

DNMT1 but not its interaction with the replication machinery is required for maintenance of DNA methylation in human cells

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DNA methylation plays a central role in the epigenetic regulation of gene expression in vertebrates. Genetic and biochemical data indicated that DNA methyltransferase 1 (Dnmt1) is indispensable for the maintenance of DNA methylation patterns in mice, but targeting of the *DNMT1* locus in human HCT116 tumor cells had only minor effects on genomic methylation and cell viability. In this study, we identified an alternative splicing in these cells that bypasses the disrupting selective marker and results in a catalytically active DNMT1 protein lacking the proliferating cell nuclear antigen-binding domain

required for association with the replication machinery. Using a mechanism-based trapping assay, we show that this truncated DNMT1 protein displays only twofold reduced postreplicative DNA methylation maintenance activity in vivo. RNA interference-mediated knockdown of this truncated DNMT1 results in global genomic hypomethylation and cell death. These results indicate that DNMT1 is essential in mouse and human cells, but direct coupling of the replication of genetic and epigenetic information is not strictly required.

Introduction

In mammals, DNA methylation is crucially involved in controlling gene expression, cell differentiation, silencing of transposable elements, X inactivation, imprinting, and neoplastic transformation (Bird, 2002; Baylin and Ohm, 2006). DNA methylation patterns are established and maintained by three major DNA methyltransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B. DNMT1 is the only mammalian DNMT that has a preference for hemimethylated CpG sites (Bestor and Ingram, 1983; Pradhan et al., 1999) and localizes at both replication foci and repair sites because of its interaction with the proliferating cell nuclear antigen (PCNA; Leonhardt et al., 1992; Chuang et al., 1997; Margot et al., 2001; Easwaran et al., 2004; Mortusewicz et al., 2005).

As a result of these observations and data from genetic manipulations in the mouse, DNMT1 is thought to be the major enzyme responsible for postreplicative maintenance of DNA methylation. Homozygous null deletions of mouse *dnm1* are

lethal early in development and result in an 80% reduction of global genomic methylation in embryonic stem cells and embryos (Lei et al., 1996). These and other studies also showed that despite growing normally, *dnm1*^{-/-} embryonic stem cells have reduced differentiation potential both in vivo and in vitro (Gaudet et al., 1998; Jackson et al., 2004). Later work using conditional deletion demonstrated that Dnmt1 is indispensable for the survival of differentiated cells (Jackson-Grusby et al., 2001).

An equivalent role of DNMT1 in human cells was questioned by the homozygous deletion of exons 3–5 of the *DNMT1* gene in HCT116 colorectal carcinoma cells (Rhee et al., 2000). This deletion encompasses the sequence encoding the PCNA-binding domain (PBD) of DNMT1 and leaves the next exon out of frame. Thus, it was expected that this deletion would eliminate DNMT1 maintenance activity and cause a dramatic drop in genomic methylation levels. Surprisingly, HCT116 cells bearing this deletion (referred to here as MT1 knockout [KO] cells) showed only a 20% reduction of global genomic methylation levels and nearly no loss of methylation at CpG islands. The issue was further complicated by studies in which DNMT1 levels were knocked down by RNAi in human tumor cell lines

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Abbreviations used in this paper: 5-aza-dC, 5-aza-deoxycytidine; DKO, double KO; DNMT, DNA methyltransferase; KO, knockout; PBD, PCNA-binding domain; PCNA, proliferating cell nuclear antigen; wt, wild type.

(Leu et al., 2003; Robert et al., 2003; Suzuki et al., 2004). In these studies, a drastic decrease of methylation at CpG islands was observed, including a study using HCT116 cells (Leu et al., 2003; Robert et al., 2003; Suzuki et al., 2004). At the same time, both the transient and stable knockdown of DNMT1 in HCT116 cells seemed to have only a minor effect on methylation levels similar to those observed in MT1KO cells (Ting et al., 2004). Simultaneous KO of *DNMT1* and *DNMT3B* in HCT116 cells (double KO [DKO] cells) resulted in a dramatic reduction of genomic methylation levels, suggesting a cooperative effect of DNMT1 and DNMT3B on the maintenance of DNA methylation (Rhee et al., 2002).

Interestingly, the combination of hypomorphic and null alleles in the mouse showed that animals expressing ~20% of Dnmt1 wild-type (wt) levels are phenotypically inconspicuous and have normal levels of DNA methylation, whereas mice expressing ~10% of Dnmt1 wt levels show severe hypomethylation, are runted, and develop aggressive T cell lymphomas (Gaudet et al., 2003). Thus, there seems to be a threshold to the amount of Dnmt1 necessary for the maintenance of genomic methylation levels. This threshold amount of 10–20% roughly corresponds to the knockdown levels routinely achieved by RNAi and is hard to detect. Moreover, KO strategies may partially be frustrated by alternative splicing yielding biologically active proteins, albeit at low levels. Indeed, the first attempt to knock out Dnmt1 in mice eliminated only part of exon 4 and lead to a partial loss of function as a result of alternative splicing and weak expression of a truncated form of Dnmt1 (Li et al., 1992, 1993; Lei et al., 1996).

