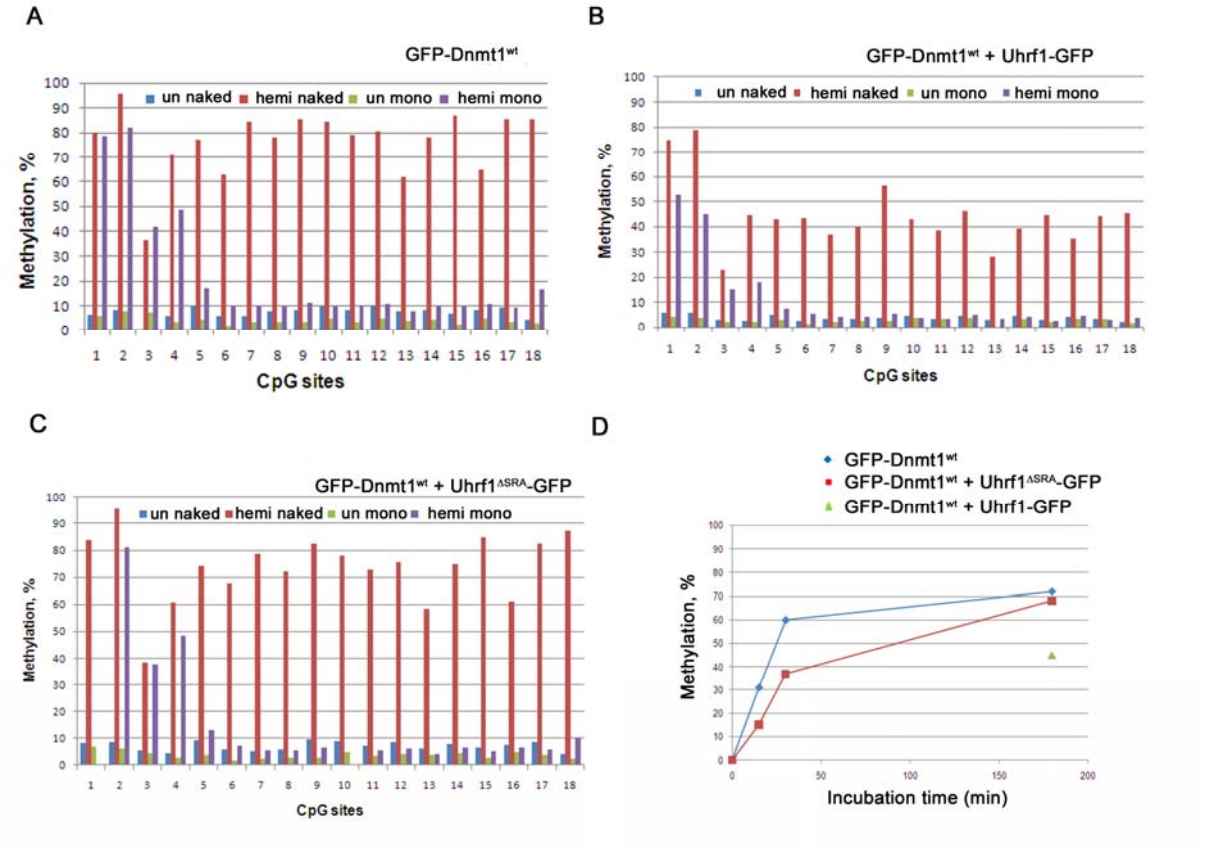

Figure 6. Uhrf1 has inhibitory function in DNA methylation by competitively binding to hemimethylated cytosine.

(A) Transiently expressed GFP-Dnmt1^{wt} were immunoprecipitated from HEK293T cells and incubated with mononucleosomal substrates and corresponding naked DNA. The methylation level of these DNA substrates was analyzed by pyrosequencing. GFP-Dnmt1^{wt} can methylate naked hemimethylated DNA substrates. In hemimethylated mononucleosomal DNA substrates, only linker DNA was methylated.

(B and C) DNA methylation level was dropped after addition of Uhrf1-GFP into methylation reaction. However, the effect was abolished by deletion of SRA domain.

(D) Time course of DNA methylation by Dnmt1 in presence of Uhrf1 and deletion mutant. The naked hemimethylated DNA substrates were incubated with GFP-Dnmt1 in the presence or absence of Uhrf1 or its deletion mutant. DNA methylation of these DNA sequence was analyzed at different time points. Notably, Uhrf1 seems to inhibit maintenance DNA methylation by competitive binding of hemimethylated CpG sites.

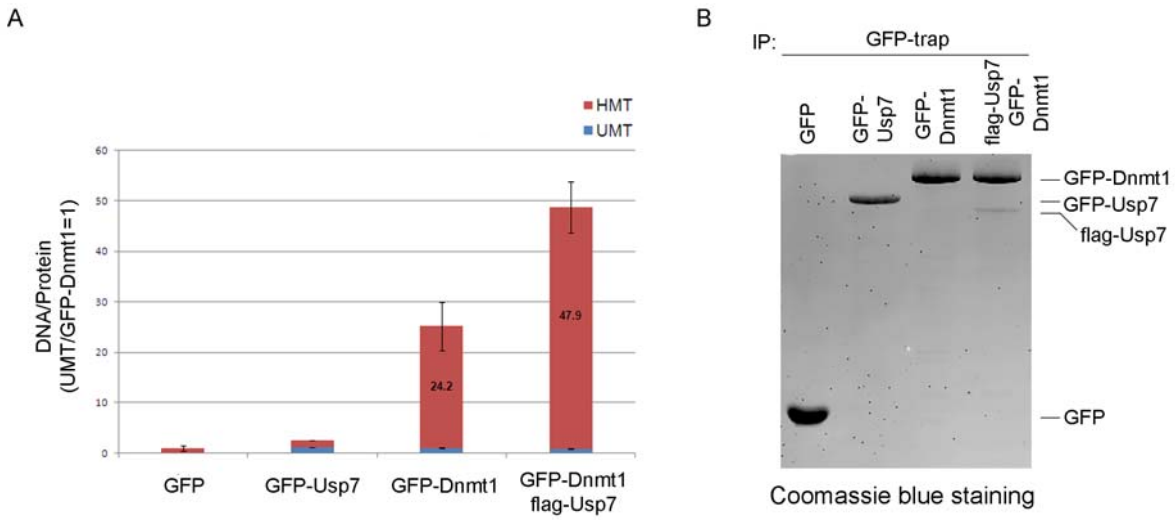

Figure 7. Usp7 stimulates Dnmt1 activity on hemimethylated DNA

(A) Trapping assays were carried out using fluorescently labeled double stranded oligonucleotide probes. The oligonucleotides have the same sequence except for containing one central CpG site with either 5-methylcytosine or cytosine. Shown are fluorescence intensity ratios of bound probe/bound GFP fusion, reflecting Dnmt1 activity. GFP was used as a control. Dnmt1 activity on hemimethylated DNA was increased two-fold in the presence of flag-Usp7.

(B) Coomassie blue staining gel showing proteins immunoprecipitated from overexpressing HEK293T cells that were used in (A).

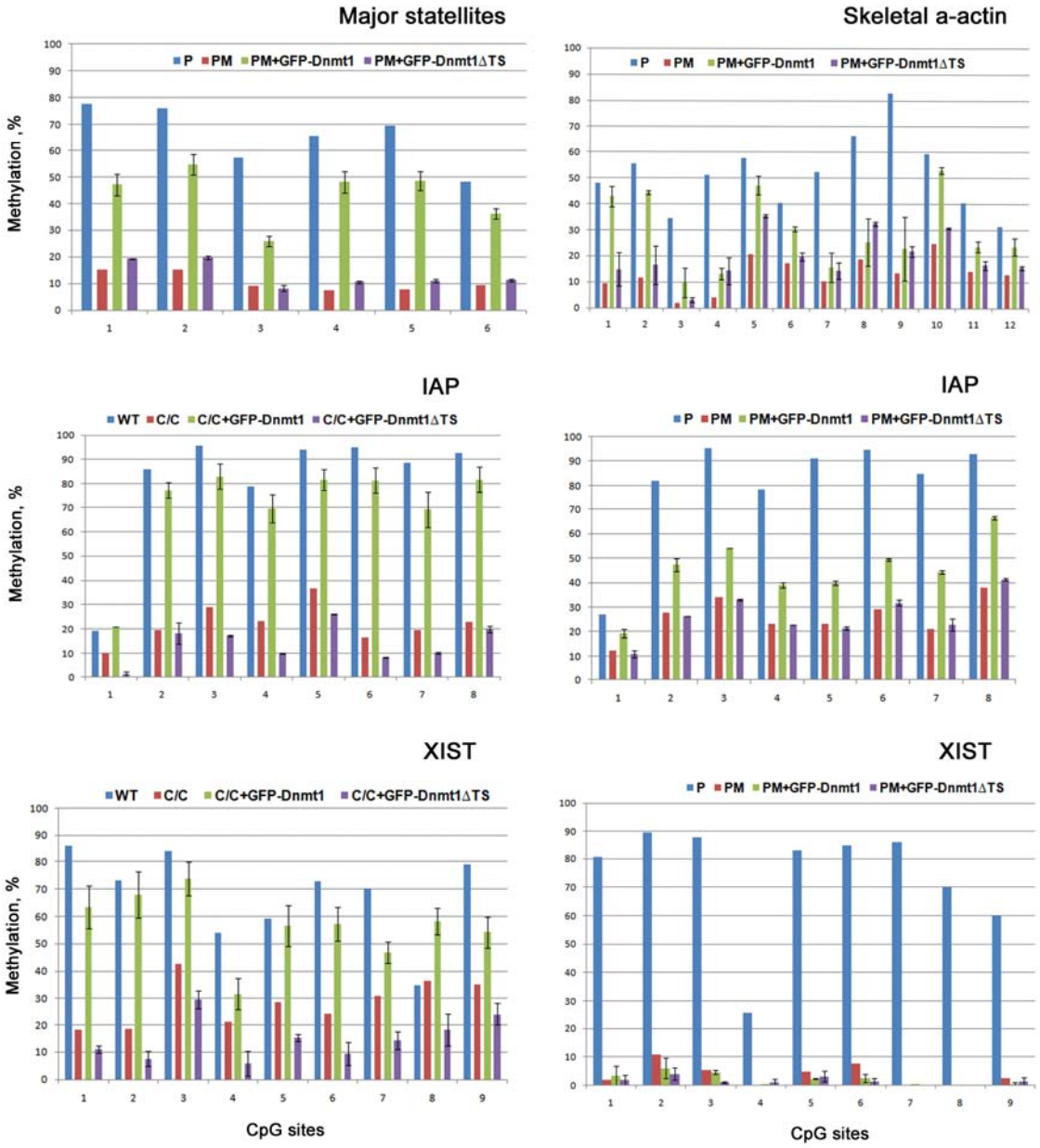

Figure S1. Additional methylation of IAP and XIST at their promoter region was analyzed by pyrosequencing.

The rescue experiments were carried out in both *dnmt1* knockout ESCs (C/C) and *p53/dnmt1* double knockout MEF cells (PM). The DNA methylation level was measured at the promoter region of the intracisternal A-particle (IAP), major satellites, skeletal α-actin and XIST (X-inactive specific transcript). $Dnmt1^{wt}$ can rescue DNA methylation similar to wild type ESCs levels (WT), but $Dnmt1^{\Delta TS}$ cannot. In MEF cells, $Dnmt1^{wt}$ is also able to rescue the DNA methylation level up to ~60% of *p53* knockout cells (P), but $Dnmt1^{\Delta TS}$ cannot.

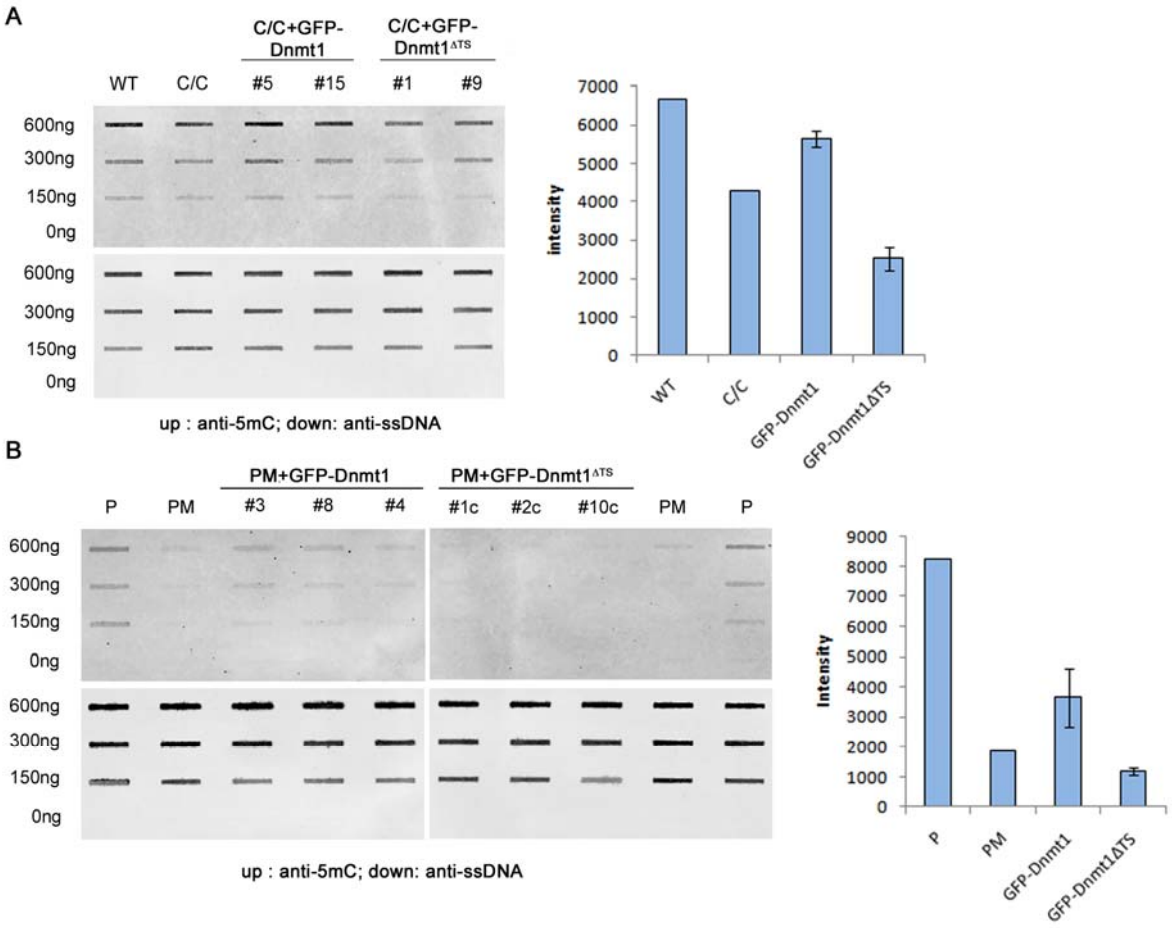

Figure S2. Measurement of global DNA methylation level in genomic DNA

The rescue experiments were carried out in both *dnmt1* knockout ESCs (C/C) and *p53/dnmt1* double knockout MEF cells (PM). Genomic DNA was isolated from different clones and loaded into nylon membrane. Methylated cytosine was detected by an anti-5mC antibody. The detection of single strand DNA here was used as an internal control.

