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Replication-independent chromatin loading of Dnmt1 during G2 and M phases

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The major DNA methyltransferase, Dnmt1, associates with DNA replication sites in S phase maintaining the methylation pattern in the newly synthesized strand. In view of the slow kinetics of Dnmt1 *in vitro* versus the fast progression of the replication fork, we have tested whether Dnmt1 associates with chromatin beyond S phase. Using time-lapse microscopy of mammalian cells expressing green-fluorescent-protein-tagged Dnmt1 and DsRed-tagged DNA Ligase I as a cell cycle progression marker, we have found that Dnmt1 associates with chromatin during G2 and M. This association is mediated by a specific targeting sequence, shows strong preference for constitutive but not facultative heterochromatin and is independent of heterochromatin-specific histone H3 Lys 9 trimethylation, SUV39H and HP1. Moreover, photobleaching analyses showed that Dnmt1 is continuously loaded onto chromatin throughout G2 and M, indicating a replication-independent role of Dnmt1 that could represent a novel and separate pathway to maintain DNA methylation.

Keywords: centromeric heterochromatin; DNA replication; DNA methylation; Dnmt1; epigenetics

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INTRODUCTION

The higher eukaryotic genome is broadly organized into two structurally distinct chromatin domains, euchromatin and heterochromatin, which functionally translate into active and repressed genetic elements, respectively. At the molecular level, such chromatin domains are characterized by two principal epigenetic marks, DNA methylation and histone modifications. In mammals, DNA methylation predominantly occurs at cytosine residues in CpG dinucleotide sites and is mostly associated with a repressive effect on gene expression (Doerfler, 1983; Bird & Wolffe, 1999; Leonhardt & Cardoso, 2000). Notably, heterochromatic domains are characterized by increased CpG methylation and trimethylation of histone H3 at Lys 9 (H3K9). Stable maintenance of these epigenetic marks through multiple cell divisions is essential for regulated gene expression, development and stability of the genome (Li *et al*, 1992; Jackson-Grusby *et al*, 2001; Peters *et al*, 2001).

In mammals, methylation of CpG dinucleotides is catalysed by two classes of enzyme—*de novo* and maintenance DNA methyltransferases. Dnmt3a and Dnmt3b belong to the former class and are required for establishment of new DNA methylation patterns during development (Okano *et al*, 1999). Dnmt1 has a preference for hemimethylated CpG sites (Bestor, 1992), which are a product of DNA replication. Corroborating this, Dnmt1, but not Dnmt3a and Dnmt3b, relocates to sites of DNA replication (replication foci, RF) as the cell enters S phase (Leonhardt *et al*, 1992; Margot *et al*, 2001). These observations led to the proposal that Dnmt1 is the maintenance methyltransferase and that its association with replication sites during S phase ensures the faithful duplication of the DNA methylation patterns post-replicatively. Three regions of its amino-terminal domain have been implicated in targeting Dnmt1 to RF, namely, the PCNA binding domain (PBD), targeting sequence (TS) and the polybromo homology domain (PBHD) (Leonhardt *et al*, 1992; Chuang *et al*, 1997; Liu *et al*, 1998). It is presently unclear which domain mediates this association or whether separate domains are required for targeting to early-replicating euchromatic regions and for late-replicating heterochromatic regions. It is also unknown how Dnmt1, which shows very slow kinetics *in vitro* (Pradhan *et al*, 1999), can cope with the replication fork speed, in particular at densely methylated heterochromatic regions, or whether Dnmt1 function extends beyond S phase. We have, therefore, undertaken a detailed analysis of the molecular basis for the association of Dnmt1 with the replication machinery and with chromatin, throughout the entire cell cycle in living cells. Our *in vivo* results show that in addition to its binding to replication sites, which is dependent exclusively on PBD, Dnmt1 associates with chromatin (preferentially, constitutive heterochromatin) from late S till early G1 by means of a separate domain called TS. This association is independent of heterochromatin-specific histone

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methylation and proteins that bind these methylated histones. Furthermore, it is independent of DNA replication as it occurs *de novo* during G2 and M phases.

RESULTS AND DISCUSSION

We have previously shown that Dnmt1 associates with sites of active DNA replication during S phase (Leonhardt *et al*, 1992). It has since been accepted that this spatial and temporal coupling of Dnmt1 with the replication machinery ensures the maintenance of DNA methylation patterns at every cell cycle. However, the slow methylation reaction kinetics of the purified Dnmt1 *in vitro* raises the question as to how the replication-coupled enzyme efficiently methylates the large number of cytosines, in particular in densely methylated heterochromatin regions. To address this question, we have investigated the cellular distribution of Dnmt1 throughout the entire cell cycle.