We carefully revisited *DNMT1* expression in MT1KO and DKO cell lines at the RNA and protein levels. Using RT-PCR and a newly developed antibody, we found that alternative splicing occurs in MT1KO and DKO cell lines that bypasses the KO cassette and allows the expression of a DNMT1 variant lacking the PBD. We show that this truncated variant is enzymatically active in vitro and in vivo and that its levels are crucial for the maintenance of global genomic methylation and cell survival.

Results and discussion

MT1KO and DKO cells express an internally deleted DNMT1 variant

We first checked for the presence of DNMT1 transcripts in MT1KO and DKO cells. Northern blot analysis showed that MT1KO cells expressed low levels of an mRNA species with a slightly lower molecular weight than full-length DNMT1 mRNA (Fig. 1 A). Consistently, reverse transcription followed by PCR amplification revealed the presence of DNMT1 mRNA species in parental HCT116 cells and *DNMT* KO derivatives (Fig. 1 B). A primer pair spanning exons 32–35 (corresponding to sequence coding for the catalytic domain) yielded different amounts of a specific PCR fragment in parental, MT1KO, and DKO cells. A second primer pair spanning exons 1–15 and including the region targeted for deletion (exons 3–5; Fig. 1 C) produced fragments with different sizes in parental and MT1KO cells, whereas no product was detectable in DKO cells. However, reamplification of these PCR reactions with a nested set of

primers located in exons 2 and 10 produced the same two fragments for MT1KO and DKO cells. Direct sequencing revealed that these fragments represent alternative splicing events, specifically from the precise joining of exon 2 with either exon 7 (smaller fragment) or exon 6 (larger fragment; Fig. 1 C). Splicing from exons 2–7 does not alter the reading frame and would result in a DNMT1 protein with an internal deletion spanning part of the DMAP1 interaction domain (Rountree et al., 2000) and PBD. In contrast, exons 2 and 6 are not in frame, and their joining results in a reading frame terminating after 30 nt in exon 6 (Fig. 1 C).

The same nested PCR approach with RNA from parental HCT116 cells produced three fragments. Direct sequencing showed that the smallest of these fragments corresponds to the transcript encoding the major DNMT1 somatic isoform, that the medium-sized fragment corresponds to the DNMT1b transcript isoform, which includes an additional 48-bp exon between exons 4 and 5 (exon 4a; Fig. 1 C; Hsu et al., 1999; Bonfils et al., 2000), and that the largest fragment represents heteroduplex molecules of these two isoforms generated during the PCR reaction. These results indicate that the alternative splicing is caused by genomic alterations of the *DNMT1* KO allele.

To verify whether the detected alternatively spliced mRNAs are actually translated in vivo, we subjected whole cell extracts from parental, MT1KO, and DKO cell lines to Western blotting with a new monoclonal antibody against DNMT1 (Fig. 1 D). A major band of ~180 kD was detected in parental HCT116 cells, corresponding to the expected size of full-length DNMT1. In contrast, a single band with an approximate molecular mass of 160 kD was detected in MT1KO and DKO cells. This size fits the predicted molecular weight of the exons 2–7 splicing isoform. Quantification after normalization with an antibody to lamin B showed that the relative abundance of the variant DNMT1 protein expressed in MT1KO and DKO cells with respect to the wt DNMT1 in parental HCT116 cells is at most 17 and 11%, respectively (Fig. 1 D). We conclude that MT1KO and DKO cell lines express decreased amounts of a mutant DNMT1 protein that originates from an alternative splicing event bypassing the deletion of exons 3–5. This mutant, which we hereafter refer to as DNMT1^{ΔE3-6}, lacks part of the DMAP1 interaction domain and PBD.

DNMT1^{ΔE3-6} is catalytically active both in vitro and in vivo and does not interact with PCNA

Next, we tested whether DNMT1^{ΔE3-6} is a functional methyltransferase. To this aim, we first transfected HEK293T cells with expression constructs for either human wt DNMT1, human DNMT1^{ΔE3-6}, mouse Dnmt1 lacking the PBD, or mouse Dnmt1^{C1229W} all fused to GFP. In the latter construct, the cysteine responsible for the transient formation of a covalent bond with the C5 position of the cytosine ring during the methylation reaction is replaced by tryptophan, resulting in a catalytically inactive enzyme (Schermelleh et al., 2005). Extracts were made from the transfected cells, and the respective GFP fusion proteins were immunopurified and tested for their methyltransferase activity in vitro. Except for GFP-Dnmt1^{C1229W}, which,