The quantification was done by Image J. GFP-Dnmt1$^{\Delta TS}$ cannot restore the DNA methylation patterns in both *dnmt1* knockout ESCs and PM MEF cells in contrast to GFP-Dnmt1^{wt} (A and B). WT and P mean the wild type ESCs and *p53* knockout MEF cells, respectively.

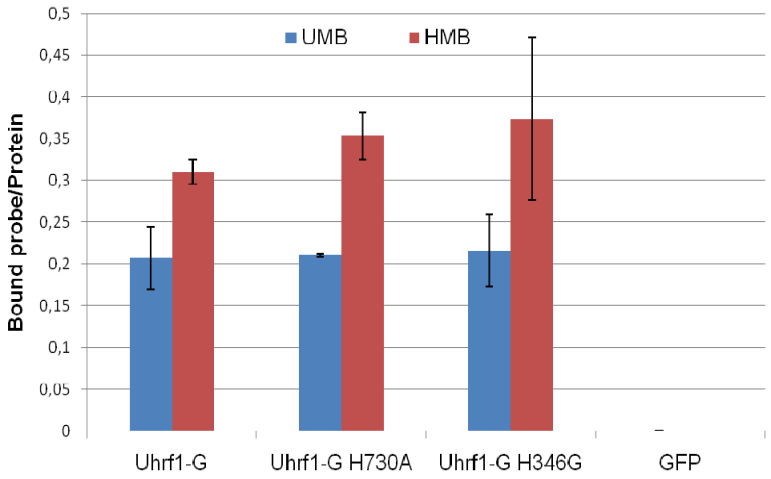

Figure S3. *In vitro* DNA binding properties of Uhrf1 and its mutants

Binding assays were carried out using two fluorescently labeled double stranded oligonucleotide probes in direct competition. The oligonucleotides have the same sequence except for containing one central either un- or hemimethylated CpG site. Shown are fluorescence intensity ratios of bound probe/bound GFP fusion. GFP was used as a control. The DNA binding properties of Uhrf1 was not affected by single amino acid substitution of histidine 730 to alanine and histidine 346 to glycine, respectively.

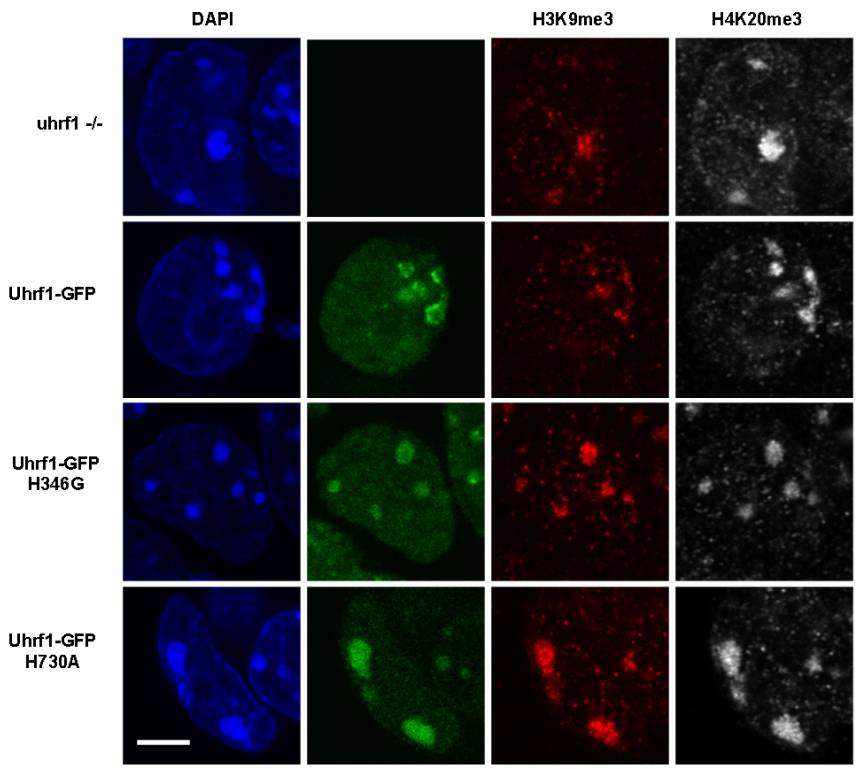

Figure S4. Failure of Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP to remethylate *uhrf1*$^{-/-}$ ESCs is independent of binding trimethylated histone H3K9 and H4K20. The trimethylated H3K9me3 and H4K20me3 were detected by anti-H3K9me3 and H4K20me3 antibodies, respectively. Uhrf1 colocalizes with heterochromatin marks, H3K9me3 and H4K20me3, reflecting that Uhrf1^{H346G}-GFP and Uhrf1^{H730A}-GFP still possess histone binding ability. The scale bar stands for 5 μm.

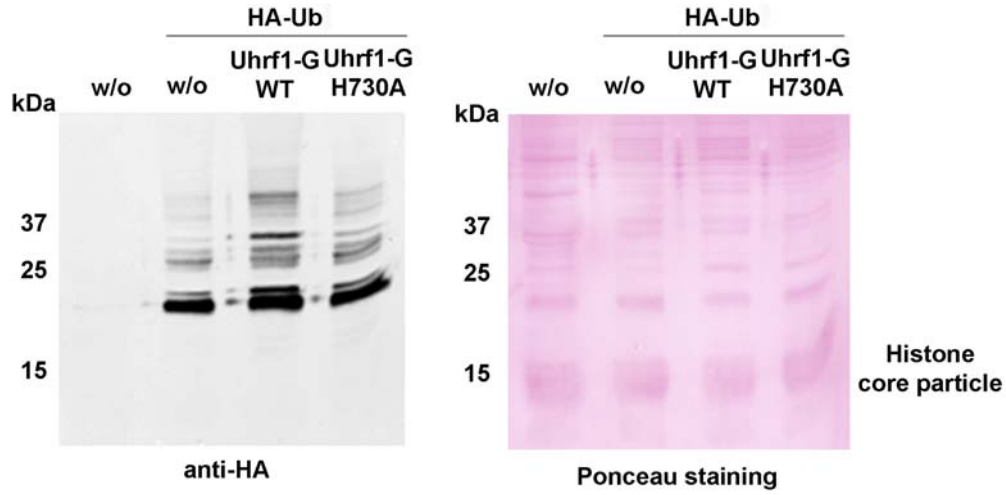

Figure S5. Substitution of histidine 730 to alanine abolished the ubiquitin ligase activity of Uhrf1.

HEK293T cells were transiently transfected with HA-ubiquitin alone or in combination with Uhrf1^{wt}-GFP or Uhrf1^{H730A}-GFP. Histone core particles were isolated and separated by SDS-PAGE. Ubiquitinated histones were visualized with a HA antibody. Ponceau staining here is used as a loading control.

A

TGCATGTATTGAACAGCGACTCGGGTTATGTGATGGACCCTATACGCGGCCGCC
CTGGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGCAAGCTCTAGCACC
GCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTAC
TCCCTAGTCTCCAGGCACGTGTCAGATATATACATCCTGTGCA

B

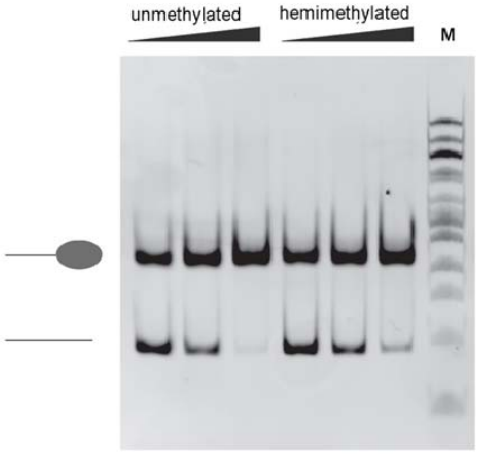

Figure S6. Reconstitution of mononucleosomes *in vitro*

(A) The 601 DNA sequence is used for reconstitution of mononucleosomes *in vitro*. This sequence contains 18 of CpG sites, 13 of them in the nucleosome position (highlighted in yellow) and 5 of them in the linker part.

(B) Titration of histone octamer and DNA to make reconstituted mononucleosome. The reconstituted mononucleosomes were checked by running polyacrylamide gel and were stained by ethidium bromide. The ratios of histone octamer/unmethylated or hemimethylated DNA are 0.75:1, 1:1 and 1.25:1, respectively. The ratio 1.25:1 of histone octamer/DNA is the best condition for making pure mononucleosomes.

2.4. REGULATION OF DNA METHYLTRANSFERASE 1 BY INTERACTIONS AND MODIFICATIONS

Regulation of DNA methyltransferase 1 by interactions and modifications

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Key words: DNMT1, Dnmts, DNA Methylation, Epigenetics, DNA Methyltransferases, post-translational modifications, protein interactions

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Abstract

DNA methylation plays a central role in the epigenetic regulation of gene expression during development and disease. Remarkably, the complex and changing patterns of genomic DNA methylation are established and maintained by only three DNA methyltransferases. Here we focus on DNMT1, the major and ubiquitously expressed DNA methyltransferase in vertebrates, to outline possible regulatory mechanisms. A list of all protein interactions and post-translational modifications reported for DNMT1 clearly shows that DNMT1 and by extension also DNA methylation in general are functionally linked with several other epigenetic pathways and cellular processes. General themes of these interactions and modifications include the activation, stabilization and recruitment of DNMT1 at specific sites and heterochromatin regions. For a comprehensive understanding of the regulation of DNA methylation it is now necessary to systematically quantify these interactions and modifications, to elucidate their function at the molecular level and to integrate these data at the cellular level.

Introduction

Multicellular organisms contain a variety of highly specialized cells that albeit their morphological and functional differences contain the same genetic information. During development different sets of genes are activated and repressed to generate the cell type-specific functionality. It becomes more and more clear that cell type-specific gene expression patterns are established and maintained by a complex interplay of transcriptions factors and epigenetic regulators. In particular, DNA and histone modifications control the composition, structure and dynamics of chromatin and thereby regulate gene expression.[1-3]

DNA methylation was the first identified epigenetic modification and has been intensively studied for half a century. DNA methylation is the post-replicative addition of methyl groups to the C5 position of cytosines catalyzed by a family of DNA methyltransferases (Dnmts). All C5 Dnmts share the same conserved motifs involved in the complex molecular mechanism of methyl group transfer best studied in the prokaryotic Dnmt M.HhaI (Fig. 1A).[4] The mechanism involves Dnmt binding to the DNA, flipping the target cytosine out of the DNA double helix, covalent bond formation of a conserved cysteine nucleophile with the cytosine C6, transfer of a methyl group from S-adenosyl-L-methionine to the activated cytosine C5 and Dnmt release by β-elimination.[4, 5]

In vertebrates, there are five members of the Dnmt family known that differ in structure and function and apart for DNMT2 all Dnmts compromise an N-terminal, regulatory domain in addition to the C-terminal, catalytic domain (Fig. 1A). The Dnmt3 subfamily, comprising DNMT3a and DNMT3b, shows activity towards unmethylated DNA and establishes *de novo* DNA methylation patterns during gametogenesis and embryogenesis.[6-8] The cofactor DNMT3L[9] specifically recognizes unmethylated histone H3K4[10] and stimulates the activity of DNMT3a and DNMT3b[11] but by itself lacks enzymatic activity.[12] Established DNA methylation patterns are maintained during DNA replication and DNA repair by the ubiquitously expressed DNMT1[13, 14] that displays a strong preference for hemimethylated CpG sites, the substrate of maintenance DNA methylation.[15] The fifth member of the Dnmt family, DNMT2, shows very weak activity towards DNA and was instead shown to methylate cytoplasmic $tRNA^{Asp}$.[16, 17]

Despite their common catalytic mechanism all three active eukaryotic Dnmts have distinct and non redundant functions which are likely mediated by their diverse regulatory domains (Fig. 1A). In

contrast to prokaryotic Dnmts, the tissue-specific *de novo* Dnmts, DNMT3a and DNMT3b are only active on a subset of target CpG sites[18] reflecting a selective control imposed by the N-terminal, regulatory domain. The ubiquitously expressed DNMT1 preferentially methylates hemimethylated CpG sites and thereby maintains DNA methylation patterns after DNA replication and DNA repair.[13-15] Remarkably, the catalytic domain of DNMT1 – although possessing all conserved motifs – is by itself not catalytically active and requires allosteric activation by the N-terminal, regulatory domain.[19-21] In this review we will focus on a detailed description of DNMT1 regulation to illustrate the extremely complex and interconnected regulation of DNA methylation.

Structure and function of DNMT1

Bioinformatic analysis suggested that mammalian DNMT1 evolved by fusion of at least three ancestral genes.[20] The N-terminal domain of DNMT1 harbors a proliferating cell nuclear antigen (PCNA) binding domain (PBD), a targeting sequence (TS domain), a zinc finger domain (CXXC) and two bromo-adjacent homology domains (BAH1/2) and is connected to the C-terminal, catalytic domain by seven lysyl-glycyl dipeptide repeats.[22, 23] The PBD domain has been shown to mediate the interaction with PCNA leading to the association of DNMT1 with the replication machinery.[13, 24, 25] The TS domain mediates association with heterochromatin and may lead to dimerization of DNMT1.[13, 25, 26] The CXXC domain of DNMT1 binds to unmethylated DNA but its deletion did not alter the activity and specificity of DNMT1.[27] Interestingly, the isolated C-terminal, catalytic domain of DNMT1, although harboring all conserved Dnmt motifs, requires an additional large part of the N-terminal domain for enzymatic activity.[19-21] Fusion of this N-terminal region of DNMT1 to the prokaryotic Dnmt M.HhaI induced a preference for hemimethylated DNA.[28] Cleavage between the regulatory and the catalytic domain stimulated the initial velocity of methylation of unmethylated DNA without substantial change in the rate of methylation of hemimethylated DNA.[29] These findings illustrate the role of the N-terminal domain in the selective activation of the catalytic domain to ensure faithful maintenance of DNA methylation patterns.

First insights into the molecular mechanism of DNMT1 regulation were provided by two recently published crystal structures comprising either a large fragment of mouse DNMT1 (aa 291-1620) (PDB ID code 3AV4) or a shorter fragment (aa 650-1602) in complex with unmethylated DNA (PDB ID code 3PT6).[30, 31] Notably, the overall structure of the eukaryotic catalytic domain is highly similar to the prokaryotic M.HhaI (PDB ID code 5mht) (Fig 1B/C).[32] In addition, both structures showed an interaction of N-terminal, regulatory domain with the C-terminal, catalytic domain[30, 31] which is consistent with prior genetic and biochemical studies.[19, 33] Remarkably, the TS domain was found deeply inserted into the catalytic pocket and would in this conformation prevent DNMT1 binding to hemimethylated DNA, the substrate for maintenance DNA methylation.[31] These structural insights indicate that DNMT1 most likely undergoes several conformational changes during the methylation reaction. Activation of DNMT1 requires displacement of the TS domain from the DNA binding pocket to allow substrate binding. Indeed, deletion of the TS domain lowered the activation energy[32] and addition of purified TS domain inhibit DNMT1 methylation activity *in vitro*[34] clearly pointing to an auto-

inhibitory function of the TS domain. These findings suggest that interacting proteins may modulate the interactions of the N-terminal domain with the catalytic domain and thereby regulate the activity of DNMT1 *in vivo*.