The cell cycle stage *in vivo* was identified using a DNA ligase I fusion with red fluorescent protein (RFP–ligase) as an S-phase marker (Cardoso *et al*, 1997). In addition, we generated a green fluorescent protein (GFP) fusion with Dnmt1 (GMT). We followed double-transfected cells over the cell cycle by taking confocal 3D image stacks every 30–60 min. Time-lapse analysis (Fig 1) showed that Dnmt1 associated with all RF but, surprisingly, its chromatin association persisted throughout G2 and M phases, in contrast to DNA ligase I. Furthermore, there was a preferential association with late-replicating heterochromatin (arrowheads), which was no longer detectable in the next G1 phase. We further confirmed that the same was true for the endogenous enzyme, using a variety of antibodies to Dnmt1 (supplementary Fig 1 online and data not shown). This prolonged association of Dnmt1 with chromatin might then allow sufficient time for fully methylating all hemimethylated sites generated during DNA replication.

To clarify how Dnmt1 binds to RF during S phase and which domain is responsible for its continued association with chromatin during G2 and M phases, we generated a series of fluorescently tagged mutants affecting the known domains of Dnmt1. First, we analysed which domain is required for association with RF and, importantly, whether there is any selective preference for early or late RF that differ in their methylation densities. The results are summarized in Fig 2. The N-terminal domain fused to GFP (MTNG) associated with RF at all stages of S phase and deletion of the PBHD alone (MTNG.1) or both TS and PBHD (MTNY.2 and MTNY.3) did not affect the ability of the fusion protein to associate with RF. Contrary to earlier reports (Liu *et al*, 1998), PBHD–YFP fusion protein did not associate with RF at any stage during S phase. Interestingly, TS colocalized with late-replicating heterochromatin. A construct carrying only the 10-amino-acid PBD and the following ten residues rich in basic amino acids (PBD–GFP) efficiently associated with RF throughout S phase, and not only during early S as suggested before (Chuang *et al*, 1997). In summary, association of Dnmt1 with RF throughout S phase depends on PBD, whereas TS shows a preference for late-replicating chromatin.

Next, we investigated which Dnmt1 domain was responsible for the continued association with chromatin in G2 and M by time-lapse analysis (Fig 3A) as well as by using the cell cycle staging strategy described in supplementary Fig 1 online (data not shown). The results of the analysis of all Dnmt1 mutants showed that TS alone is responsible for the persistent association with chromatin throughout G2 and M. Similar results were obtained in other cell types and species (supplementary Fig 2 online). Deletion of the TS domain completely abolishes Dnmt1 association with chromatin during G2 and M, but does not prevent the binding of Dnmt1 to RF (Fig 3B). We then tested whether TS interacts preferentially with constitutive heterochromatin (centromeric) or also with facultative heterochromatin (inactive X) by detecting the latter with antibodies against trimethylated K27 residue of histone H3 (Peters *et al*, 2003; Plath *et al*, 2003). TS–GFP showed a strong preference for centromeric heterochromatin and only very low levels were detectable at facultative heterochromatin (arrows,