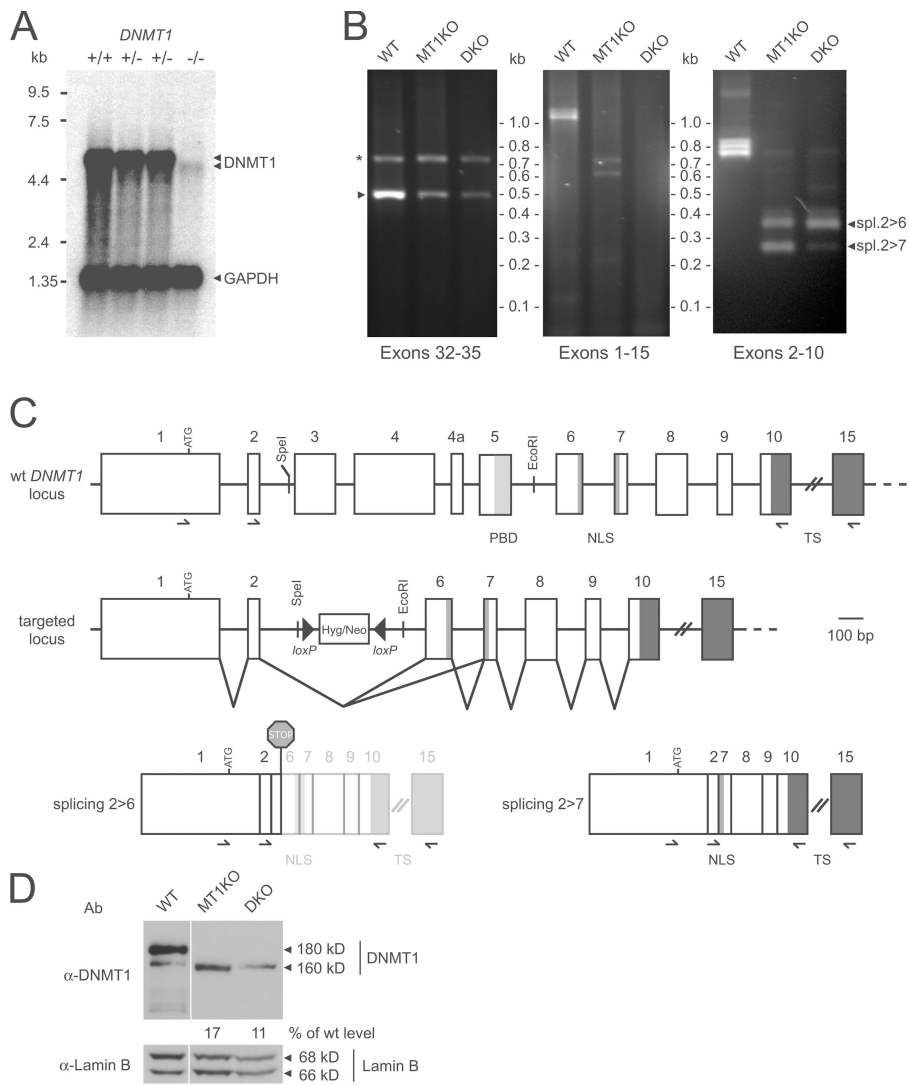


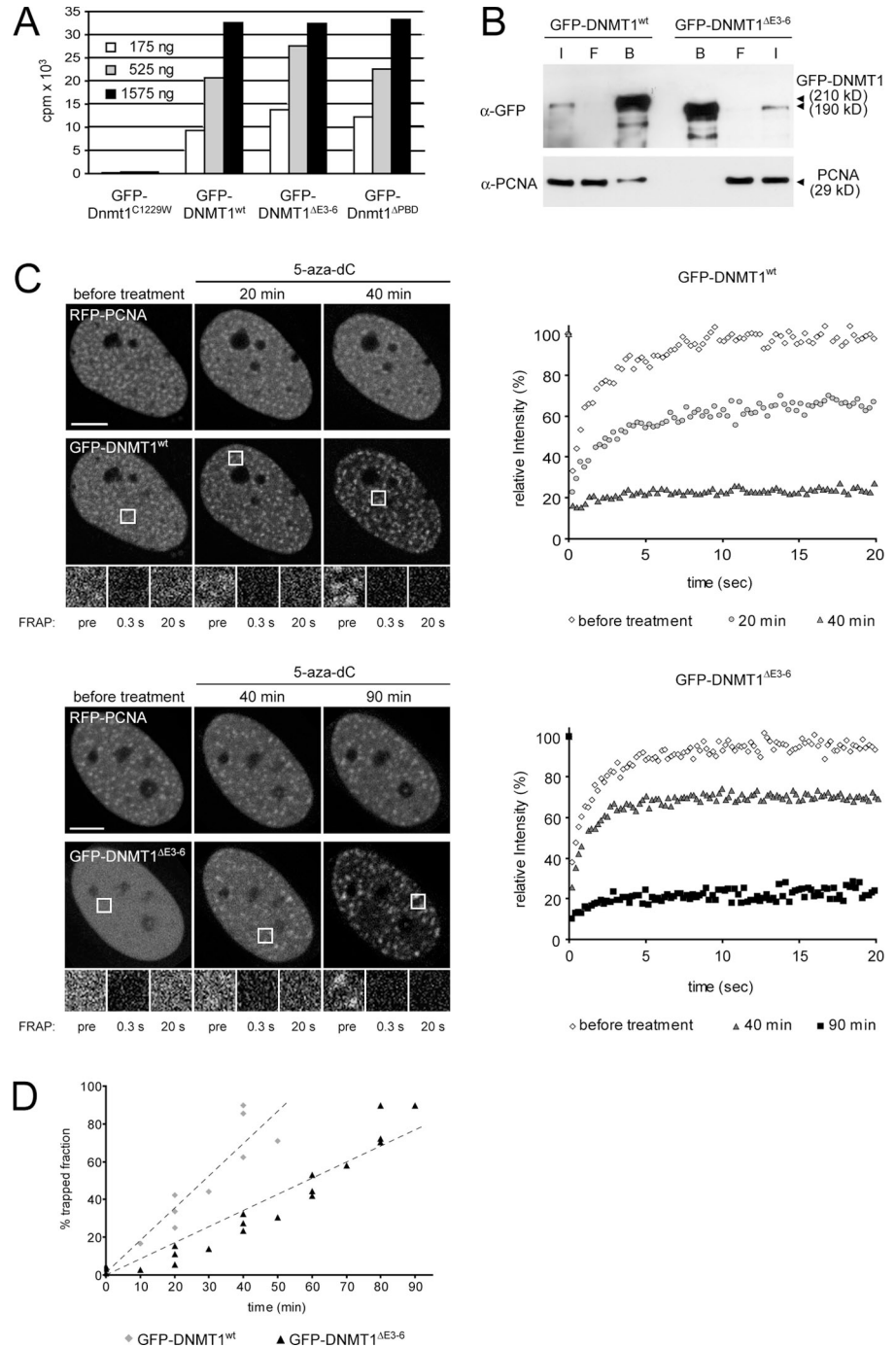
Figure 1. *DNMT1*^{-/-} (MT1KO) and *DNMT1*^{-/-}; *DNMT3B*^{-/-} (DKO) HCT116 cell lines express an internally deleted DNMT1 variant. (A) Northern blot analysis of HCT116 cell lines, including parental cells (+/+), two independent *DNMT1*^{-/-} clones, and one *DNMT1*^{-/-} clone (MT1KO). The blot was hybridized with a DNMT1 cDNA probe and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a loading control. The positions of the two transcripts and molecular weight markers are indicated. (B) RT-PCR analysis of parental (WT), *DNMT1*^{-/-} (MT1KO), and *DNMT1*^{-/-}; *DNMT3B*^{-/-} (DKO) HCT116 cells. The amplified regions are indicated at the bottom of each panel. In the left panel, the arrowhead indicates the specific PCR fragment, and the asterisk indicates an unspecific product. The right panel shows a nested PCR, where PCR products shown in the middle panel were used as templates. The two alternative splice forms expressed in MT1KO and DKO cells (right) and the positions of molecular weight markers are indicated. (C) Schematic drawing of the wt (top) and targeted *DNMT1* alleles (middle) and alternatively spliced transcripts from the latter allele (bottom). Exons are shown as open rectangles (numbered on top) and are drawn to scale, whereas noncoding sequences are shown as lines and are not in scale. Sequences coding for major peptide domains are shown in different shades of gray, and their names are reported at the bottom. The positions of