Regulation of DNMT1 activity by interacting factors

Over the last two decades, a large variety of proteins was found to interact with DNMT1 ranging from DNA methyltransferases, DNA binding proteins, chromatin modifiers and chromatin binding proteins to tumor suppressors, cell-cycle regulators and transcriptional regulators (Fig. 2 and Table 1).

The discrepancy between the high processivity of DNA replication (~ 0.035 seconds per nucleotide)[35] and the low turnover rates (70-450 seconds per methyl group) of recombinant DNMT1[36] *in vitro* suggests that additional mechanisms increase the activity of DNMT1 *in vivo*. Indeed, DNMT1 associates with the replication machinery[13] by direct binding to PCNA, a homotrimeric ring serving as a common loading platform for replication factors.[24, 37-39] Binding of DNMT1 to PCNA enhances the methylation activity about twofold, but is not strictly required for maintaining DNA methylation *in vivo*. [40, 41] Thus, the transient association between DNMT1 and PCNA alone cannot bridge the gap between the slow *in vitro* kinetics of DNMT1 and the fast progression of the replication fork *in vivo*.

Although DNMT1 can bind hemimethlytated CpG sites by itself it also interacts with methyl-CpG binding proteins like MeCP2[42], MBD2/3[43] and the UHRF family.[23, 44] MeCP2 binds DNA, induces chromatin compaction[45, 46] and interacts with DNMT1 via its transcription repressor domain (TRD).[42] MeCP2 and MBD2, that specifically recognize fully methylated CpG sites[47] and MBD3 also form complexes with histone deactelyases HDAC1 and HDAC2 which in turn interact with DNMT1.[42, 48-51] This set of interactions explain the correlation between DNA hypermethylation with histone hypoacetylation at transcriptional inactive regions[52] suggesting a role in transcriptional repression.[53] This complex may also comprise the DNMT1 association protein (DMAP1)[51, 54] and the transcriptional co-regulator Daxx[55] mediating repression in an HDAC-independent manner.[51, 54] In addition, the interaction with methyl-CpG binding proteins and HDACs may enrich DNMT1 in highly methylated heterochromatic regions to increase local methylation efficiency and/or enhance heterochromatin formation at these highly methylated regions.

Recently, UHRF1 has emerged as an essential co-factor for maintenance DNA methylation. The genetic ablation of *uhrf1* in embryonic stem cells (ESCs) leads to genomic hypomethylation similar to *dnmt1 -/-* ESCs. [56, 57] UHRF1 co-localizes and interacts with DNMT1 throughout S-phase and preferentially binds hemimethylated DNA via a SET and Ring associated (SRA) domain and the H3K9me3 heterochromatin mark via a tandem Tudor domain.[58-62] The crystal structure of the SRA

domain in complex with hemimethylated DNA revealed that the 5-methylcytosine is flipped out of the DNA double helix, a configuration that would stabilize the UHRF1-DNA interaction.[58-62] These results suggest that DNMT1 does not directly bind its substrate, the hemimethylated DNA, but is rather recruited by UHRF1. Recently, also the second member of the UHRF family, UHRF2, was shown to interact with DNMT1 and repressive epigenetic marks. [63] The similar but slightly different structure and function of UHRF1 and UHRF2 on one hand and their opposite expression pattern on the other hand suggest non-redundant functions during development.[63] In addition, UHRF1, UHRF2 as well as DNMT1 bind to the *de novo* Dnmts DNMT3a and DNMT3b.[63-68] These results demonstrate a complex interplay between methyl-CpG binding proteins and Dnmts in establishing genomic DNA methylation patterns.

Besides the interaction with methyl-CpG binding proteins, DNMT1 also associates with a number of proteins involved in establishment and maintenance of heterochromatin structure. DNMT1 interacts with the major eukaryotic histone methyltransferases Suv39H1[69] and EHMT2[70-73] (also known as G9a) that are essential for H3K9 methylation.[66, 74] Genetic ablation of *ehmt2*[74, 75] and *suv39h*[66] in mouse ESCs leads to DNA hypomethylation at specific loci in genomic DNA and altered DNA methylation of pericentric satellite repeats, respectively. Interestingly, also the H3K9me3 binding heterochromatin protein HP1β was shown to interact with DNMT1[69] but localization of DNMT1 at late replicating chromocenters seemed to be independent of SUV39H1/2 and HP1β.[25] In addition, DNMT1 interacts with Polycomb group (PcG) proteins EZH2[76, 77] and EED[76] that are subunits of the PRC2/EED-EZH2 complex, which methylates H3K27. EZH2 was shown to recruit DNMT1 to target genes and thereby mediates promoter methylation.[76]

In addition to the histone modifying enzymes, also chromatin remodeling ATPases like LSH are required for maintaining DNA methylation in mammals. LSH is related to the SNF2 family of chromatin-remodeling ATPases and forms a complex with DNMT1, DNMT3b and HDACs suggesting a role in transcriptional repression.[78, 79] Similarly, BAZ2A (also known as TIP5), the large subunit of the nucleolar remodeling complex (NoRC), forms a complex with DNMT1, DNMT3s, HDACs and hSNF2H mediating recruitment to rDNA[80]. One component of this complex, the chromatin remodeler hSNF2H, increases the binding affinity of DNMT1 to mononucleosomes.[81] The interaction with these chromatin remodeling factors may help DNMT1 accessing substrate sites in heterochromatic regions.

Besides indirect connections to transcriptional regulation, also direct interactions of DNMT1 with transcription regulators and factors have been described. CXXC1 (CFP1), a component of the SetD1A/B methyltransferase complex, binds to DNMT1 and *Cxxc1*-deficient ESCs display reduced levels of global DNA methylation.[82] Moreover, DNMT1 was shown to interact with the transcription factors SP1[83], SP3[83] and STAT3.[84] A STAT3-DNMT1-HDAC1 complex binds to the promoter of *shp1,* encoding a negative regulator of cell signaling, inducing cell transformation.[84] Also RIP140, a co-repressor for nuclear receptors, interacts with DNMT1 and DNMT3s in gene repression.[85]

Additional DNMT1 interactions have been reported with various tumor suppressor genes including WT1[77], Rb[50, 86], p53[87] and hNaa10p.[88] The Wilms tumor suppressor protein (WT1) recruits DNMT1 to the *Pax2* promoter resulting in DNA hypermethylation.[77] The retinoblastoma (Rb) tumor supressor gene product associates with DNMT1 and the transcription factor E2F1 resulting in transcriptional repression of E2F1-responsive promoters.[50] The interaction with p53 stimulates DNMT1 activity leading to hypermethylation.[87] Finally, the tumor suppressor hNaa10p recruits DNMT1 to promoters of tumor suppressor genes and increases the DNA binding affinity of DNMT1.[88] Besides a role in normal gene regulation also connections with deregulated gene expression in cancer were reported. The leukemia-promoting PML-RAR fusion recruits DNMT1 to target promoters inducing gene hypermethylation, an early step of carcinogenesis.[89]

For the sake of completeness, the following DNMT1 interacting proteins should be mentioned even though the function of these interactions is still unclear. The first described interaction partners of DNMT1 were Annexin V[90] and the molecular chaperone p23[91]. Also the function of DNMT1 interactions with RGS6, a protein negatively regulating the heterotrimeric G protein signalling[92], with PARP-1[93, 94] and with HESX1[95] remain elusive. The diversity of proteins reported to interact with DNMT1 provides a first insight into the complexity of the epigenetic network and illustrates the central role of DNMT1 in these regulatory pathways.

Regulation of DNMT1 activity by modifications

While most of the interacting factors regulate DNMT1 activity by recruitment to specific DNA sequences and target genes some of them have been described to modulate DNMT1 activity by post-translational modifications (Fig. 3). Recent publications described different post-translational modifications that influence the stability, abundance and activity of DNMT1.

DNMT1 was shown to undergo cell cycle dependent changes in acetylation and ubiquitination. [96-98] On the one hand, DNMT1 is acetylated by KAT5 and subsequently ubiquitinated by the E3 ubiquitin ligase UHRF1 marking DNMT1 for proteolytic degradation.[97] On the other hand, DNMT1 is deubiquitinated by USP7 (also known as Herpes virus-associated ubiquitin-specific protease Hausp) and deacetylated by HDAC1 protecting DNMT1 from proteolytic degradation.[97, 98]

Actually, the first post-translation modification described was the phosphorylation of Dnmt1 at Ser515 (DNMT1 Ser509)[99] (Fig 3A) that was suggested to modulate the interaction between the regulatory and catalytic domain.[100] In addition, Dnmt1 is phosphorylated at Ser146 (unique for mouse) by the casein kinase 1δ/ε decreasing the DNA binding affinity[101] and at not further mapped sites in the regulatory domain by the family of PKCs.[102] Notably, it has been reported that the phosphorylation of DNMT1 disrupts the DNMT1/PCNA/UHRF1 interaction promoting global DNA hypomethylation in human gliomas.[103] Phosphorylation of Ser143 by AKT1 during early and mid-S phase has been described to stabilize DNMT1.[104] S143 phosphorylation in turn blocks methylation of the adjacent Lys142 by SETD7 that marks DNMT1 for proteolytic degradation during late S-phase and G2.[104, 105]

Besides Lys142, SET7/9 can also methylate Lys1096 of Dnmt1 (Lys1094 of DNMT1), which destabilizes the enzyme. The corresponding demethylation by KDM1A (also known as lysine specific demethylase LSD1 and AOF2) in turn increases the stability of Dnmt1.[106] Consistently, the genetic ablation of *kdm1a* in ESCs led to progressive loss of DNA methylation[106] and caused cellular differentiation.[107] Finally, DNMT1 is also sumoylated by the ubiquitin-conjugating enzyme UBC9 leading to increased catalytic activity on genomic DNA *in vitro.*[108]

Perspective

This ever-increasing list of interacting factors and post-translational modifications reported for Dnmt1 impressively illustrates the complexity of the regulation of DNA methylation *in vivo*. As many of these interactions and modifications were identified in different cells and species using different and mostly qualitative methods, it is now essential to systematically and quantitatively analyze which of them occur in which combination, in which phase of the cell cycle, at what stage of cellular differentiation, in which cell types and in which fraction of the cellular DNMT1 protein pool. Clearly, the availability of first structural data on DNMT1 helps to elucidate the function of these interactions and modifications in the regulation of DNMT1 at the molecular level.[30, 31, 109] The hardest part, however, is the integration of all these data to comprehend DNMT1 regulation in the context of living cells taking into account the complexity and dynamics of its natural substrate, the chromatin, as well as the competition or cooperation with countless other cellular proteins and processes.

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Figure Legends

Figure 1.

Structural insights into the DNA methyltransferase 1 (DNMT1). **A**. Schematic outline of the domain architecture of mammalian Dnmts in comparison to the prokaryotic Dnmt M.HhaI. All Dnmts harbor highly conserved motifs (I-X) in the C-terminal, catalytic domain. In addition, DNMT1 harbors a proliferating cell nuclear antigen (PCNA) binding domain (PBD) followed by the targeting sequence (TS domain), a zinc finger domain (CXXC), two bromo-adjacent homology domains (BAH1/2). DNMT3a and DNMT3b comprise a PWWP domain and a PHD domain that is also found in DNMT3L. **B**. Crystal structure of the prokaryotic cytosine-(C5) methyltransferases M.HhaI in complex with hemimethylated DNA (PDB ID code 5mht).[32] **C**. Crystal structure of the large fragment (291-1620) of mouse DNMT1 (PDB ID code 3AV4)[31] in superimposition with the prokaryotic structure (B) showing the expected steric clash between the TS domain and DNA binding in the catalytic pocket. The single domains are color-coded as in (A)**.**

Figure 2.

Overview of DNMT1 interacting proteins. Interacting proteins range from DNA methyltransferases, DNA binding proteins, chromatin modifiers and chromatin binding proteins to tumor suppressors, cell-cycle regulators and transcriptional regulators. Proteins involved in the post-translation modification of DNMT1 are highlighted in green.

Figure 3.

Regulation of DNMT1 by post-translational modifications. **A**. Schematic outline of the domain architecture of DNMT1. The lysine residues K142 and K1094 are methylated and the serine residues S143 and S509 phosphorylated. In addition, the murine Dnmt1 is phosphorylated at S146. The conservation of known modified sites are shown in sequence logos by WebLogo[110]. The input to WebLogo was a ClustalW[111] alignment of DNMT1 from different species (*Homo sapiens* P26358.2; *Bos Taurus* DAA27999.1; *Mus musculus* P13864.5; *Rattus norvegicus* Q9Z330.2; *Gallus gallus* Q92072.1; *Paracentrotus lividus* Q27746.1 and *Danio rerio* AAI63894.1). **B**. Crosstalk between DNMT1 and interacting proteins leading to post-translational modifications of DNMT1. Following

abbreviations are use: Ac = Acetylation; Ub = Ubiquitination; SUMO = Sumoylation; P = Phosphorylation; Me = Methylation. Dotted line indicates a hypothetical dephosphorylation.

Table 1.

Overview of DNMT1 interacting proteins. The function of proteins is according to the UniProt database[112, 113] and the applied method is according to the BioGRID[114] database apart from the fluorescent two-hybrid (F2H) assay.[115] All protein names are according to the human nomenclature unless noted otherwise. Proteins involved in the post-translation modification of DNMT1 are highlighted in green.

