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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).

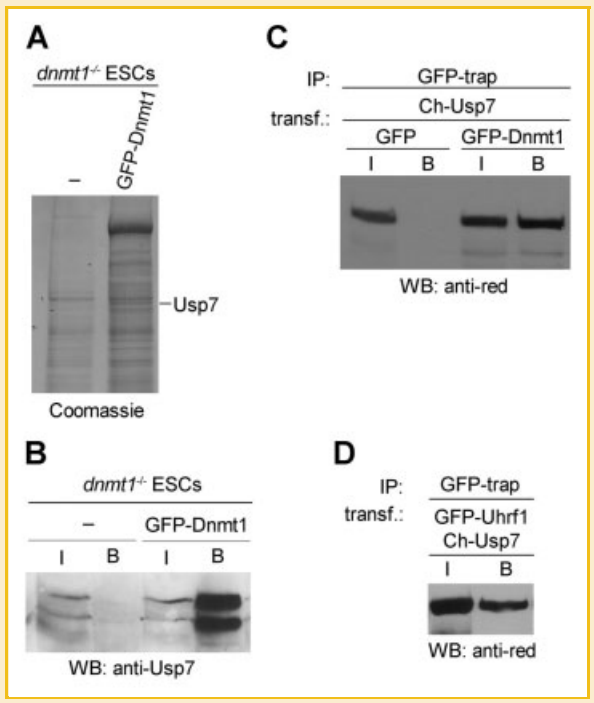

Fig. 1. Usp7 interacts with Dnmt1 and Uhrf1. A: GFP-trap pulldowns from *dnmt1*$^{-/-}$ ESCs and a clone of the same cells stably expressing GFP-Dnmt1. A colloidal Coomassie blue stained SDS–PAGE of bound fractions is shown and the position of Usp7 as identified by mass spectrometry is shown. B: GFP-trap 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.

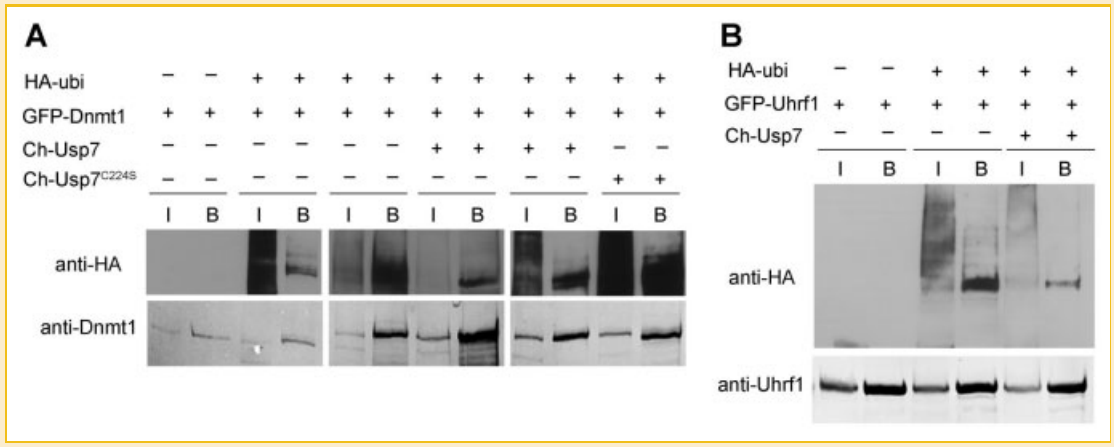

Fig. 2. Usp7 deubiquitinates both Dnmt1 and Uhrf1. GFP-trap pulldowns from HEK293T cells expressing the indicated combinations of HA-ubiquitin, Ch-Usp7, Ch-Usp7^{C224S}, and either GFP-Dnmt1 (A) or GFP-Uhrf1 were probed with an anti-HA antibody to detect ubiquitinated proteins and either anti-Dnmt1 (A)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

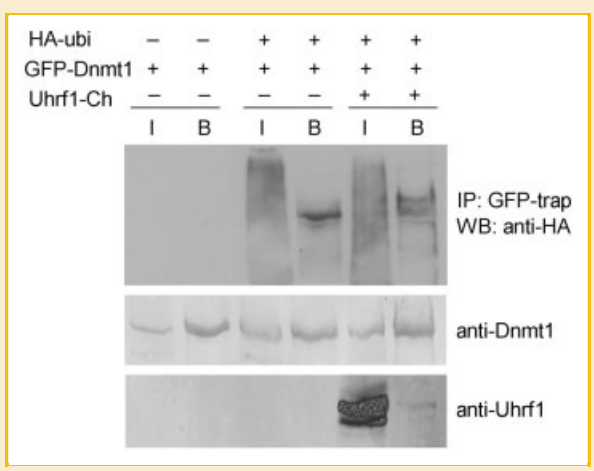

Fig. 3. Uhrf1 ubiquitinates Dnmt1. GFP-trap pulldowns from HEK293T cells expressing the indicated combinations of HA-ubiquitin, GFP-Dnmt1 and Uhrf1-Ch were probed with anti-HA and anti-Uhrf1 antibodies as in Figure 2B.

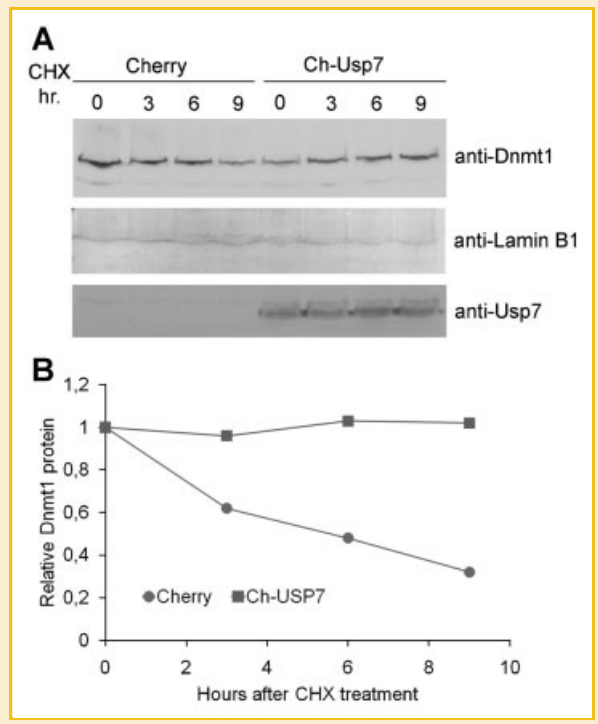

Fig. 4. USP7 stabilizes Dnmt1. HEK293T cells expressing either Cherry or Ch-Usp7 were treated with cycloheximide (CHX) for the indicated time periods before harvesting. A: Endogenous Dnmt1 levels were detected with an anti-Dnmt1 antibody, while anti-Lamin B1 and anti-Usp7 blots served as loading controls.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