primers used for RT-PCR, translational start (ATG) codons, and termination (STOP) codons are indicated. (D) Western blot analysis of parental HCT116 cells and *DNMT1* KO derivatives with antibodies to DNMT1 and lamin B1 (as a loading control). The molecular mass of major bands is indicated. The 170-kD band detected with the anti-DNMT1 antibody in the parental cells lane has a higher molecular mass than the band detected in MT1KO and DKO lanes and likely represents a DNMT1 degradation product. The percentages of normalized signal levels from the DNMT1 variant in MT1KO and DKO cells with respect to wt DNMT1 levels in HCT116 cells (100%) are shown at the bottom of the corresponding lanes.

as expected, had no substantial catalytic activity, all GFP-fused enzymes showed very similar levels of methyltransferase activity (Fig. 2 A). This result shows that DNMT1^{ΔE3-6} is catalytically active in vitro. Importantly, GFP-DNMT1^{ΔE3-6} that was transiently expressed in human cells localized to the nucleus (Fig. 2 C), which is consistent with the identification of multiple functional NLSs in mouse Dnmt1 (Cardoso and Leonhardt, 1999). This opened the possibility that the residual DNMT1^{ΔE3-6} could contribute to maintenance of the relatively high genomic methylation levels in MT1KO cells. As the deletion in DNMT1^{ΔE3-6} eliminates the PBD, we checked whether GFP-DNMT1^{ΔE3-6} is still capable of interacting with PCNA. Endogenous PCNA coimmunoprecipitated with GFP-DNMT1^{wt} expressed in HEK293T cells but not with GFP-DNMT1^{ΔE3-6} (Fig. 2 B).