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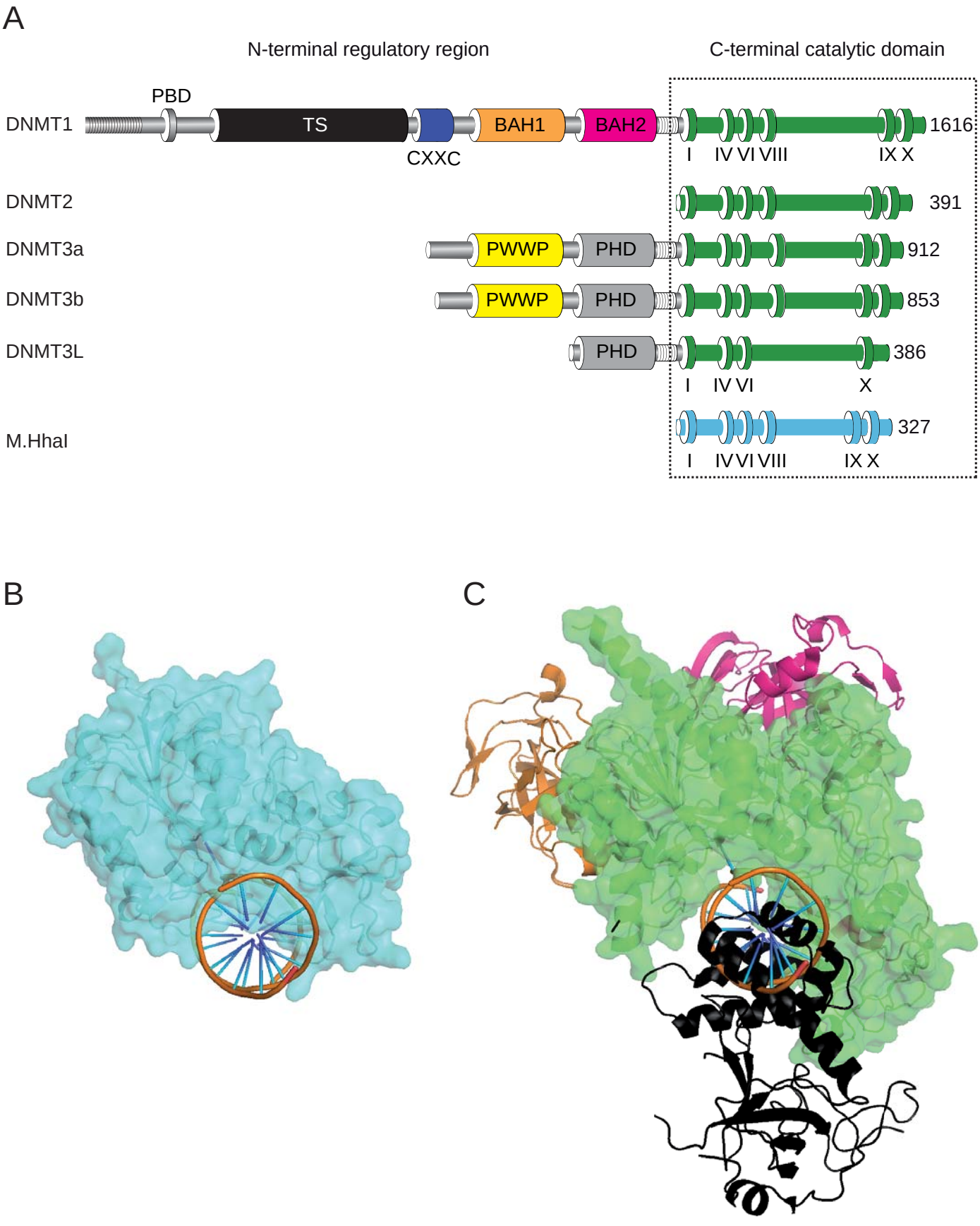
Figure 1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N-terminal regulatory region
C-terminal catalytic domain
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DNMT1
TS
CXXC
BAH1
BAH2
1616
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DNMT2
391
DNMT3a
PWWP
PHD
912
DNMT3b
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DNMT3L
PHD
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M.HhaI
327
I IV VI VIII IX X
B
C

Figure 2

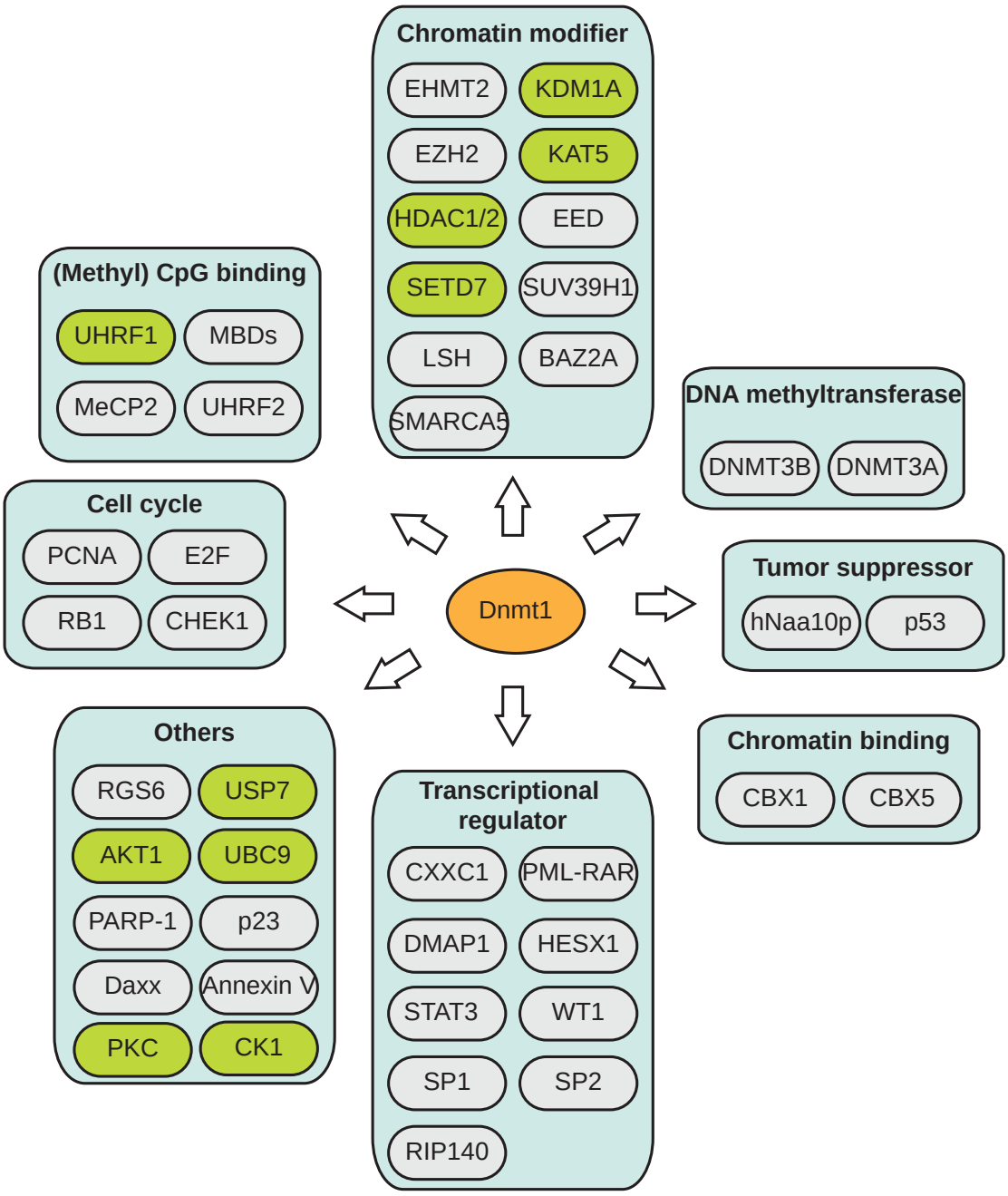
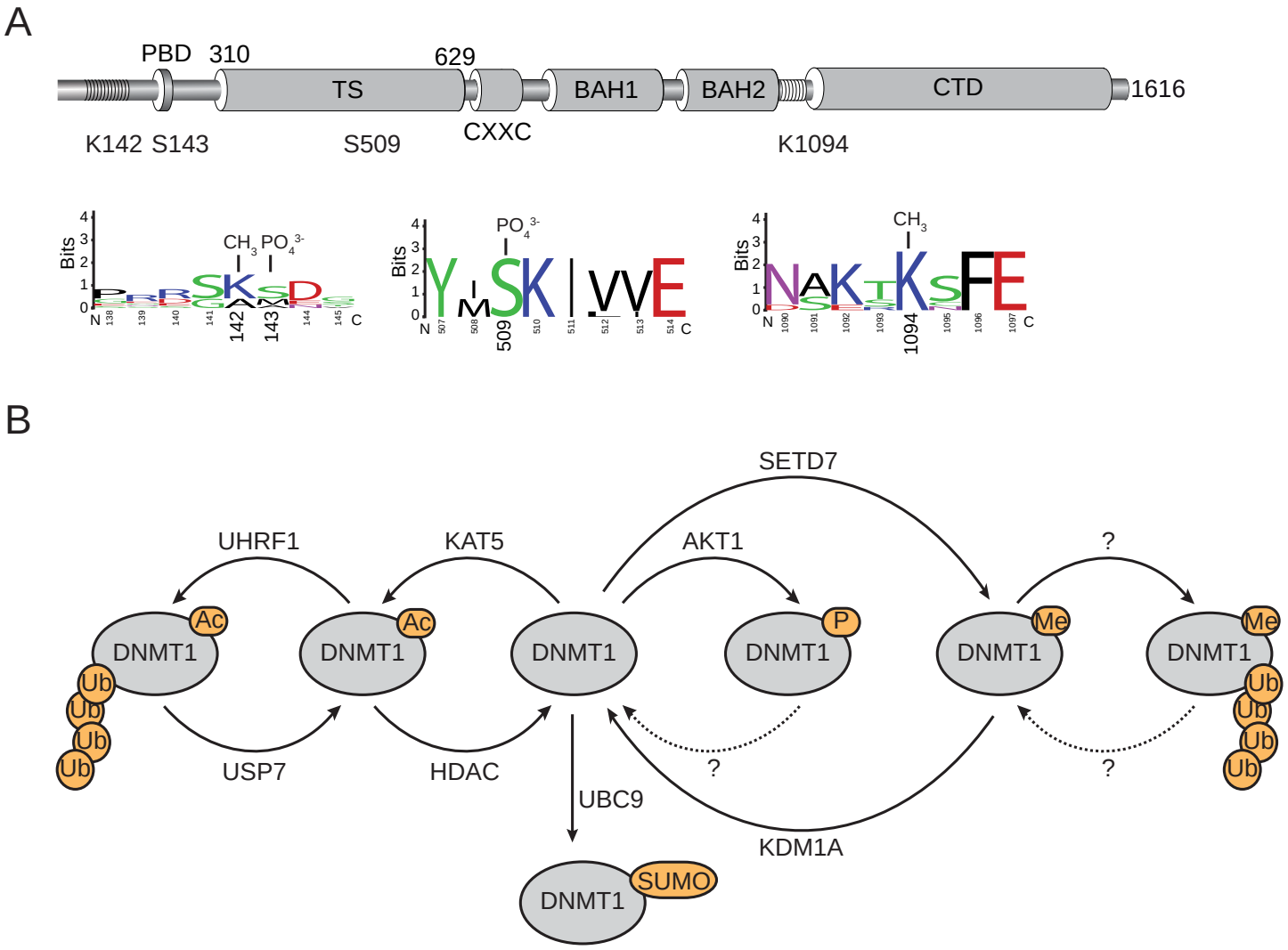

Chromatin modifier
EHMT2
KDM1A
EZH2
KAT5
HDAC1/2
EED
SETD7
SUV39H1
LSH
BAZ2A
SMARCA5
(Methyl) CpG binding
UHRF1
MBDs
MeCP2
UHRF2
DNA methyltransferase
DNMT3B
DNMT3A
Cell cycle
PCNA
E2F
RB1
CHEK1
Dnmt1
Tumor suppressor
hNaa10p
p53
Others
RGS6
USP7
AKT1
UBC9
PARP-1
p23
Daxx
Annexin V
PKC
CK1
Transcriptional regulator
CXXC1
PML-RAR
DMAP1
HESX1
STAT3
WT1
SP1
SP2
RIP140
Chromatin binding
CBX1
CBX5

Figure 3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CXXC
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143
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CH3
1094
B
SETD7
UHRF1
KAT5
AKT1
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DNMT1
Ac
P
Me
Ub
USP7
HDAC
UBC9
KDM1A
SUMO

Table 1

Interacting Protein	Function	Method	Reference
Trancriptional regulator			
DMAP1	Involved in transcription repression and activation Component of the NuA4 histone acetyltransferase (HAT) complex	Yeast 2-hybrid (Y2H) Affinity Capture-Western (AC/WB) Reconstituted Complex	*Rountree et al. 2000*
PML-RAR	Transcriptional regulator of retinoic acid (RA) target genes; induces gene hypermethylation and silencing by recruiting Dnmt1	AC/WB	*Di Croce et al., 2002*
HESX1	Required for the normal development of the forebrain, eyes and other anterior structures; Possible transcriptional repressor	Y2H AC/WB	*Sajedi et al., 2008*
CXXC1 (CFP1)	Transcriptional activator that exhibits a unique DNA binding specificity for [AC]CpG[AC] unmethylated CpG motifs	AC/WB	*Butler et al., 2008*
SP1	Transcription factor	AC/WB	*Esteve et al.; 2007*
SP3	Transcription factor	AC/WB	*Esteve et al.; 2007*
STAT3	Transcription factor that binds to the interleukin-6 (IL-6)-responsive elements	AC/WB Co-localization	*Zhang et al.; 2005*
WT1	Transcription factor that plays an important role in cellular development and cell survival	AC/WB	*Xu et al.; 2011*
RIP140	Modulates transcriptional activation and repression	AC/WB	*Kiskinis et al.; 2007*
Chromatin modifier			
HDAC1/2	Deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4)	AC/WB Co-fractionation Reconstituted Complex Y2H	*Fuks et al.,2000 Rountree et al.; 2000 Achour et al.;2008 Kimura et al.; 2003 Robertson et al.;2000 Myant et al.; 2008;*
KDM1A (AOF2; LSD1)	Histone demethylation of H3K4me and H3K9me; Demethylation of DNMT1	AC/WB	*Wang et al., 2008*
SUV39H1	Histone methyltransferase that specifically trimethylates H3K9	AC/WB	*Fuks et al., 2003*
EHMT2 (G9a)	Histone methyltransferase that specifically mono- and dimethylates H3K9	AC/WB	*Estève et al., 2006 Kim et al.,2009 Tachibana et al 2002; Peters et al., 2003*
EZH2	Polycomb group (PcG) protein. Catalytic subunit of the PRC2/EED-EZH2 complex	AC/WB Reconstituted complex	*Vire et al., 2006; Xu et al; 2011*
EED	Polycomb group (PcG) protein. Component of the PRC2/EED-EZH2 complex	AC/WB Co-localization	*Vire et al., 2006*
SETD7	Histone methyltransferase that specifically monomethylates H3K9	AC/WB Biochemical Activity	*Esteve et al.; 2009*