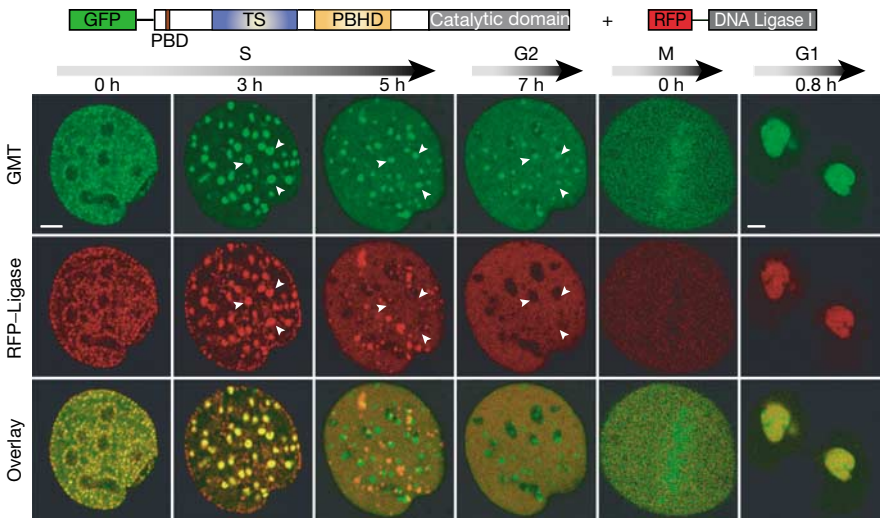
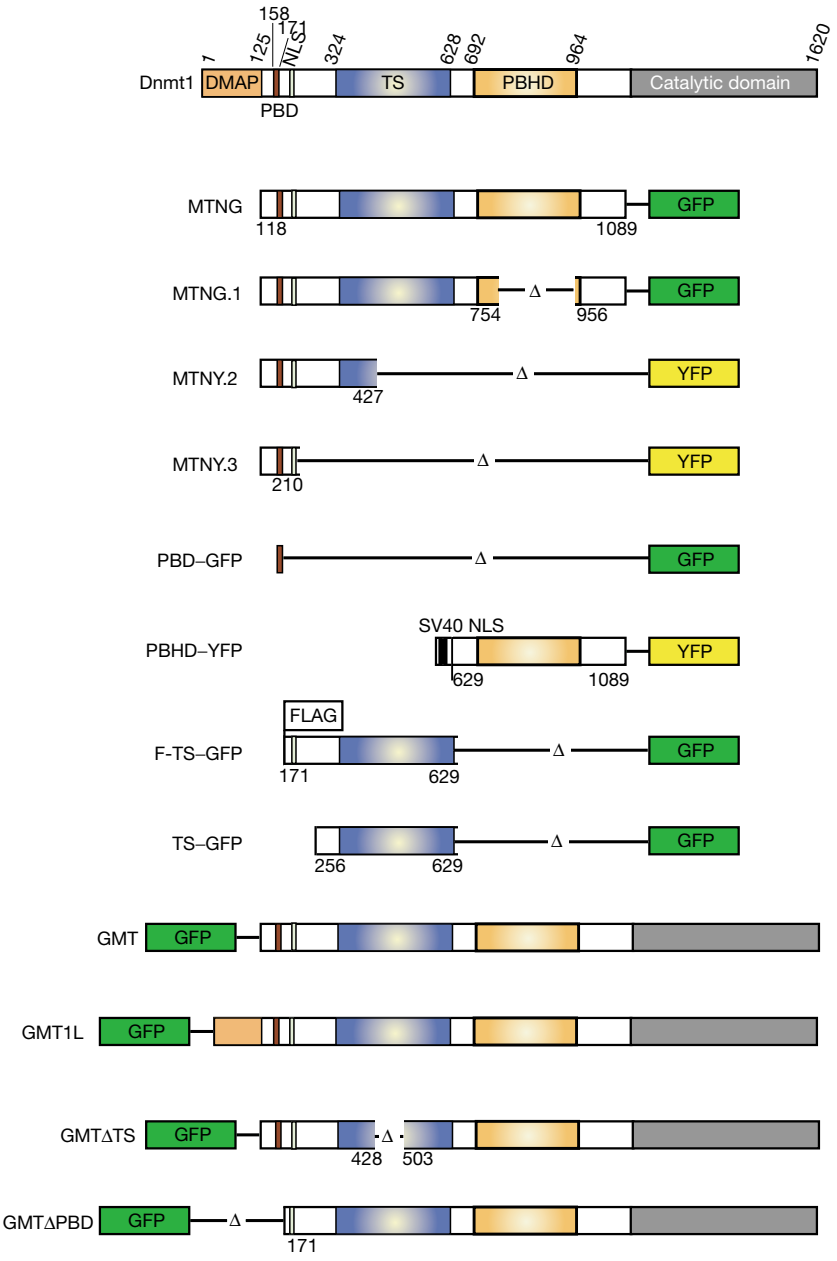


Fig 1 | Dnmt1 shows cell-cycle-dependent association with chromatin. Live-cell imaging of C2C12 cells expressing full-length Dnmt1 fusion to GFP (GMT) and RFP–ligase. Selected frames from this time series are shown. At the time imaging was started (0 h), this cell was in mid-S phase. The arrowheads indicate preferential association of GMT with heterochromatic regions during late S and G2. (The images from mitosis and G1 in this assembly are from a different cell.) Scale bar, 5 μm.



Association with RF		
Early S	Mid-S	Late S
+	+	+
+	+	+
+	+	+
+	+	+
+	+	+
–	–	–
–	–	+*
–	–	+*
+	+	+
+	+	+
+	+	+
–	–	+*

Fig 2 | Mapping of Dnmt1 domains responsible for replication foci association. DMAP corresponds to the DMAP1 transcriptional repressor interacting domain (Rountree *et al*, 2000). For other domains, see text. *Association takes place not only in S phase (see Fig 3).

Fig 3C). During mitosis, there was still a preferred accumulation at centromeric heterochromatin although TS was also detected along the entire chromosomes (Fig 3A and supplementary Fig 2 online). As histone H3 trimethylation at Lys 9 is a characteristic feature of centromeric heterochromatin (Peters *et al*, 2003), we tested whether TS association is dependent on this modification or on SUV39H/HP1 binding by expressing TS–GFP in *Suv39h1* and *Suv39h2* double-null primary mouse embryonic fibroblasts (PMEFs; Lachner *et al*, 2001). These cells contain HP1 proteins, but their heterochromatin association is abrogated (Fig 3D). In contrast, we found a clear localization of TS–GFP at chromatin with the same preferential centromeric heterochromatin association in *Suv39h1/2* double-null PMEFs (Fig 3D), which clearly shows that this association is independent of HP1, SUV39H1, SUV39H2 and H3K9 trimethylation.