To test whether DNMT1^{ΔE3-6} is catalytically functional in vivo, we used a recently developed trapping assay (Schermele et al., 2005). HeLa cells were cotransfected with expression

constructs for RFP-PCNA and either GFP-DNMT1^{wt} or GFP-DNMT1^{ΔE3-6}. 24 h after transfection, cells were incubated in the presence of 5-aza-deoxycytidine (5-aza-dC). During replication, this cytosine analogue is incorporated into newly synthesized DNA. When DNMT1 engages in the methylation of 5-aza-dC, a covalent complex is formed that cannot be resolved. As a consequence, DNMT1 is immobilized (trapped) at these sites. The trapping rate can be measured by FRAP as a time-dependent decrease of the mobile fraction of GFP-DNMT1 fusions and reflects their enzymatic activity in vivo. In contrast to GFP-DNMT1^{wt}, GFP-DNMT1^{ΔE3-6} did not accumulate at replication foci during early to mid S phase (Fig. 2 C). This is consistent with our coimmunoprecipitation results and data obtained with a mouse Dnmt1 mutant lacking the PBD (Easwaran et al., 2004). However, upon the addition of 5-aza-dC, a time-dependent focal accumulation of GFP-DNMT1^{ΔE3-6} was observed. FRAP analysis revealed that GFP-DNMT1^{ΔE3-6} is trapped with only an approximately twofold lower efficiency than GFP-DNMT1^{wt}

Figure 2. GFP-DNMT1^{ΔE3-6} is catalytically active in vitro and is capable of postreplicative DNA methylation in vivo. (A) In vitro DNMT assay of GFP-DNMT1 fusion variants transiently expressed in HEK293T cells and immunopurified with an anti-GFP antibody. (B) Coimmunoprecipitation of endogenous PCNA with either GFP-DNMT1^{wt} or GFP-DNMT1^{ΔE3-6} expressed in HEK293T cells. Input (I), flow through (F), and bound (B) fractions are shown; 5% of input and flow through fractions with respect to bound fractions was loaded. The molecular masses of the two GFP fusions are indicated on the right. (C and D) Trapping assay for GFP-DNMT1^{wt} and GFP-DNMT1^{ΔE3-6} transiently expressed in HeLa cells. (C) Images on the left show cells in early S phase according to the RFP-PCNA pattern and expressing the indicated GFP-DNMT1 variant. The distribution of fluorescent fusions is shown before and at indicated time points after the addition of 30 μM 5-aza-dC. Regions targeted by photobleaching are indicated by boxes and are shown magnified at the bottom before and 0.3 (first postbleaching time point) and 20 s after bleaching. Bars, 5 μm. Plots on the right show FRAP curves of GFP fusions at targeted regions before and at indicated time points after the addition of 5-aza-dC from the corresponding cells shown on the left. (D) Trapping assays for GFP-DNMT1^{wt} or GFP-DNMT1^{ΔE3-6} were performed as in C on three cells for each construct, and the estimated immobilized fractions were plotted with respect to the time of incubation with 5-aza-dC.



(Fig. 2, C and D). These data strongly suggest that the DNMT1^{ΔE3-6} enzyme expressed in MT1KO and DKO cells is fully catalytically active, localizes to the nucleus, and is capable of postreplicative methylation maintenance despite the loss of interaction with PCNA.

The level of DNMT1^{ΔE3-6} in MT1KO cells is critical for cell survival and maintenance of global genomic methylation

We then sought to establish the contribution of DNMT1^{ΔE3-6} to the maintenance of genomic methylation levels in MT1KO cells. To this aim, we knocked down DNMT1^{ΔE3-6} by RNAi for

a prolonged period of time and analyzed global genomic methylation levels (Fig. 3). At each collection point, a relatively modest but substantial decrease in DNMT1^{ΔE3-6} protein levels was achieved (Fig. 3 A). Progressively fewer cells were found in cultures treated with DNMT1 siRNA with respect to cultures treated with control siRNA (Fig. 3 B). Microscopic inspection of cultures after 8 and 12 d of treatment with DNMT1 siRNA revealed large numbers of dead cells and cells with very long and thin cytoplasmic protrusions, whereas cells treated with control siRNA exhibited a normal morphology and death rate (Fig. 3 C). Interestingly, both of these phenotypes are reminiscent of the highly hypomethylated DKO cells. Global genomic