KAT5 (TIP60)	Catalytic subunit of the NuA4 histone acetyltransferase complex	AC/WB	*Du et al.; 2010*
LSH	Chromatin remodeling	AC/WB	*Myant and Stancheva, 2008*
SMARCA5 (SNF2H)	Helicase that possesses intrinsic ATP-dependent nucleosome-remodeling activity	AC/WB	*Robertson et al.; 2004*
BAZ2A (TIP5)	Essential component of the NoRC (nucleolar remodeling complex) complex	Reconstituted Complex	*Zhou et al.; 2005*
(Methyl) CpG binding proteins			
MeCP2	Methylated CpG binding protein	AC/WB Reconstituted complex	*Kimura and Shiota, 2003*
MBD2/MBD3	Methylated CpG binding protein	AC/WB	*Tatematsu et al., 2000*
UHRF1	Hemimethylated CpG binding protein; essential for maintenance DNA methylation	Y2H AC/WB Reconstituted complex Co-localization	*Bostick et al., 2007* *Sharif et al., 2007* *Arita et al., 2008; Awakumov et al.; 2008; Qian et al., 2008* *Achour et al,; 2008* *Meilinger et al.; 2009*
UHRF2	E3 ubiquitin-protein ligase which mediates ubiquitination and subsequent proteasomal degradation of PCNP; Hemimethylated CpG binding protein	AC/WB	*Pichler et al.; 2011*
Tumor suppressor			
p53	Tumor suppressor in many tumor types	AC/WB	*Esteve et al., 2005*
hNaa10p	Tumor suppressor Stimulate Dnmt1 activity	AC/WB	*Lee et al., 2010*
DNA methyltransferase			
DNMT3a	Required for genome wide de novo methylation and is essential for the establishment of DNA methylation patterns during development	AC/WB Reconstituted Complex	*Fatemi et al., 2002* *Kim et al., 2002*
DNMT3b	Required for genome wide de novo methylation and is essential for the establishment of DNA methylation patterns during development	AC/WB Reconstituted Complex	*Rhee et al., 2002* *Kim et al., 2002* *Lehnertz et al.; 2003* *Geiman 2004*
Chromatin binding protein			
HP1β	Recognizes and binds methylated H3K9	Reconstituted Complex	*Fuks et al.; 2003*
CBX5	Recognizes and binds methylated H3K9	AC/WB	*Lehnertz et al.; 2003*
Cell cycle regulator			
PCNA	Targeting Dnmt1 to replication foci	AC/WB Reconstituted Complex Co-localization	*Leonhardt et al.,1992* *Chuang et al.; 1997* *Rountree et al.; 2000*
RB1	Cell cycle regulator	AC/WB Reconstituted Complex Co-fractionation	*Robertson et al., 2000* *Pradhan et al.; 2002*
E2F1	Transcription factor Play a role in controlling cell cycle entry	Co-fractionation	*Robertson et al., 2000*
CHEK1 (CHK1)	checkpoint mediated cell	AC/WB	*Palii et al.; 2008*

	cycle arrest in response to DNA damage or the presence of unreplicated DNA		
Others			
RGS6	Regulator of G-protein signaling	Reconstituted Complex	*Liu et al; 2004*
USP7	Hydrolase that deubiquitinates target proteins	AC/Mass Spectrometry AC/WB F2H	*Sowa et al.; 2009* *Qin et al.; 2010* *Du et al.; 2010*
UBC9	E2 ubiquitin-conjugating enzyme	AC/WB	*Lee et al.; 2009*
AKT1	RAC-alpha serine/threonine-protein kinase	AC/WB Co-localization	*Esteve et al.; 2010*
PARP-1	Poly [ADP-ribose] polymerase 1	AC/WB Reconstituted Complex	*Reale et al.; 2005* *Zampieri et al.; 2009*
p23	Molecular chaperone	AC/WB	*Zhang and Verdine 1996*
Daxx	Adapter protein in a MDM2-DAXX-USP7 complex	AC/WB Co-localization	*Muromoto et al.; 2004*
Annexin V	Anticoagulant protein	AC/WB	*Ohsawa et al.; 1996*
PKCs	Protein kinase C	AC/WB Co-localization	*Lavoie et al.; 2011*
CK1 δ/ε	Casein kinase 1δ/ε	AC/WB	*Sugiyama et al.; 2010*

3. Discussion

3.1. The regulation of Dnmt1 in living cells is still unclear

3.1.1. Regulation of Dnmt1 by posttranslational modification during the cell cycle

Over the past decades many interacting proteins were reported to regulate Dnmt1. In this thesis, the regulation of Dnmt1 function by its interacting partners Usp7 and Uhrf1 was investigated. We showed that Uhrf1 and Usp7 control the stability of Dnmt1 by controlling its ubiquitination state. At the same time Du *et al* described a mechanism for the control of DNMT1 stability involving destabilization of DNMT1 by Tip60-mediated acetylation and UHRF1-mediated ubiquitination as well as stabilization by HDAC1-mediated deacetylation and Usp7-mediated deubiquitination, thus confirming and extending our results (Du *et al.*, 2010).

Tight control of Dnmt1 expression may be critical for preservation of normal DNA methylation levels and patterns. Low levels of Dnmt1 resulted in global hypomethylation, tumor formation and cell death (Gaudet et al., 2003; Eden et al., 2003; Yang et al., 2003). In contrast, the overexpression of *dnmt1* leads to hypermethylation (Biniszkiewicz et al., 2002). Recently, Pradhan *et al* pointed out that the abundance of DNMT1 peaks in early S phase and decreases during mid and late S phase (Esteve et al, 2009). More recent work from the same group suggests that the abundance of DNMT1 during the cell cycle is regulated by posttranslational modifications (Esteve et al., 2010; Fig. 10). Although the ubiquitination of Dnmt1 by Uhrf1 was observed, it remains elusive whether this modification also participates in the regulation of Dnmt1 abundance during the cell cycle.

The cell cycle dependant localization of Dnmt1 is mediated by its N-terminal regulatory domain. Immunostaining and coimmunoprecipitation experiments revealed that the PCNA-binding domain (PBD) and the targeting sequence domain (TS) are responsible for targeting Dnmt1 to replication sites and pericentric heterochromatin, respectively (Leonhardt et al., 1992; Easwaran et al., 2004, Schermelleh et al., 2007). Interestingly, we observed that the association of Dnmt1 with constitutive (pericentric) persists from the later part of mid S phase, when these sequences are replicated through to the G2 phase (Easwaran et al., 2004). This prolonged association of Dnmt1 with pericentric heterochromatin may be required to maintain the high levels of methylation of this genomic compartment, especially considering

the relatively low turnover rate of Dnmt1 as measured *in vitro* (Pradhan et al., 1999). However, the biological function and mechanism of this phenomenon is still not fully understood. A possibility is that the posttranslational modification of Dnmt1 might affect its localization as well as its stability during the cell cycle.

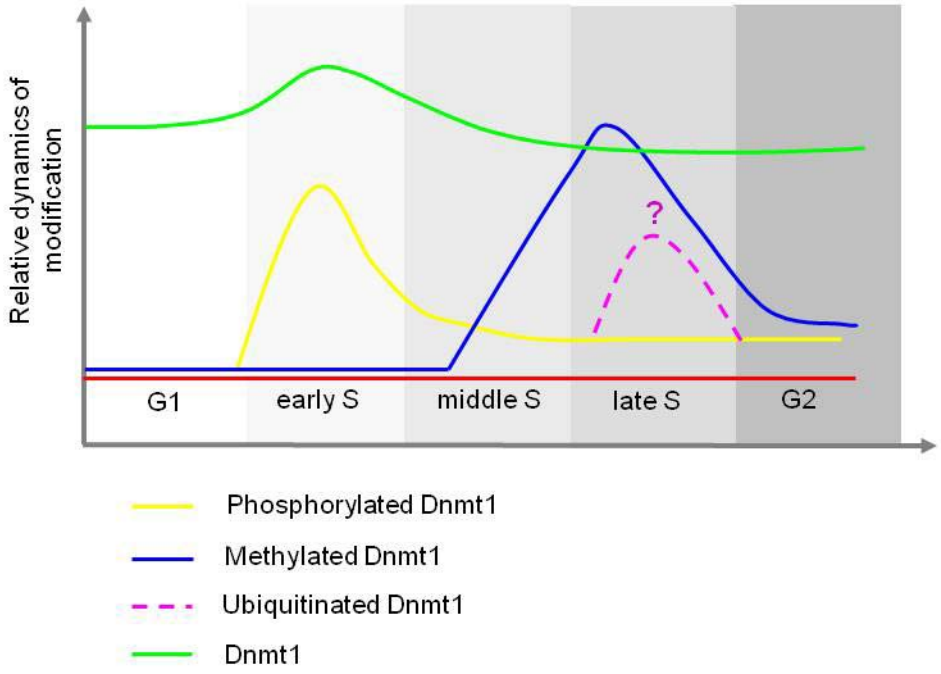

Figure 10. Dnmt1 abundance is regulated by posttranslational modification throughout the cell cycle
Dnmt1 protein levels peak in early S phase and drop after cells enter to mid S phase (green curve). Dnmt1 is modified by phosphorylation, methylation, acetylation, ubiquitination and sumoylation. However, so far cell cycle profiles of Dnmt1 posttranslational modification were investigated only for phosphorylation at Ser143 and methylation at Lys142. Phosphorylation of Dnmt1 at Ser143 occurs in early S phase (yellow curve), whereas Dnmt1 is methylated at Lys142 in late S phase to promote its degradation (blue curve). The ubiquitination of Dnmt1 is supposed to occur at late S phase.

Therefore, several interesting questions remain to be investigated, including whether DNA methylation levels are affected either globally or at specific loci upon depletion of *usp7*, how control of Dnmt1 stability by ubiquitination/acetylation correlates with that imparted by the recently described methylation/phosphorylation switch at lysine 142/serine 143 (Esteve et al., 2010), whether Dnmt1 acetylation and ubiquitination are cell cycle dependent, and where ubiquitination occurs.

To investigate whether the regulation of Dnmt1 stability by ubiquitination is cell cycle stage dependent, I first checked the colocalization of Dnmt1 and Usp7 during DNA replication. My data showed that in mouse embryonic fibroblasts (MEF) Usp7 accumulates at pericentric heterochromatin at the time of its replication (later part of mid S phase) suggesting that Usp7 might specifically protect Dnmt1 bound to constitutive heterochromatin to allow for maintenance of the heavily methylated repeats in this genomic compartment (Fig. 11).

However, this association of Usp7 with heterochromatin was not observed in ESCs (Fig. 12), suggesting that Usp7 might play different roles in undifferentiated pluripotent stem cells and differentiated cells.

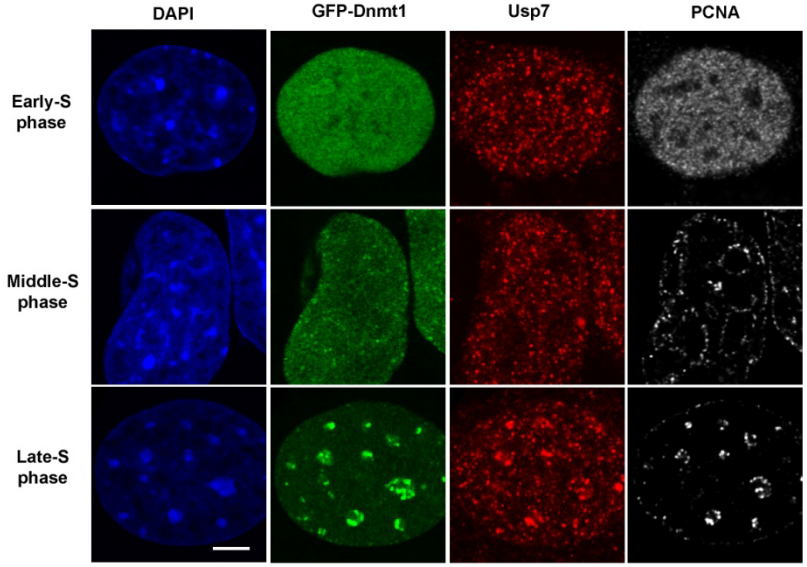

Figure 11. Colocalization of Dnmt1 and Usp7 during S phase in MEF cells
The *p53* and *dnmt1* double knockout MEF cells stably expressing GFP tagged Dnmt1 were used in this study. The endogenous PCNA was stained using an anti-PCNA antibody to identify replication foci and to distinguish S phase stages. Nuclear staining was performed with 4', 6-diamidino-2-phenylindole (DAPI) stain. GFP-Dnmt1 accumulates at replication sites throughout S phase where it colocalizes with PCNA. In contrast, the endogenous Usp7 stained with an anti-Usp7 antibody shows a fully dispersed nuclear distribution in early and mid S phase stages, whereas in late S phase an association with heterochromatin is observed. The scale bar stands for 5 µm.

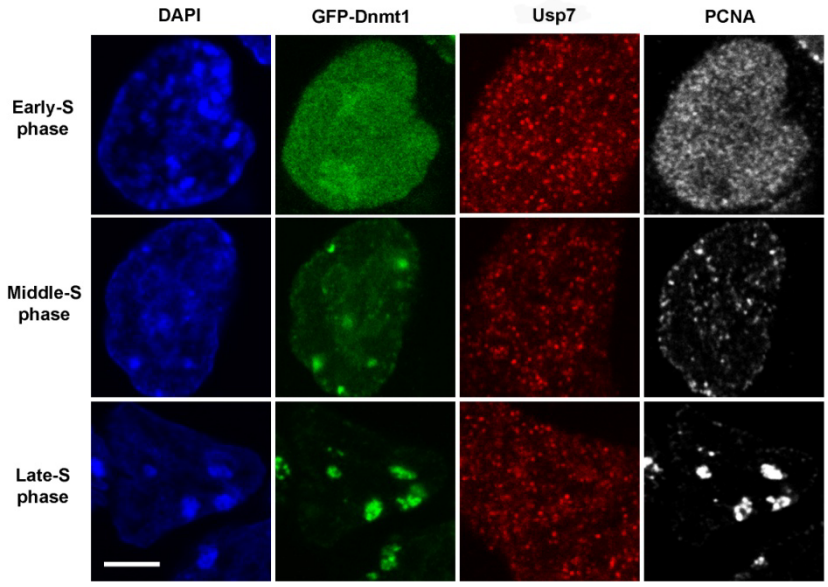

Figure 12. Colocalization of Dnmt1 and Usp7 during S phase stages of the cell cycle in ESCs
The *dnmt1* knockout ESCs stably expressing GFP tagged Dnmt1 were used in this study. The endogenous PCNA was stained using an anti-PCNA antibody to identify replication foci and to distinguish S phase stages. Nuclear staining was performed with DAPI stain. GFP-Dnmt1 accumulates at replication sites throughout S phase where it colocalizes with PCNA. In contrast, the endogenous Usp7 stained with an anti-Usp7 antibody shows a fully dispersed nuclear distribution during S phase. The scale bar stands for 5 µm.

Interestingly, Du *et al* showed biochemical evidence from synchronized human colorectal cancer cells that Dnmt1 strongly associates with Usp7 during early and mid S phases and starts being degraded in late S phase when its association with Uhrf1 is highest (Du et al., 2010). Thus, Usp7 associates with and stabilizes Dnmt1 till the end of pericentric heterochromatin replication. Starting around late S phase Usp7 dissociates with Dnmt1 and may be gradually replaced by Uhrf1, which drives Dnmt1 degradation by ubiquitinating it, possibly as methylation of pericentric DNA sequences is gradually completed. Therefore, my colocalization data actually complement the results of Du *et al*.