We then investigated whether Dnmt1 association with chromatin during G2 and M occurs independently of DNA replication. We tested whether the enzyme is *de novo* loaded during G2 or stays stably bound from S phase by fluorescence photobleaching and recovery analyses (FRAP). In Fig 4A, a cell nucleus is shown before bleaching with dispersed distribution of RFP-tagged PCNA (used, like DNA ligase I, as a cell cycle

progression marker; Leonhardt *et al*, 2000) and strong association of GFP-tagged Dnmt1 to heterochromatin, indicating that the cell is in G2 phase. Both fluorophores were bleached in the indicated area (inset), which included a heterochromatic chromocentre (Fig 4A). As expected, RFP–PCNA in non-S-phase cells is highly mobile and redistributed very fast after photobleaching, showing in the first post-bleach image a dispersed distribution in the nucleoplasm (Sporbert *et al*, 2002). Interestingly, GFP-tagged Dnmt1 showed essentially complete fluorescence recovery at the bleached heterochromatin focus in about 2 min (half-time of recovery was 11 s in Fig 3B and 11.7 s for the average curve in Fig 4C), clearly arguing against a unique loading in S phase with static chromatin association throughout G2 and M phases. *De novo* loading of Dnmt1 was also observed in M phase (data not shown). This clearly shows that there is continuous *de novo* chromatin binding of Dnmt1 throughout G2/M, which occurs independently of DNA replication.

In summary, Dnmt1 associates throughout S phase with all sites of active DNA synthesis by means of PBD but, in addition, is continuously loaded onto chromatin (preferentially constitutive heterochromatin) from late S throughout G2 and M by means of an independent TS (Fig 5).

Speculation

We propose that the prolonged association of Dnmt1 with chromatin could allow enough time for the maintenance of the dense methylation of centromeric heterochromatin and might additionally act as a marking and loading platform for subsequent chromatin modifiers. Recent studies in fungi, plants and metazoans have debated the interdependence of DNA methylation and histone modifications in setting up silent heterochromatin domains (Tamaru & Selker, 2001; Soppe *et al*, 2002; Lehnertz *et al*, 2003; Tariq *et al*, 2003). The inherent ability of Dnmt1 to bind to centromeric heterochromatin independently of SUV39H-mediated H3K9 trimethylation and HP1 suggests separate pathways for establishment and maintenance of stable heterochromatin domains. Furthermore, our data suggest a novel function for Dnmt1 that goes beyond the known replication-coupled maintenance of DNA methylation patterns. The replication-independent recruitment of Dnmt1 by means of TS to chromatin could represent a novel and independent pathway to maintain DNA methylation at every cell division.