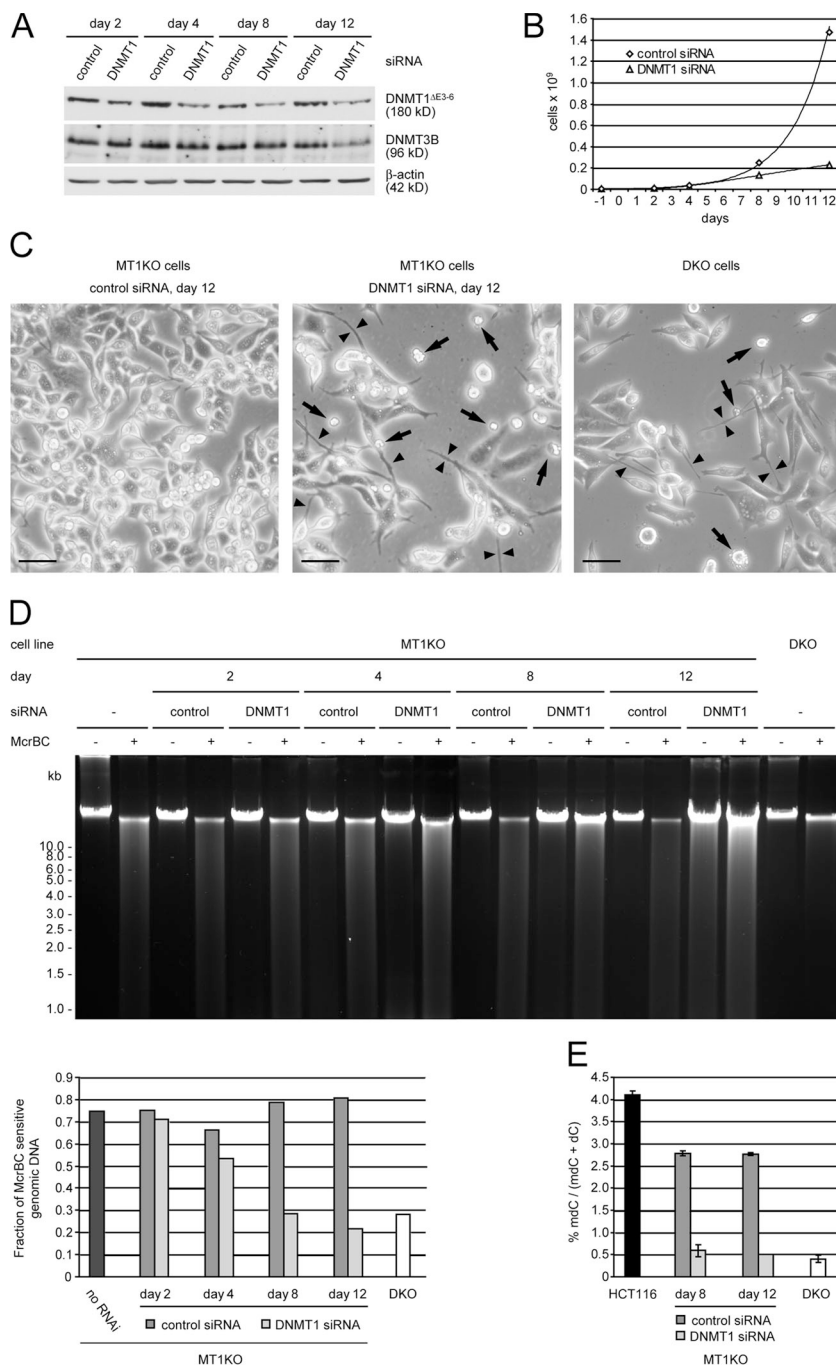


Figure 3. Prolonged knockdown of DNMT1^{ΔE3-6} in MT1KO cells leads to genomic demethylation and cell death. Equal numbers of MT1KO cells were plated and transfected with either DNMT1 or control siRNA the next day (day 0) and every second day for 12 d. On days 2, 4, 8, and 12, cells were harvested, aliquots were collected for protein extracts and DNA isolation, and, except for day 12, equal numbers were replated. (A) Levels of DNMT1^{ΔE3-6} and DNMT3B were analyzed by Western blotting of whole cell extracts collected at the indicated days of siRNA treatment. Detection of β-actin was used to control for loading. (B) Cell numbers were plotted taking into consideration the splitting factor at each passage. (C) Phase-contrast images of MT1KO cultures treated with either control (left) or DNMT1 (middle) siRNA for 12 d and an image of DKO cells (right). Arrows point to dead cells, and arrowheads point to cells with thin and extended cytoplasmic protrusions that are present only in MT1KO cells treated with DNMT1 siRNA and in DKO cells. Bars, 50 μm. (D and E) Assays for the determination of global genomic methylation levels from the indicated cell lines and treatments. (D) Genomic DNA was digested with the M_{cr}BC endonuclease, which selectively recognizes methylated sequences. The top panel shows electrophoretic separation of the digests. Progressive demethylation in cells treated with DNMT1 siRNA is indicated by the increasingly similar patterns between mock digestions (–) and samples incubated with the enzyme (+). The bottom panel shows quantification of the M_{cr}BC-sensitive (methylated) DNA fraction from the samples shown in the top panel. (E) HPLC quantification of global 5-methyl-2'-deoxycytidine (mC) content. Bars and error bars represent mean values and SEM from five measurements, respectively, except for HCT116 parental cells and MT1KO cells treated with DNMT1 siRNA for 12 d (six and two measurements, respectively).

methylation was assayed by restriction with the endonuclease M_{cr}BC, which selectively digests methylated sequences, and by HPLC analysis (Fig. 3, D and E). Progressively lower genomic methylation was detected from days 4 to 12 of treatment with DNMT1 siRNA, with cells treated for 8 and 12 d retaining only ~10% of the methylated cytosines present in parental HCT116 cells, which is similar to the level found in DKO cells. Interestingly, the levels of DNMT3B remained unaffected until day 8 of RNAi treatment when global genomic methylation was already drastically decreased (Fig. 3, A, D, and E). A slight reduction of DNMT3B levels was observed only after 12 d of RNAi treatment and is likely caused by secondary effects. Thus, a prolonged reduction of DNMT1^{ΔE3-6} levels, although moderate,

caused a drastic decrease of genomic methylation in the presence of normal levels of DNMT3B.