3.1.2. Usp7, Uhrf1 and Dnmt1 form a dynamic complex

Usp7 is identified as a novel interacting protein of Dnmt1 by co-immunoprecipitation in combination with mass spectrometry analysis. Due to the interaction between Dnmt1 and Uhrf1 was reported in 2007 (Achour et al., 2007; Bostick et al., 2007), I showed that Usp7 also associates with Uhrf1, suggesting that a dynamic complex including Dnmt1, Uhrf1 and Usp7 might exist in mammalian cells. Using immunostaining to monitor protein localization in cells, the distribution of Uhrf1 in cell nucleus dynamically changes during the cell cycle, showing a colocalization with Dnmt1 at replication sites, especially at heterochromatin during mid and late S phases, whereas Usp7 shows a dispersed distribution in the nucleus during DNA replication, but concentrated at heterochromatin with Dnmt1 only at late S phase, suggesting that the complex of Dnmt1/Usp7 and Dnmt1/Uhrf1 might exist at early and mid S phases *in vivo*, and a complex of Dnmt1, Usp7 and Uhrf1 might be formed at late S phase. Uhrf1 and Usp7 were reported to modulate Dnmt1 stability by ubiquitination and deubiquitination, but it is still unknown whether Usp7 is also involved in maintenance of DNA methylation.

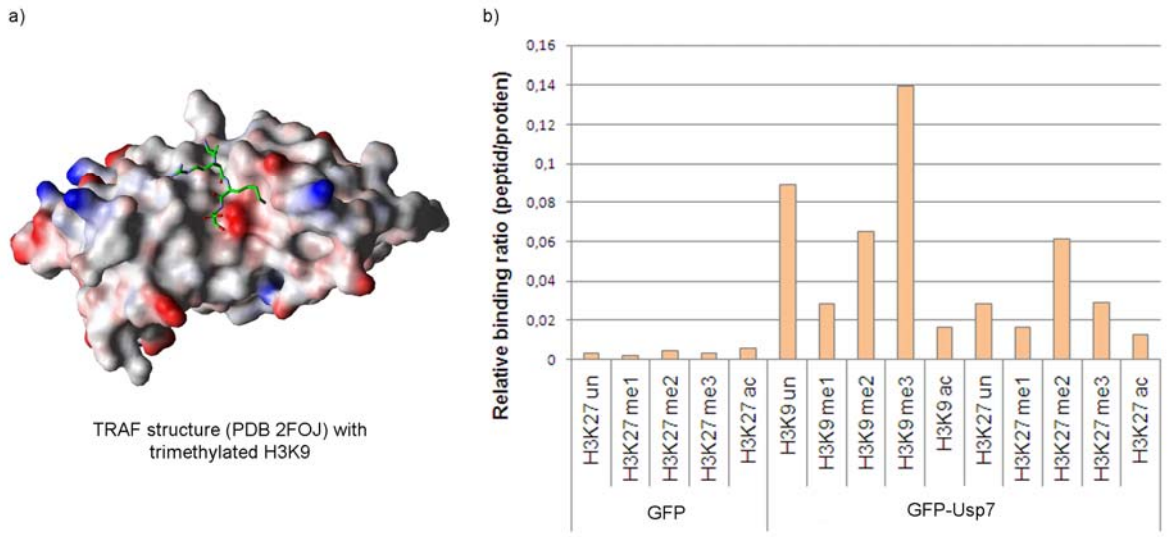

Figure 13. Structure and histone binding properties of GFP-Usp7
a) Side view of the TRAF domain as surface representation in complex with trimethylated lysine 9 (green stick model). The image was generated with PyMOL. The crystal structure of TRAF domain (PDB 2FOJ) is available in the PBD database (Saridakis et al., 2005). The trimethylated lysine 9 could fix into the cage in the surface of TRAF domain. b) The binding specificity of GFP-Usp7 for histone H3 N-terminal peptides. The histone H3 peptides containing either inactive chromatin marks trimethylated H3K9 or H3K27, or active marks acetylated H3K9 or H3K27 were tested in the binding specificity of GFP-Usp7. Y-axis presents fluorescence intensity ratios of bound probe to bound GFP fusion. GFP-Usp7 shows a preferential binding for trimethylated histone H3K9.

With an *in vitro* peptide binding assay Usp7 showed a preferential binding to trimethylated histone H3K9 and weakly binds to trimethylated histone H3K27 (Fig. 13b) in contrast to active chromatin marks, acetylated H3K9 and H3K27. The association of Usp7 with histone peptide might depend on its TRAF domain, since the trimethylated lysine 9 could fix in the cage of TRAF (Fig. 13a). Additionally, Usp7 was observed to mediate the deubiquitination of histone H2A and H2B (van der Knaap et al., 2005). Therefore, a possible mechanism that Uhrf1 and Usp7 coordinately signal for Dnmt1 binding and release from heterochromatin might be speculated (Fig. 14). In this proposed mechanism, Uhrf1 might initially modify histones by ubiquitination at unknown lysine sites as a mark for down-stream molecular binding, which subsequently modulate the accessibility of chromatin. Dnmt1 thereby can access its target sites. Then Usp7 may activate the reverse process to catalyze the removal of ubiquitin moiety from histone, resulting in possible chromatin rearrangement. Usp7 could accumulate at heterochromatin via the Dnmt1-Uhrf1 complex, or its TRAF domain.

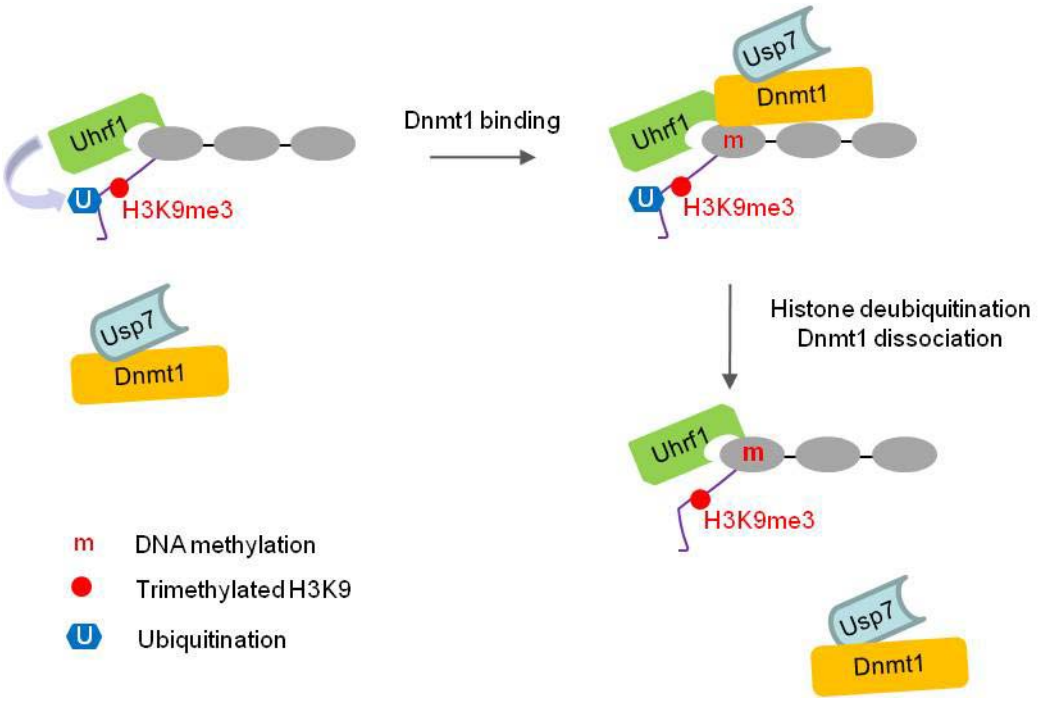

Figure 14. Proposed mechanism of Usp7 and Uhrf1 in recruiting Dnmt1 to chromatin
Uhrf1 recognizes and binds to chromatin through its SRA and tandem Tudor domains. Uhrf1 could transfer ubiquitin moiety (U) to histone H3 (Citterio et al., 2004) that might be as a signal for recruiting Dnmt1 and Usp7 to DNA containing CpG sites. After methylating (m) the cytosine, Usp7 catalyzes the deubiquitination of histone H3 to activate chromatin reorganization that might allow Dnmt1 dissociation from chromatin.

Uhrf1 is a chromatin binding protein and can tightly bind to chromatin containing trimethylated H3K9 and hemimethylated CpG sites. Due to a reduction of DNA methylation level was observed in *uhrf1* knockout mice (Bostick et al., 2007 and Sharif et al., 2007), Uhrf1 is addressed as essential cofactor of Dnmt1 for maintenance of DNA methylation. Considering Usp7 is also involved in Dnmt1-Uhrf1 complex, it is reasonable to speculate that Usp7 plays a role not only in protein stability, but also in the regulation of Dnmt1 activity. By *in vitro* trapping assay, here Usp7 was shown to increase the trapping rate of Dnmt1 at hemimethylated DNA. These observations were complement to the results of Du's. It was shown that global DNA methylation was not affected in *usp7*$^{-/-}$ human tumor cell lines. However, the DNA methylation level at imprinting gene *H19* was decreased to 65% by disruption of *usp7* (Du et al., 2010). Interestingly, *in vitro* Uhrf1 showed an inhibitory function in Dnmt1 activity resulting from its SRA domain, implicating that Uhrf1 might play multiple roles in the regulation of Dnmt1 activity. On one hand, Uhrf1 is essential for recruiting Dnmt1 to replication foci. But on the other hand, it plays a role in inhibition of Dnmt1 activity. Therefore, it could be interesting to clarify whether the opposite effect on DNA methylation by Usp7 and Uhrf1 is ubiquitination dependent or independent.

3.2. Uhrf1 ubiquitin activity and chromatin states

Based on the preferential binding of both hemimethylated CpG and H3K9me3 (Rottach et al., 2009), Uhrf1 was proposed to connect DNA methylation and histone modification. Additionally, Uhrf1 is a histone modifier, as it harbors a Ring domain which possesses ubiquitin E3 ligase activity. Thus, it might have function in modulating chromatin structure through its ubiquitin activity. *In vitro*, H2A, H2B and H3 were identified as substrates of Uhrf1 with similar efficiency (Citterio et al., 2004). Here, I confirmed that overexpression of Uhrf1 increases ubiquitination of histones *in vivo*, however it is still unclear which histones and which sites are modified. However, it was pointed out that the histone H3 could be the best substrate for Uhrf1 under conditions that more closely mimic the physiological conditions (Citterio et al., 2004). Interestingly, in this study I also showed that the ubiquitination activity of Uhrf1 was required for maintenance of DNA methylation by Dnmt1. Therefore, the ubiquitination of histone H3 by Uhrf1 might be required for maintaining DNA methylation.

In contrast to ubiquitination of H2A and H2B, the H3 ubiquitination is little characterized. In mammalian cells, the ubiquitination of H3 was first reported in meiotic and postmeiotic germ cells of rat testes (Chen et al., 1998). It was speculated that the ubiquitination of H3 could contribute to induce nucleosomal replacement and depletion, and thereby may open up the higher order nucleosome structure to increase the chromatin accessibility for the transcription machinery. Further, $E3^{Histone}$ was characterized as a HECT domain E3 ligase in mammalian spermatids, which is responsible for the ubiquitination of histone H3 (Liu et al., 2005). Moreover, CUL4-DDB-ROC1 was also identified as a histone ubiquitin ligase of histone H3 in human cells and the ubiquitylation of histone H3 is activated by UV irradiation, suggesting it participates in the cellular response to DNA damage (Wang et al., 2006). The RAG1 and RAG2 proteins are the only lymphoid specific factors. Recently, RAG1 was shown to preferentially interact with and promotes monoubiquitination of histone H3 by its Ring domain (Grazini et al., 2009). Monoubiquitination of histone H3 by RAG1 is involved in regulation the phase of chromosomal V(D)J recombination.

Ubiquitination of histone might serve as signal for repression or activation of other histone modifications, such as histone methylation and acetylation. Uhrf1-mediated H3 ubiquitination therefore might coordinate with other posttranslational histone modifications, like trimethylated histone H3K9 to modulate chromatin property. Also a cross-talk between

H3 ubiquitination and histone H4 acetylation might exist in cells, because depletion of Uhrf1 results in an increase of histone H4 acetylation at lysine 5, 8, 12 and 16 at pericentromeric heterochromatin region (Papait et al., 2007). Indeed it was shown that acetylated histone H4 contributes to open up condensed chromatin, suggesting that deacetylation of histone H4 is essential for maintaining chromatin compaction and silent state (Chen et al., 2000). In addition, it was found that Tip60 (a histone acetyltransferase with specificity toward lysine 5 of histone H2A) and HDAC1 (a histone deacetylase 1) present in a complex with Uhrf1 (Achour et al., 2009). Therefore, it can be speculated that a cross-talk between histone ubiquitination mediated by Uhrf1 and acetylation of histone exists *in vivo* and modulates the chromatin accessibility for subsequent chromatin modifiers, like DNA methyltransferases. To investigate this hypothesis, the exact site of ubiquitination in histone H3 need to be mapped to study its combination and the correlation of H3 ubiquitination and histone acetylation. An interesting question, how Dnmt1 access its target sites which are packaged into condensed chromatin, could be answered.

In addition, a functional PHD domain resides in Uhrf1 between the tandem Tudor and the SRA domain, which has been proposed as a reader of histone modification and binds to chromatin. The binding preference for histone modifications, including the methylated or acetylated H3K4 and H3K9, were investigated by *in vitro* peptide binding assay. In contrast to previous studies (Karagianni et al., 2008), the PHD domain of Uhrf1 showed no binding to any peptides tested (unpublished data by Patricia Wolf). Moreover, the point mutant of Uhrf1 substituted histidine at amino acid 346 to glycine failed to rescue DNA methylation patterns in *uhrf1* $^{-/-}$ ESCs, suggesting that the PHD domain of Uhrf1 is also involved in maintenance of DNA methylation. Since the tandem Tudor domain contributes to the binding of Uhrf1 to trimethylated histone H3K9, the PHD and tandem Tudor domains might coordinately bind to "bivalent" histone modifications (Fig. 15), a still unknown histone modification and the trimethylated histone H3K9. Since the PHD domain of Uhrf1 was reported to be involved in large-scale reorganization of pericentromeric heterochromatin, it was proposed that PHD domain might also contribute to open up the chromatin structure (Papait et al., 2008).