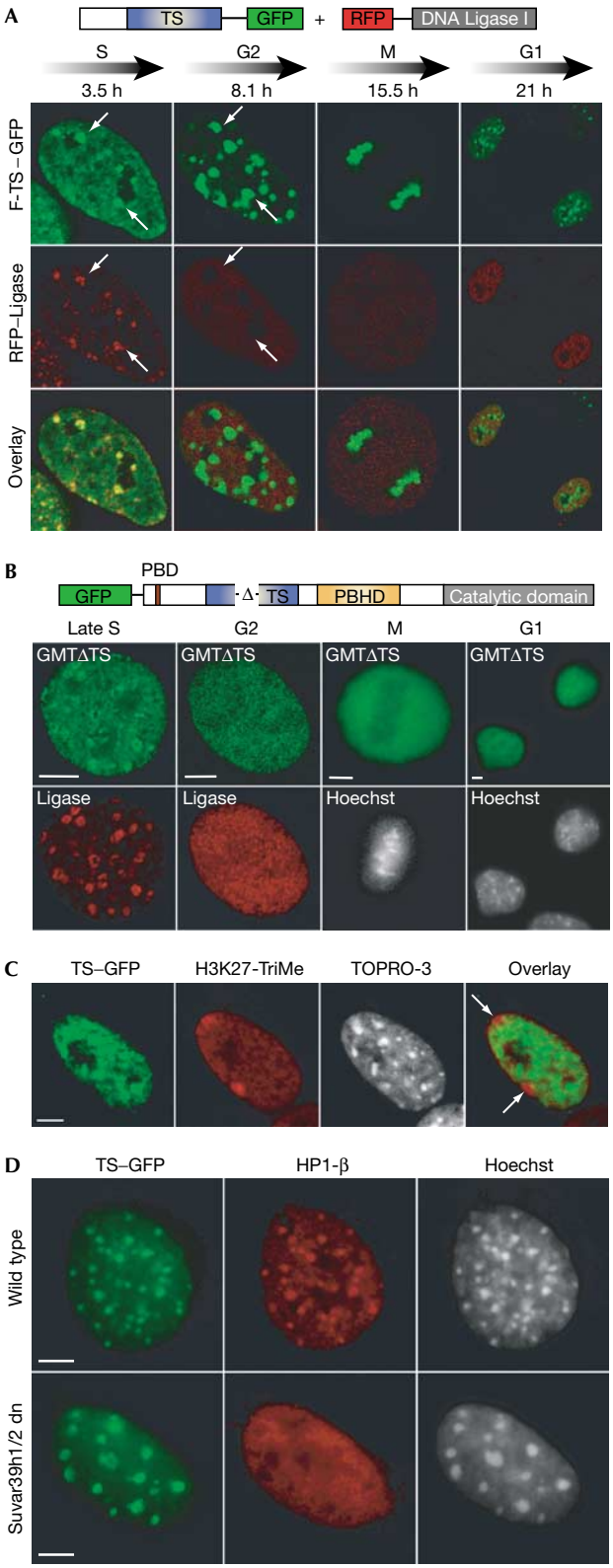


Fig 3 | TS mediates association of Dnmt1 with chromatin, preferentially constitutive heterochromatin, which is independent of H3K9 trimethylation. (**A**) Live-cell imaging of C2C12 cells expressing F-TS–GFP and RFP–ligase. Selected frames from this time series are shown. After 3.5 h from start of imaging, the cell has entered late S phase (deciphered from the pattern of RFP–ligase) and the large F-TS–GFP foci (arrows) show colocalization with RFP–ligase. (**B**) C2C12 cells expressing GFP-tagged Dnmt1 lacking TS (GMTΔTS) analysed at the different cell cycle stages (for strategy, see supplementary Fig 1 online. (**C**) TS preferentially associates with constitutive heterochromatin and not with facultative heterochromatin. C2C12 cell expressing TS–GFP was immunostained with anti-trimethylated H3K27 antibody labelling the inactive X chromosome and DNA was visualized with TOPRO-3. These C2C12 cells are polyploid and have two inactive X chromosomes (H.P. Easwaran *et al*, unpublished observations). (**D**) PMEFs from wild-type and *Suvar39h1/2* double-null mice expressing TS–GFP and FLAG-tagged HP1β were stained with anti-FLAG antibody and centromeric heterochromatin was visualized with Hoechst 33258. Scale bars, 5 μm.

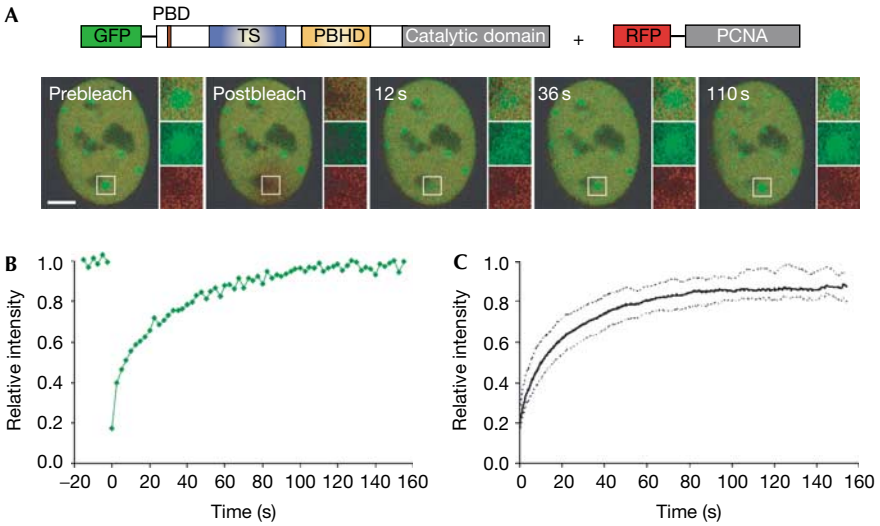


Fig 4 | Recovery of GFP-tagged Dnmt1 after photobleaching indicates *de novo* loading of Dnmt1 onto chromatin throughout G2. (**A**) FRAP series of a typical G2 nucleus expressing GMT (green) and RFP–PCNA (red). An area containing a heterochromatic focus was bleached (inset). Scale bar, 5 μm. (**B**) Recovery curve of the FRAP experiment shown in (**A**). Half-time of recovery is 11 s. (**C**) FRAP data of five nuclei were averaged. Mean curve (solid line) and standard deviation (dotted lines) are shown. Half-time of recovery of the mean curve is 11.7 s.

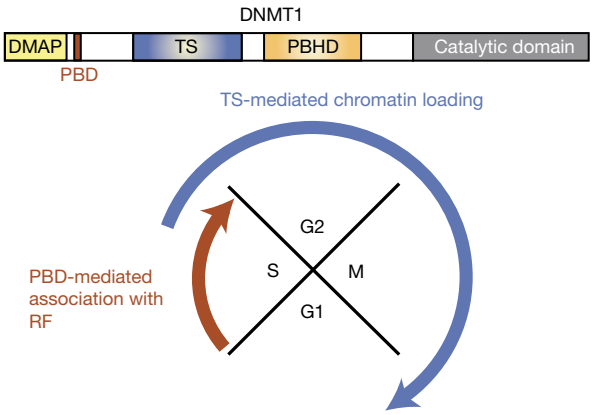


Fig 5 | Summary of the role of PBD and TS in controlling the dynamic association of Dnmt1 with chromatin during the cell cycle. PBD directs association of Dnmt1 with RF throughout the S phase and TS mediates association with chromatin, preferentially heterochromatin, during G2 and M.

METHODS

Cell culture and transfection. Mouse C2C12 myoblasts and HeLa cells were cultured in DMEM supplemented with 20% and 10% FCS, respectively. *Suv39H1/2* double-knockout PMEFs were maintained as described (O'Carroll *et al*, 2000). Cells were transfected with plasmid DNA using the calcium phosphate–DNA co-precipitation method followed by a glycerol shock and incubated overnight before performing immunostainings or live-cell analyses.