These results indicate that the residual level of the DNMT1 mutant present in MT1KO cells provides most of the methyltransferase activity responsible for maintaining relatively high methylation levels in this cell line. Interestingly, DKO cells express a similarly reduced amount of DNMT1^{ΔE3-6} (Fig. 1 D), which could explain their very low level of DNA methylation. Thus, the situation revealed here for the human MT1KO and DKO cell lines is reminiscent of transgenic mice bearing hypomorphic and null *dnmt1* alleles. Although homozygotes for the hypomorphic allele were phenotypically normal and showed nearly no molecular alterations, the combination of

a hypomorphic allele with a null allele resulted in severe hypomethylation, growth defects, and cancer (Gaudet et al., 2003). Analogously, the contrasting results previously obtained with DNMT1 knockdown experiments in human cell lines are likely caused by the varying efficiency of RNAi that may or may not lower DNMT1 below the threshold level that is sufficient for the maintenance of normal methylation levels. Also, the reduced proliferation rate and viability of severely hypomethylated cells may lead to an enrichment of cells with less efficient knockdown of DNMT1, which may explain the variable results obtained in different RNAi studies. The drastically decreased viability of MT1KO cells that we observed upon the knockdown of DNMT1 is also consistent with the rapid cell death of mouse fibroblasts after the conditional KO of *Dnmt1* (Jackson-Grusby et al., 2001). While this manuscript was in preparation, a residual DNMT1 activity crucial for cell survival was independently identified in MT1KO cells (Egger et al., 2006). These results indicate that DNMT1 plays a similar and prominent role in the maintenance of DNA methylation in mouse and human cells and that the dependence on DNMT1 for the survival of differentiated cells is similar in these species and likely in all mammals.

We showed that the DNMT1^{ΔE3-6} mutant expressed in MT1KO cells is enzymatically active and displays only a two-fold reduced postreplicative methylation rate *in vivo* despite lacking the domain responsible for interaction with PCNA. We also showed by RNAi-mediated knockdown experiments that the expression level of this truncated DNMT1 is critical for the maintenance of genomic methylation. Collectively, these results demonstrate that the interaction of DNMT1 with the replication machinery is not strictly necessary for the maintenance of DNA methylation but improves its efficiency. As most cell types express an excess of DNMT1, this improved efficiency may not be critical for cell survival but may contribute to the faithful maintenance of epigenetic information and stable gene expression patterns in differentiated cells and developing organisms.

Materials and methods

Cell culture and transfection

HeLa, HEK293T, and HCT116 cells and their derivatives were maintained in DME supplemented with 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamycin. All HCT116 cell lines were supplied by B. Vogelstein and K. Schuebel (Johns Hopkins University, Baltimore, MD). Cells were transfected with Transfectin (Bio-Rad Laboratories) according to the manufacturer's instructions.

Northern blotting and RT-PCR analysis

5 μg poly (A)⁺ RNA from the indicated cell lines were subjected to Northern blotting and probed with a 1.1-kb BamHI fragment from DNMT1 cDNA according to standard procedures. For RT-PCR, total RNA was isolated from HCT116, MT1KO, and DKO cells with TRIzol reagent (Invitrogen), and reverse transcription was performed with the First Strand cDNA Synthesis kit (GE Healthcare) by priming with random hexamer oligonucleotides. PCR amplifications were performed in a 50-μl final volume containing 1.5–2.0 U SAWADY Taq DNA polymerase (PegLab), 1× buffer (20 mM Tris, pH 8.55, 16 mM [NH₄]₂SO₄, 2 mM MgCl₂, and 0.1% Tween 20), 0.2 μM of each primer, and 0.2 mM of each deoxynucleotide triphosphate. For primary PCR reactions, cDNA template from ~0.75 μg of total RNA was used with either primers HMT1catF (5'-TGCAACATCCTGCTGAAGCTGG-3'; forward, exon 32) and HMT1catR (5'-GACCCGAGCTCAACCTGGTTATG-3'; reverse, exon 35) or HMT1mF (5'-GTCTGCTGAAGCCTCCGAGATG-3'; forward, exon 1) and HMT1e15R (5'-TTTGAGGTCAGGGTCGTCAGG-3'; reverse, exon 15) as well as the following touchdown cycling profile:

94°C for 2 min; 10 cycles at 94°C for 20 s; 68–60°C descending by 2°C every two cycles for 15 s; 72°C for 30 s plus a 3-s increment at each cycle; 30 cycles at 94°C for 20 s; 58°C for 15 s; and 72°C for 1 min plus a 5-s increment at each cycle. For nested PCR reactions, 1 μl of primary reaction product was used as a template with primers HMT1e2F (5'-AAAGATTGGAAAGAGACAGCTTAACAG-3'; forward, exon 2) and HMT1e10R (5'-TCTCCATCTTCGCTCCTCAG-3'; reverse, exon 10) and a cycling profile consisting of 25 cycles of 94°C for 20 s, 58°C for 15 s, and 72°C for 30 s plus a 5-s increment at each cycle.