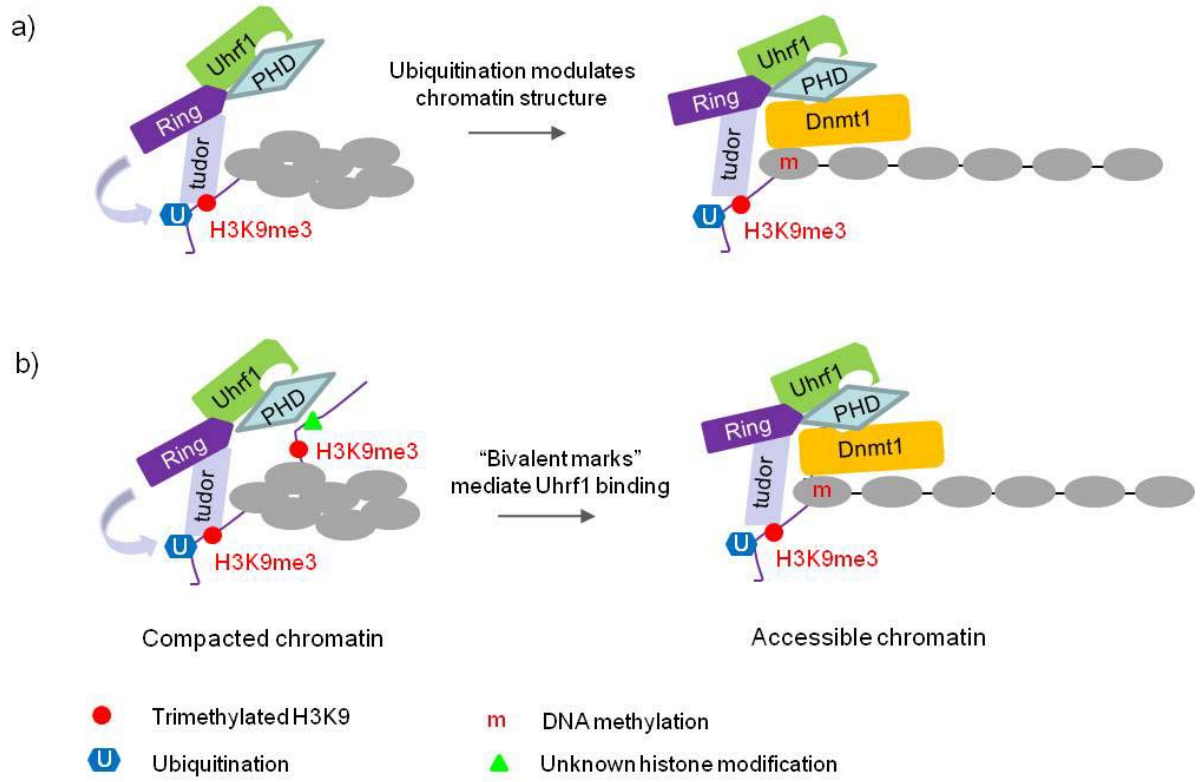

Figure 15. Uhrf1 might modulate chromatin accessibility through the PHD and the Ring domain
a) The tandem Tudor domain and the SRA domain recognize the histone H3K9me3 and the hemimethylated cytosines, respectively, that mediate Uhrf1 binding to chromatin. The Ring domain of Uhrf1 could modify histone H3 by ubiquitination (Citterio et al., 2004), which might be a signal for chromatin remodeling factor binding, to modulate chromatin accessibility. This histone modification and subsequent chromatin decondensation could help Dnmt1 access its target sites. b) The PHD domain of Uhrf1 might also be involved in modulating chromatin accessibility through recognizing an unknown histone modification.

3.3. Chromatin accessibility of Dnmt1 and histone modification

In eukaryotic cells, DNA is assembled to the chromatin units of nucleosome, which contain in addition to 147 base-pairs (bp) of DNA also a histone octamer of H2A, H2B, H3 and H4 proteins (Felsenfeld and Groudine, 2003). The linker DNA between two neighboring nucleosomes is ~70 bp in higher eukaryotes. DNA packaged in nucleosomes prevents transcription factors and DNA interacting proteins from access their target sites. However, the reduced accessibility of the target sites on nucleosomal DNA could be overcome by chromatin remodeling factors, which can reposition nucleosomes using energy generated from ATP hydrolysis.

DNA methyltransferases were shown to methylate CpG sites in nucleosomal DNA *in vitro* (Takeshima et al., 2006; Gowher et al., 2005; Okuwaki and Verreault, 2004). Dnmt3a showed higher DNA methylation activity than Dnmt3b towards the naked DNA and the naked part of nucleosomal DNA. Dnmt3b can methylate the DNA within the nucleosomal core region although the activity is low, while Dnmt3a does not. The ability of DNMT1 to methylate nucleosomal DNA is highly dependent on the nature of DNA template (Okuwaki and Verreault, 2004). Here I also showed that Dnmt1 cannot methylate the hemimethylated CpG sites which are packaged into nucleosomes. Nucleosomes without any histone modifications protect DNA from DNA methylation by Dnmt1. Therefore, access of Dnmt1 to nucleosomal DNA substrate may require chromatin remodeling activities. For example, DNA methylation patterns of several highly repeated sequences including the rDNA arrays, a Y-specific satellite and subtelomeric repeats were changed by mutating the *ATRX* gene that encodes a component of ATP-dependent chromatin remodeling factor of the SWI2/SNF2 family (Gibbons et al., 2000). Recently, it was reported that Snf2H can enhance Dnmt3a/3b activity on nucleosomal DNA by reposition of nucleosomes in the presence of ATP (Felle et al., 2011). Access of Dnmt1 to nucleosomal DNA templates may rely on chromatin remodeling factors, which recognize specific histone modification patterns. Therefore, it is reasonable to speculate that histone modification patterns may control access of Dnmt1 to nucleosomes. In addition, Dnmt1 may also directly read the histone modification. As first evidence, using a histone peptide binding approach we found that the isolated TS domain of Dnmt1 shows a preferentially binding to the heterochromatin mark, the trimethylated histone H3K9, in contrast to the acetylated H3K9 (unpublished data by Patricia Wolf). In addition, several proteins including HP1 and Uhrf1 were identified as adaptor proteins to link DNA

methylation to histone modification, suggesting an indirect way which targets Dnmt1 to nucleosomes.

A series of epigenetic signals, including methylation, phosphorylation and acetylation at histone tails, influence higher order chromatin structure. The acetylation and deacetylation of histones dynamically change chromatin arrangement. By neutralizing negative charges, histone acetylation results in an opening of chromatin states (Gorisch et al., 2005), whereas deacetylation of histones catalyzed by HDAC is thought to generate a more compact chromatin state. Interestingly, it was shown that HDAC1 interacts with Dnmt1, suggesting that HDAC1 may link DNA methylation to histone deacetylation. In addition, a recent study indicates that DNA is more accessible in chromatin containing H3K4me and hyperacetylated H4K16, which localize at active promoters and gene rich areas (Bell et al., 2010). In contrast, DNA is hardly accessible when histone H3 is methylated at lysine 27 (H3K27me). Moreover, it was reported that nucleosomes with dimethylated H3K36 are refractory to nucleosome disassembly (Rao et al., 2005). In addition to histone modification, also the histone variants were shown to be involved in modulating chromatin accessibility (Raisner et al., 2005; Zhang et al., 2005).

Although in the last two decades DNA methylation and histone modifications have been extensively characterized as two crucial epigenetic systems, little is known how Dnmt1 access the genomic DNA when it is packaged into nucleosome in mammalian cells and which histone posttranslational modifications might be involved in this process. Therefore, to fully understand the mechanism of maintenance of DNA methylation, it is important to elucidate the binding preference of Dnmt1 toward nucleosomal templates that contain different histone modifications. To elucidate this question, it is necessary to generate hemimethylated DNA sequences. Gerasimaite *et al* engineered a hemimethylase M.HhaI that specifically recognize GCG sites (Gerasimaite et al., 2009) that can be applied to prepare the hemimethylated DNA sequences.

To investigate this question, an alternative approach can be used, that is to precipitate the histones with Dnmt1 and analyze their modifications. For this purpose, I digested chromatin with micrococcal nuclease into mononucleosomes and thereby analyze mononucleosomes that would be coprecipitated with Dnmt1. For this aim, I used *dnmt1* null embryonic ESCs, that stably expressing GFP tagged Dnmt1. My preliminary data showed that histones can be coprecipitated with Dnmt1, but their specific posttranslational modification signature will have to be identified by mass spectrometry (Fig. 16).

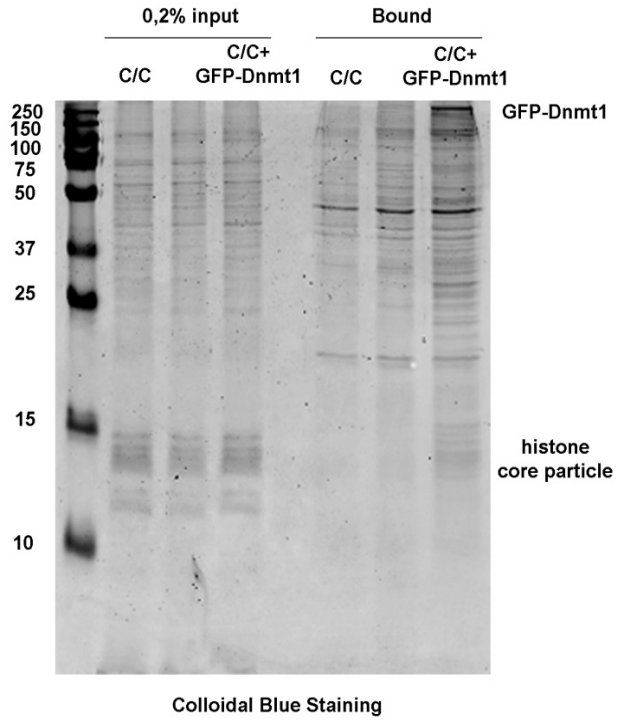

Figure 16. Isolation of histones associated with DNA methyltransferase 1
Mononucleosomes isolated from an ES cell line stably expressing GFP tagged Dnmt1 in *dnmt1* knockout cells (C/C+GFP-Dnmt1) were coprecipitated with Dnmt1 by using the GFP trap. The bound fraction was separated in a 5-12% of gradient gel and visualized by colloidal blue staining. Histone core particles were coprecipitated with Dnmt1 in C/C+GFP-Dnmt1 ESCs, but not in the negative control, the *dnmt* $^{-/-}$ ESCs (C/C). For preparation of mononucleosomes, nuclei are digested with 40 U/ml of micrococcal nuclease at 37°C for 5 min.

3.4. Dynamics of DNA methylation

3.4.1. Tets, a novel protein family in epigenetics

The Tet family includes three members: Tet1, Tet2 and Tet3. Tet enzymes are 2-oxoglutarate (2OG)-and Fe (II)-dependent dioxygenases with homology to JBP1/2 proteins and AlkB, which are enzymes responsible for hydroxylation of thymine in Trypanosomes and methyladenine in all kingdoms of life, respectively. Tet proteins modify 5mC to 5hmC.

Tet1 protein is relative large protein harboring a CXXC-type zinc finger domain, a Cys-rich region and a catalytic domain. Surprisingly, the CXXC domain of Tet1 is highly similar to the zinc finger of Dnmt1. The isolated CXXC domain of Dnmt1 (aa 643-700) possesses DNA binding activity with a preference for unmethylated DNA by *in vitro* DNA binding assay. Deletion of the CXXC domain did not affect Dnmt1 activity and DNA binding properties, suggesting that it is dispensable for the catalytic activity of Dnmt1 (Frauer et al., 2011) and might play other roles, such as mediating protein-protein interaction. However, in contrast to our results, Pradhan *et al* showed that the CXXC domain of human Dnmt1 is necessary for enzymatic activity (Pradhan et al., 2008). A deletion of DNMT1 (aa 648-690) was characterized with a radioactive methyltransferase activity assay. The $DNMT1^{\Delta CXXC}$ activity on hemimethylated DNA was significantly reduced. Moreover, stable expression of $DNMT1^{\Delta CXXC}$ in COS-7 cell line resulted in a reduction of DNA methylation at rDNA repeat sequences (25%). Therefore, the authors proposed a dominant negative effect of $DNMT1^{\Delta CXXC}$. The discrepancy with our results might be due to the differences in the construction of deletion mutants or to the use of Dnmt1 from different mammalian species. Basing on sequence comparison by mouse and human Dnmt1 sequences, the deletion of DNMT1 used by Pradhan group might affect the folding of surrounding protein sequences.

The CXXC domain of Tet1 (aa 561-614) was constructed and tested whether it can bind to DNA. Interestingly, we found that the CXXC domain of Tet1 has no DNA binding affinity. Consistently, based on our structural model of CXXC domains, the CXXC domain of Tet1 is different from the structure of the Dnmt1 CXXC domain in the region of the KFGG motif. In contrast to our result, it was shown that the CXXC domain of TET1 (aa 528-674) can bind to both unmethylated and methylated CpG-rich DNA sequences (Zhang et al., 2011; Xu et al., 2011). This discrepancy with our data might result from the use of different assay and DNA substrate or from the differences in the construction of CXXC fragments. Additionally, by

overexpression of CXXC deletion mutant of Tet1 in HEK293T cells, the 5hmC level is not affected, suggesting that the CXXC domain is dispensable for catalytic activity of Tet1 *in vivo* (Frauer et al., 2011). Thereby, further work is required to confirm and identify the region(s) of Tet proteins responsible for DNA binding.

In mammalian zygotes, genomic methylation patterns are erased from the paternal genome even before the first replication cycle. Although the enzyme responsible for removal of methyl group from 5mC is so far not identified, it was proposed that the conversion of 5mC to 5hmC is one initial step for active DNA demethylation. Two recent studies detected high levels of 5hmC in the paternal genome from the one to the four cell stages (Iqbal et al., 2011; Wossidlo et al., 2011). In the same studies Tet3 transcripts were specifically detected in oocytes and zygotes, with a rapid decline already at the two cell stage. Thus, Tet3 is likely the enzyme responsible for converting 5mC to 5hmC in the paternal genome of zygotes (Iqbal et al., 2011; Wossidlo et al., 2011). The generation of specific antibodies against Tet proteins would help to investigate the expression pattern of Tet proteins during embryonic development and in adult tissues.

In addition to elucidate the expression patterns, the regulation of Tet proteins could receive more attentions. To study the regulation of Tet proteins, it can be performed to identify the potential interaction partners. Several methods can be used to search for potential Tet interacting proteins, including the traditional yeast two-hybrid and affinity purification in combination with mass spectrometry.

3.4.2. 5hmC, a novel DNA modification

5mC and 5hmC are two modified cytosine bases. Whereas 5mC has been extensively studied for two decades, 5hmC has only recently been shown in mammalian genomes. The oxidation of 5mC to hmC catalyzed by Tet proteins is supposed to mediate gene reactivation. It was reported that catalytic mutations in Tet2 results in genomic hypermethylation in multiple myeloproliferative neoplasms (Tefferi et al., 2010), suggesting that Tet proteins might promote DNA demethylation. Moreover, overexpression of Tet proteins in HEK293T cells led to a reduction of 5mC content (Tahiliani et al., 2009). These observations suggest the possibility that 5hmC represents an intermediate of an active DNA demethylation process.