Expression constructs. Plasmid constructs encoding translational fusions of mouse Dnmt1, human DNA ligase I and human PCNA with GFP/YFP/RFP were derived from the following vectors: pEGFP-N2, pEGFP-C1, pDsRed1-C1 (Clontech, Palo Alto, CA, USA) and pEVRF0. An SV40-NLS was included for efficient nuclear targeting of deletion proteins, which lost the native NLS. The PBD insert for PBD–GFP fusion protein was generated by synthesizing sense and antisense oligonucleotides corresponding to both strands of the PBD coding region. The mouse FLAG-tagged HP1β expression construct was as described (Nielsen *et al*, 2001). We tested correct expression of the fusion proteins in COS7 or 293T cells by western blotting as described before (Leonhardt *et al*, 1992).

Immunofluorescence. Cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 0.25% Triton X-100 for 10 min. The following primary antibodies (diluted in buffer containing 0.2% gelatin) were used: anti-2x-trimethyl H3K27 (6523) rabbit antibody (Peters *et al*, 2003) and anti-FLAG M2 mouse monoclonal antibody (Kodak, Rochester, NY, USA). Secondary antibodies (Molecular Probes, Leiden, the Netherlands) were conjugated to Alexa Fluor 568. The DNA was counterstained with Hoechst 33258 or TOPRO-3 and cells were mounted in Mowiol with 2.5% DABCO.

A detailed description of the strategy and methods to identify cells at the different cell cycle stages is included in supplementary Methods online. More than 100 cells from each cell cycle stage were analysed to determine the localization of the wild-type and mutant Dnmt1 proteins.

Fixed-cell microscopy. Stained cells were analysed using a Zeiss LSM 510 Meta confocal microscope equipped with a ×63/1.4 NA Plan-Apochromat objective. Ar laser (488 and 514 nm), HeNe laser 1 (543 nm) and HeNe laser 2 (633 nm) were used to excite the fluorophores. Hoechst 33258-counterstained nuclei were analysed on an Axioplan 2 microscope and images acquired with a cooled CCD camera (SensiCam) using Zeiss Axiovision software and appropriate filter sets.

Live-cell microscopy and FRAP analysis. Live-cell analysis was performed by plating the cells on 40-mm-diameter glass coverslips before transfection. At 1 day after transfection, the coverslip was mounted onto the microscope stage and maintained at 37 °C using

an FCS2 live-cell chamber and temperature controller (Bioptechs, Butler, PA, USA).

For time-lapse analysis, images were acquired with a Zeiss LSM 510 using the 488 and 543 nm laser lines at low power (1–5%). Four *z*-sections at 1 μm intervals were imaged every 30–60 min and the cells were followed throughout the cell cycle. After image acquisition, the different *z*-sections at each time point were aligned manually to correct for movements in the *z*-plane.

For FRAP analysis, a region of interest was selected and photobleached by an intense 488 nm Ar laser beam (set to 100% transmission) for 1 s, after which confocal image series were recorded at 2.5 s time intervals. Mean fluorescence intensities of the bleached region were corrected for background and for total nuclear loss of fluorescence over the time course. FRAP data of five nuclei were averaged and the mean curve as well as the standard deviation was calculated and displayed using the GraphPad Prism™ software. The half-time of recovery was calculated from the single as well as the average curves.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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REFERENCES

Bestor TH (1992) Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J* **11:** 2611–2617

Bird AP, Wolffe AP (1999) Methylation-induced repression—belts, braces, and chromatin. *Cell* **99:** 451–454

Cardoso MC, Joseph C, Rahn HP, Reusch R, Nadal-Ginard B, Leonhardt H (1997) Mapping and use of a sequence that targets DNA ligase I to sites of DNA replication *in vivo*. *J Cell Biol* **139:** 579–587

Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF (1997) Human DNA–(cytosine-5) methyltransferase–PCNA complex as a target for p21WAF1. *Science* **277:** 1996–2000

Doerfler W (1983) DNA methylation and gene activity. *Annu Rev Biochem* **52:** 93–124

Jackson-Grusby L *et al* (2001) Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. *Nat Genet* **27:** 31–39

Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410:** 116–120

Lehnertz B, Ueda Y, Derijck AA, Braunschweig U, Perez-Burgos L, Kubicek S, Chen T, Li E, Jenuwein T, Peters AH (2003) Suv39h-mediated histone h3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr Biol* **13:** 1192–1200

Leonhardt H, Cardoso MC (2000) DNA methylation, nuclear structure, gene expression and cancer. *J Cell Biochem* **35**(Suppl)**:** 78–83

Leonhardt H, Page AW, Weier HU, Bestor TH (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* **71:** 865–873

Leonhardt H, Rahn HP, Weinzierl P, Sporbert A, Cremer T, Zink D, Cardoso MC (2000) Dynamics of DNA replication factories in living cells. *J Cell Biol* **149:** 271–280

Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69:** 915–926

Liu Y, Oakeley EJ, Sun L, Jost JP (1998) Multiple domains are involved in the targeting of the mouse DNA methyltransferase to the DNA replication foci. *Nucleic Acids Res* **26:** 1038–1045

Margot JB, Cardoso MC, Leonhardt H (2001) Mammalian DNA methyltransferases show different subnuclear distributions. *J Cell Biochem* **83:** 373–379

Nielsen AL, Oulad-Abdelghani M, Ortiz JA, Remboutsika E, Chambon P, Losson R (2001) Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. *Mol Cell* **7:** 729–739

O'Carroll D *et al* (2000) Isolation and characterization of Suv39h2, a second histone H3 methyltransferase gene that displays testis-specific expression. *Mol Cell Biol* **20:** 9423–9433

Okano M, Bell DW, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* **99:** 247–257

Peters AH *et al* (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107:** 323–337

Peters AH *et al* (2003) Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol Cell* **12:** 1577–1589

Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, de la Cruz CC, Otte AP, Panning B, Zhang Y (2003) Role of histone H3 lysine 27 methylation in X inactivation. *Science* **300:** 131–135

Pradhan S, Bacolla A, Wells RD, Roberts RJ (1999) Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of *de novo* and maintenance methylation. *J Biol Chem* **274:** 33002–33010

Rountree MR, Bachman KE, Baylin SB (2000) DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat Genet* **25:** 269–277

Soppe WJ, Jasencakova Z, Houben A, Kakutani T, Meister A, Huang MS, Jacobsen SE, Schubert I, Fransz PF (2002) DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. *EMBO J* **21:** 6549–6559

Sporbert A, Gahl A, Ankerhold R, Leonhardt H, Cardoso MC (2002) DNA polymerase clamp shows little turnover at established replication sites but sequential *de novo* assembly at adjacent origin clusters. *Mol Cell* **10:** 1355–1365

Tamaru H, Selker EU (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* **414:** 277–283

Tariq M, Saze H, Probst AV, Lichota J, Habu Y, Paszkowski J (2003) Erasure of CpG methylation in *Arabidopsis* alters patterns of histone H3 methylation in heterochromatin. *Proc Natl Acad Sci USA* **100:** 8823–8827

Supplementary information

Supplementary Methods

Immunofluorescence and cell cycle analyses.

We detected replication foci (RF) in S phase cells by incubating cells in medium with 100 µM BrdU for 15 min (pulse labeling). The cells were then washed two times with PBS and fixed with 3.7% formaldehyde in PBS. Cells were permeabilized with 0.25% Triton-X-100 for 10 min followed by washing three times with PBS. Non-specific binding of antibodies was prevented by blocking in 0.2% gelatin (fish skin; Sigma) for 30 min. The cells were then incubated for 1 h at 37˚C with mouse monoclonal anti-BrdU antibody (Beckton-Dickinson) or rat monoclonal anti-BrdU antibody (Harlan Sera-lab) along with other desired primary antibodies. The primary antibodies were diluted in buffer containing 0.2% gelatin, 200 U/ml DNaseI (Roche), 0.5 mM ß-mercaptoethanol, 0.33 mM $MgCl_2$, 33 mM Tris-Cl pH 8.1. The following primary antibodies were used: rabbit anti-PATH52 (against Dnmt1) (Leonhardt et al., 1992), rabbit anti-Dnmt1 (N-terminal, gift from I. Suetake and S. Tajima) (Suetake et al., 2001), mouse monoclonal anti-PCNA (with methanol fixed cells, Clone PC10, Dako), rabbit anti-human DNA Ligase I (N-terminal peptide) (Cardoso et al., 1997). Secondary antibodies (Jackson Immuno Research and Molecular Probes) conjugated to the following fluorophores were used: fluorescein isothiocyanate (FITC), Texas Red (TR), Cy5, Alexa Fluor 568, and Alexa Fluor 647. DNA was counterstained with Hoechst 33258 or TOPRO-3 and cells were mounted in Mowiol with 2.5% DABCO.

Cells in the different stages of S phase were identified by the typical patterns of replication foci as described previously (Leonhardt et al., 2000).

Cells in G2 phase were identified by performing a BrdU pulse-chase. Cells were pulse labeled with 10 µM BrdU for 15 min (pulse labeling) and washed twice with pre-warmed

DMEM containing 100μM thymidine. BrdU incorporation was chased by incubating the cells in conditioned medium containing 100μM thymidine for 2-3h. The cells were then fixed with 3.7% formaldehyde for 15min and immunostained for Dnmt1, replication proteins (DNA Ligase I or PCNA) and BrdU as described before. A fixation step with ice cold 100% methanol for 3min was included before formaldehyde fixation only when anti-PCNA (Clone PC10, Dako) was used. Cells showing BrdU incorporation and diffused PCNA or DNA Ligase I are in G2 phase.