Diverse methods were developed to monitor 5mC content and/or patterns, including procedures based on hydrolysis to single nucleotides/nucleosides and separation by thin-layer chromatography(TLC), high performance liquid chromatography (HPLC) or mass

spectrometry (MS), COBRA (combined bisulfate restriction analysis), bisulfite sequencing, pyrosequencing after bisulfite treatment and 5mC antibody-dependent detection methods. Except for the methods based on physical separation (TLC, HPLC coupled to MS), these techniques cannot distinguish 5mC from 5hmC (Jin et al., 2010). Therefore, a major challenge is to develop methods that could accurately distinguish 5mC and 5hmC in the genomic context. Recently, several methods based on glucosylation of 5hmC by a specific glucosyltransferase were reported, and could be used to quantify and map the distribution of 5hmC in mammalian genomic DNA (Szwagierczak et al., 2010; Robertson et al., 2011; Pastor et al., 2011; Song et al., 2011). Moreover, a sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels was developed (Le et al., 2011; Münzel et al., 2010). In addition, antibodies specific for 5hmC would allow genome-wide mapping of 5hmC by DNA immunoprecipitation followed by either microarray hybridization or high throughput parallel sequencing. 5mC occurs predominantly in the context of CpG dinucleotides. As methods that can discriminate 5hmC from 5mC at single base pair resolution are currently not available, it is not known whether 5hmC is also predominantly found in CpG dinucleotides or present more frequently also in other sequence contexts.

Although genomic 5hmC is proposed as an intermediate of active DNA demethylation, currently, its function is not clear. The identification of proteins that may specifically bind 5hmC would be an important step towards understanding the role(s) of this modified nucleotide. The binding proteins of 5mC, including MBD1, MBD2, MBD3 and MeCP2 did not bind to 5mC when it was hydroxylated (Jin et al., 2010), suggesting that the hydroxylation of 5mC blocks MBD proteins binding, resulting in gene reactivation. To identify proteins that recognize 5hmC, cell extracts could be incubated with immobilized DNA oligonucleotides containing 5hmC as bait (Fig. 17a). I used this approach to test whether Uhrf1 binds DNA containing 5hmC. Preliminary results showed that Uhrf1 binds DNA oligonucleotides containing an asymmetrically hydroxymethylated CpG site and the binding is even less efficiently than the same oligonucleotide sequence without modification (Fig. 17b). This experiment was just conducted once and thereby has to been repeated. In addition, an internal normalization has to be considered to confirm this result. In contrast to this result, it was reported that Uhrf1 preferentially binds to hydroxymethylated cytosine (Frauer et al., 2011). The discrepancy might result from the use of different DNA substrate or different experimental set-ups, like uses complex extracts versus purified proteins. For the

understanding of Uhrf1 regulation and function it will be important to identify the molecular basis for this opposite binding properties.

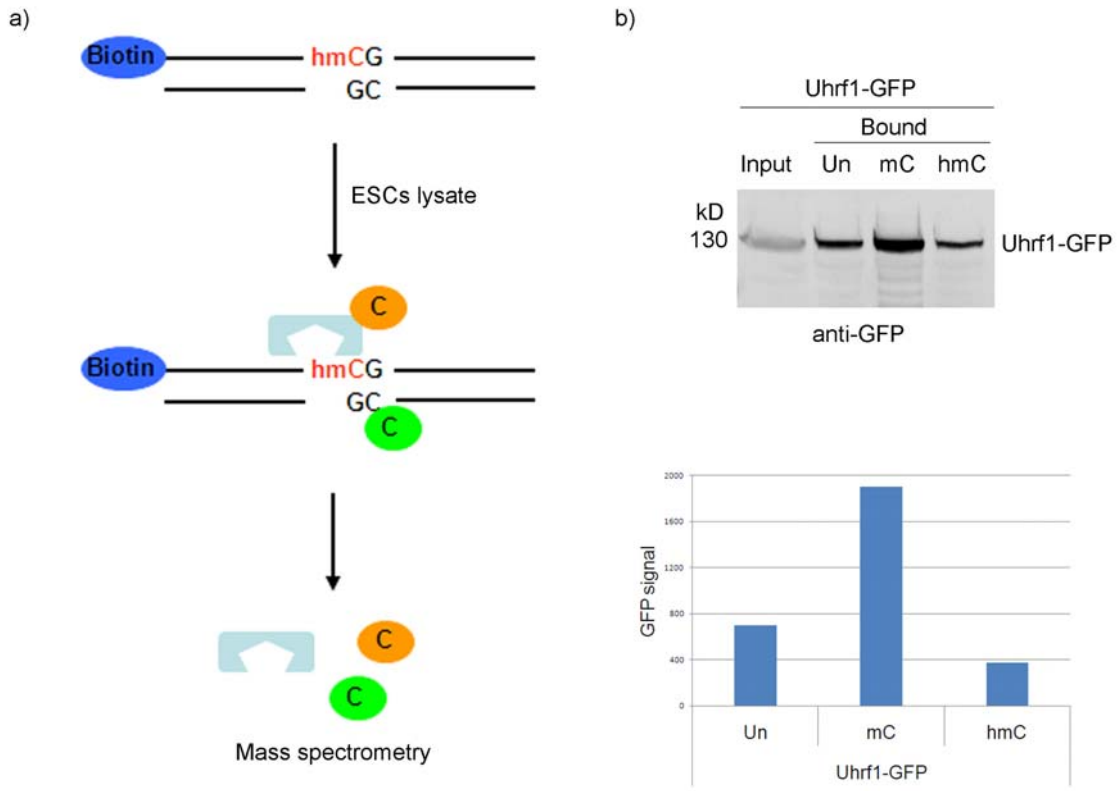

Figure 17. Strategy for identification of proteins which could recognize 5hmC
a) Strategy for identifying proteins that specifically bind 5hmC. A biotin labeled bait DNA containing either un-, hemi- or hydroxyl-methylated cytosine is incubated with ESCs lysate. The proteins co-precipitated with bait DNA are identified by mass spectrometry. b) DNA oligonucleotides were incubated with cell lysate from HEK293T cells that transiently over-expressed Uhrf1-GFP. GFP fusion protein in bound fraction was measured with a fluorimeter and detected by western blotting using anti-GFP antibodies. Uhrf1 shows a preferential binding of hemi-methylated DNA over hydroxyl-methylated as well as unmethylated DNA.

4. Annex

4.1. Reference

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4.2. Abbreviations

2OGFeDO: 2-oxoglutarate and iron (II) dependent dioxygenase superfamily

5-aza-dC: 5-aza-2´-deoxycytidine

5hmC: 5 hydroxymethylcytosine

5mC: 5-methylcytosine

AdoMet: S-Adenosyl-LMethionine

Akt1: threonine-protein kinase

BAH: bromo adjacent homology domain

BER: base excision repair

CpG: cytosine-phosphatidyl-guanine

C-Myc: Myc proto-oncogene protein

CTD: catalytic domain

DNA: deoxyribonucleic acid

Dnmt: DNA methyltransferase

DUB: de-ubiquitinase

Dot1: histone H3 methyltransferase Dot1

ESCs: embryonic stem cells

E2F: transcription factor E2F

Elp3: elongator complex protein 3

E3: ubiquitin ligase

GFP: green fluorescent protein

Gadd45: growth arrest and DNA damage inducible protein 45α

H3K9me: H3 lysine 9 methyltransferase

HMTs: histone methyltransferases

HDAC: histone deacetylase

HP1: heterochromatin protein 1

ICF: immunodeficiency, centromere instability, facial anomalies syndrome

$(KG)_7$: lysine-glycine repeats

MBD: methyl-CpG binding domain protein

MEF: Mouse Embryonic Fibroblast

MLL: mixed lineage leukemia

Np95: nuclear protein with 95 kilodalton

NTD: N-terminal domain

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

Oct4: octamer binding transcription factor 4

PBD: PCNA binding domain

PCNA: proliferating cell nuclear antigen

PDB: protein data bank

PHD: plant homeodomain

PWWP: proline-tryptophan-proline motif

PRC: polycomb repressive complex

Ring: really interesting new gene

SILAC: stable isotope labeling with amino acids in cell culture

SAGA: Spt-Ada-Gcn5-Acetyltransferase complex

S phase: synthesis phase

SRA: SET and Ring associated

TDG: thymidine glycosylase

Tip60: histone acetyltransferase Tip60

TKO: *dnmt1, dnmt3a and dnmt3b* knockout embryonic stem cells

TRD: target recognition domain

TS: targeting sequence

Ubl: ubiquitin-like

Uhrf1: ubiquitin-like, containing PHD and RING finger domains, 1

ZnF: zinc finger

4.3. Contributions

Declaration of contributions to "Usp7 and Uhrf1 control ubiquitination and stability of the maintenance DNA methyltransferase Dnmt1"

This project was conceived by Fabio Spada, Heinrich Leonhardt and me. I performed the immunoprecipitation experiment and identified Usp7 as a novel interacting protein with Dnmt1. To elucidate the role of Usp7 and Uhrf1 in the regulation of Dnmt1, I established the *in vivo* and *in vitro* ubiquitination assay and *in vivo* protein stability assay. I carried out the immunostaining assay to clarify the colocalization of Dnmt1 and Usp7 during the cell cycle. I prepared the figures and composed the first draft of the manuscript. The final version of the manuscript was revised by Fabio Spada and Heinrich Leonhardt.

Declaration of contributions to "Different Binding Properties and Function of CXXC Zinc Finger Domains in Dnmt1 and Tet1"

This study was initiated by Fabio Spada, Stefan Hasenöder and Heinrich Leonhardt. Carina Frauer and Andrea Rottach picked up the project, laid out the project aims and conceived the study together with Heinrich Leonhardt and Fabio Spada. I contributed the pyrosequencing data of wt and *dnmt1*$^{-/-}$ ES cells for single copy genes.

Declaration of contributions to "Uhrf1 plays multiple roles in the regulation of DNA methyltransferase 1"

This study was initially conceived by Fabio Spada and Heinrich Leonhardt and me. Karin Fellinger mapped the domains which are involved in the interaction between Dnmt1 and Uhrf1 (Fig. 1). Carina Frauer performed the *in vitro* methyltransferase assay and purified the Dnmt1 protein from insect cells (Fig. 2D). Garwin Pichler carried out the *in vitro* DNA binding assay to check the binding affinity of Uhrf1 mutants (Fig. S3). I performed all the other experiments. I generated several ES cell lines which stably expressed the mutants of Dnmt1 or Uhrf1. I checked the colocalization between Dnmt1 and Uhrf1 mutants and measured the DNA methylation. In addition, to making reconstitutive mononucleosome substrates, I prepared the 601 DNA fragment, including un-, hemi- and fully-methylated forms and Henrike Klinker made the reconstitutive mononucleosomes. I established the *in vitro* methyltransferase assay to test Dnmt1 activity on chromatin template in the presence or absence of Uhrf1. I prepared the figures and composed the first draft of the manuscript.

Declaration of contributions to "Regulation of DNA methyltransferase 1 by interactions and modifications"

The manuscript was written together with Garwin Pichler and Heinrich Leonhardt.

4.4. Declaration

Declaration according to the "Promotionsordnung der LMU München für die Fakultät Biologie"

Betreuung Hiermit erkläre ich, dass die vorgelegte Arbeit an der LMU von Herrn Prof. Dr. Heinrich Leonhardt betreut wurde.

Anfertigung Hiermit versichere ich ehrenwörtlich, dass die Dissertation selbstständig und ohne unerlaubte Hilfsmittel angefertigt wurde. Über Beiträge, die im Rahmen der kumulativen Dissertation in Form von Manuskripten in der Dissertation enthalten sind, wurde im Kapitel 4.3 Rechenschaft abgelegt und die eigenen Leistungen wurden aufgelistet.

Prüfung Hiermit erkläre ich, dass die Dissertation weder als ganzes noch in Teilen an einem anderen Ort einer Prüfungskommission vorgelegt wurde. Weiterhin habe ich weder an einem anderen Ort eine Promotion angestrebt noch angemeldet noch versucht eine Doktorprüfung abzulegen.

München, den 18. Juli. 2011

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Weihua Qin

4.5. Acknowledgements

Looking back, I am surprised and at the same time very grateful for all I have received during these years. It has certainly shaped me and has directed me to where I am now. All these years of studying have been I learned are full of such gifts.

First of all, I would like to thank Professor Heinrich Leonhardt for giving me the unique opportunity to conduct my PhD thesis in his lab. Thank you for your encouragement and invaluable suggestions when I was thinking of giving up sometimes. He has enlightened me with his wide knowledge and his deep intuitions about what might be possible and what is necessary to get there.

I am very grateful to the Chinese Scholarship Council, without its support the completion of this dissertation would not have been possible. I really do appreciate that the scholarship supported me for one more year when it was required.

I am very grateful to Dr. Fabio Spada for his supervision, ideas and enthusiasm. Thank you for your introduction about culturing embryonic stem cells, immunostaining and so on during my first two months in this lab. For sure, the experience helped me to get used to this new lab as soon as possible.

I would like to thank all members of Heinrich's group for the friendly and supportive atmosphere that contributed essentially to the final outcome of my studies. I would like to thank Anja Gahl for the lab running smoothly and the advice for cultivating my plant "Banana". I want to thank Susanne Breitsameter for some documents preparation. I also owe thanks to Andrea Buchfellner for friendly talking and helping.

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Last, but certainly not least, I would like to thank my family for their support and belief in me throughout these years.

5. Curriculum Vitae

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Current work address:	Ludwig Maximilians University (LMU), Munich Department of Biology II, LMU Biocenter Großhaderner Str. 2, 82152 Planegg-Martinsried, Germany

Education:

Present – 10/2007:	PhD student in biology Ludwig Maximilians University, Munich Department of biology II, Under the supervision of Prof. Heinrich Leonhardt
03/2005 – 09/2002:	Master student in Biochemical engineering Shanghai Jiao Tong University, Shanghai, China College of Life Science and Biotechnology, Under the supervision of Prof. Chengxi Cao
07/2000–09/1996:	Bachelor student in Ecology Si Chuan University, Chengdu, China College of Life Science

Scientific Experience:

	Ludwig Maximilian University, Munich
Present – 10/2007	Department of biology II, PhD thesis under the supervision of Prof. Heinrich Leonhardt: "Studies of interacting proteins controlling DNA methyltransferase 1"

08/2007 – 04/2005	Institutions of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, CAS, Under the supervision of Prof. Guoliang Xu

I participated in the project of "RIM-BP3, a Dnmt3a interacting protein, is a manchette-associated protein essential for spermiogenesis"

For this project, I contributed to analyze the phenotype of *rim-bp3* knockout mice. I checked the expression pattern of Rim-bp3 during spermiogensis and found that Rim-bp3 co-localized with tubulin at the manchette of sperm. By SEM and TEM technology, the defect spermiogenesis in *rim-bp3* knockout mice was identified. Additionally, I initially clarified the role of Rim-bp3 in spermiogenesis through identification and characterization of the Rim-bp3 interacting protein, Hook1.

I participated in gene targeting of functional Dnmt3a regulators through aggregation strategy instead of blastocyst injection and succeed in making chimeric mice

03/2005 – 09/2003	Shanghai Jiaotong University, Shanghai, China Department of biochemistry engineering, group of Prof. Chengxi Cao

I participated in the project to predict the conditions of zwitterionic stacking by transient moving chemical reaction boundary created with weak electrolyte buffers in capillary electrophoresis

I quantitatively studied the selective stacking of zwitterions in large-volume sample matrix by moving reaction boundary in capillary electrophoresis

Publications

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