Association of Dnmt1 with mitotic chromosomes was detected by fixing cells with ice cold absolute methanol for 10min followed by immunostaining with anti-PATH52 Ab in buffer containing DNaseI as used for BrdU detection. DNMT1 was detected in mitotic chromatin with anti-PATH52 antibody only in methanol fixed cells treated with DNaseI, which may explain why this localization has been overlooked in previous studies. Rabbit anti-Dnmt1 (N-terminal) detected Dnmt1 at mitotic chromatin also in formaldehyde fixed cells.

G1 cells were obtained by isolating mitotic cells by mechanical shake off. C2C12 cells (50% confluent) were vigorously agitated and the loosely attached mitotic cells were harvested by centrifugation at 228xg for 5min and resuspended in 2ml medium. The cells were then seeded on coverslips and incubated for 2h, followed by pulse labeling with BrdU and immunostaining as described. None of the cells stained BrdU positive indicating that they were still in G1.

Supplementary Figure 1

Subnuclear localization of Dnmt1 at different stages during the cell cycle.

*(*A*)* Strategy used to identify cells in defined cell cycle phases (see Supplementary Methods for detailed description). *(*B and C*)* Subnuclear localization of endogenous Dnmt1 at different stages during the cell cycle detected using two different anti-Dnmt1 antibodies - PATH52 *(*B*)*

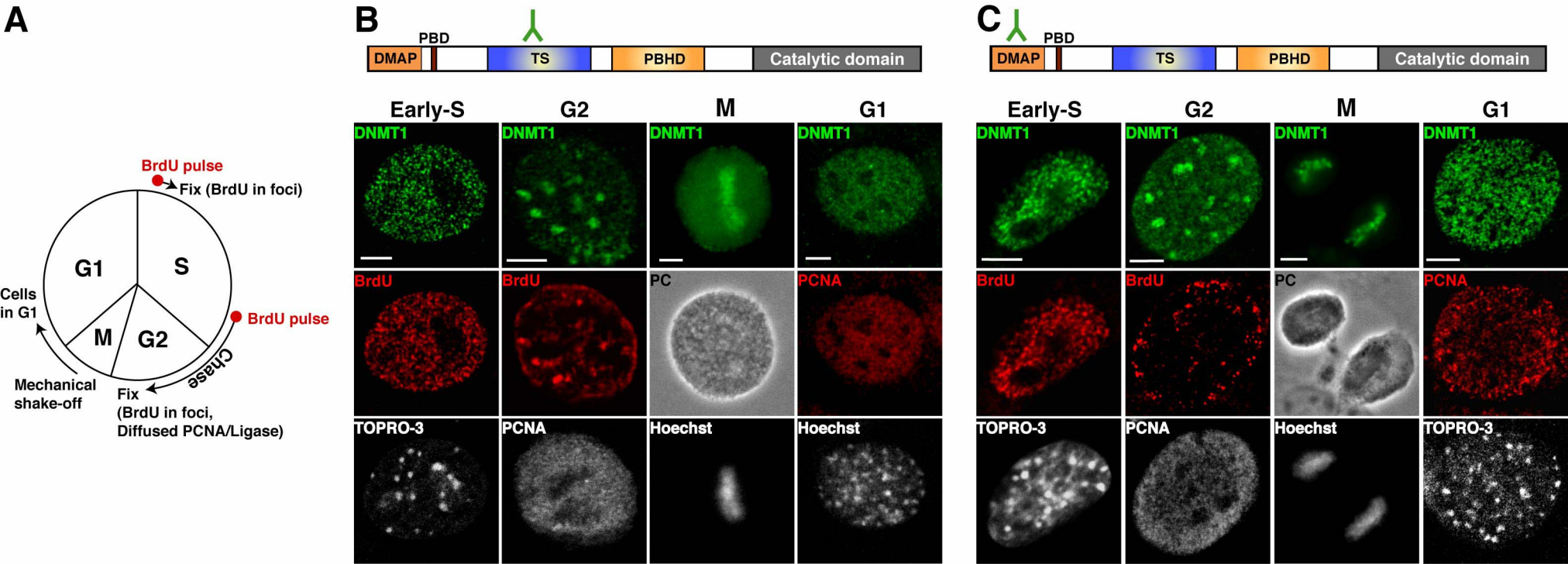
and N-terminal *(C)* (the epitopes recognized by the antibodies are shown at the top). DNA and centromeric heterochromatin were detected with either Hoechst 33258 dye or TOPRO-3, which bind strongly to mouse centromeric heterochromatin (Leonhardt et al., 1992). PC stands for phase contrast. Scale bar: 5 μm.

Supplementary Figure 2

Association of TS with chromatin is conserved in human cells.

HeLa cells expressing TS-GFP (green) and FLAG-tagged HP1ß were immunostained with anti-FLAG antibody (red) and DNA counterstained with TOPRO-3. TS-GFP associates preferentially with constitutive heterochromatin. In metaphase and anaphase, HP1ß does not associate with the condensed chromosomes while TS-GFP does. Scale bar: 5 μm.

Easwaran et al. Supplementary Figure 1



Easwaran et al. Supplementary Figure 2