Antibodies

Monoclonal antibodies were raised against purified recombinant DNMT1 and 6×His-tagged PCNA. Approximately 50 μg of antigen was injected both i.p. and subcutaneously into Lou/C rats using CPG2006 (TIB MOL-BIO) as adjuvant. After an 8-wk interval, a boost was given i.p. and subcutaneously 3 d before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed according to standard procedures. DNMT3B was detected with a mouse monoclonal antibody that recognizes the conserved catalytic domain within the DNMT3 family (clone 64B1446; Imgenex). The lamin B1 (H90), β-actin (clone AC-15), and GFP antibodies were obtained from Santa Cruz Biotechnology, Inc., Sigma-Aldrich, and Roche, respectively.

Plasmid construction

Expression constructs for mouse GFP-Dnmt1^{ΔPBD}, GFP-Dnmt1^{C1229W}, human GFP-DNMT1^{wt}, and RFP-PCNA were described previously (Easwaran et al., 2004; Schermelleh et al., 2005; Sporbert et al., 2005). The GFP-DNMT1^{ΔE3-6} expression construct was derived from the GFP-DNMT1^{wt} construct by overlap extension PCR (Ho et al., 1989).

Coimmunoprecipitation and DNMT assay

Extracts were prepared from transfected HEK293T cells in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF, and 0.5% NP-40), diluted with lysis buffer without NP-40, and incubated with an anti-GFP antibody for 30 min at 4°C with constant mixing. Immuno-complexes were pulled down with protein A-Sepharose beads (GE Healthcare), and the beads were washed extensively with dilution buffer containing 300 mM NaCl. For coimmunoprecipitation, beads were resuspended in SDS-PAGE loading buffer. For *in vitro* methyltransferase assay, beads were further washed with and resuspended in assay buffer (100 mM KCl, 10 mM Tris, pH 7.6, 1 mM EDTA, and 1 mM DTT), and 30 μl methylation mix (0.1 μCi [³H]S-adenosyl-methionine, 1.67 pmol/μl hemimethylated double-stranded 35-mer oligonucleotide, and 160 ng/μl BSA in assay buffer) was added. After incubation at 37°C for 2.5 h, reactions were spotted onto DE81 cellulose paper filters (Whatman), and radioactivity was measured by liquid scintillation.

Live cell trapping assay

The DNMT trapping assay was performed essentially as described previously (Schermelleh et al., 2005). In brief, transfected cells were incubated with 30 μM 5-aza-dC (Sigma-Aldrich) for the indicated periods of time before photobleaching experiments. FRAP analysis was performed with a confocal laser-scanning microscope (TCS SP2; Leica) equipped with a 63× 1.4 NA plan-Apochromat oil immersion objective (Leica). GFP and RFP were excited with a 488-nm Ar laser and a 561-nm diode laser, respectively. Image series were recorded with a frame size of 256 × 256 pixels, a pixel size of 100 nm, and laser power set to 1–5% of transmission with a detection pinhole size of 1 Airy U. For FRAP analysis, regions of interest were photobleached with an intense Ar laser beam (laser set to maximum power at 100% transmission of all laser lines) for 0.5 s. Image series were recorded before and after bleaching at 0.27-s intervals (typically 20 prebleach and 50–100 postbleach frames). Mean fluorescence intensities of the bleached region were corrected for background and total loss of nuclear fluorescence over the time course and were normalized to the mean of the last 10 prebleach values.

RNAi

Equal numbers of MT1KO cells were plated and transfected the next day (day 0) and every second day with 40 nM of either DNMT1 ShortCut siRNA Mix (New England Biolabs, Inc.) or a control siRNA (AllStars Negative Control siRNA; QIAGEN) using HiPerFect transfection reagent (QIAGEN). On days 2, 4, and 8, cells were harvested, aliquots were collected for protein extracts and DNA isolation, and equal numbers were replated and simultaneously transfected. On days 6 and 10, cells were transfected without splitting. The medium was never changed in between transfections.

Global genomic methylation analysis

Genomic DNA was isolated by the phenol-chloroform extraction method. For the McrBC nuclease assay, 0.5- μ g aliquots were incubated with or without 10 U of the enzyme (New England Biolabs, Inc.) in buffer supplied by the manufacturer for 1 h at 37°C, and digests were separated by agarose gel electrophoresis. ImageJ software (<http://rsb.info.nih.gov/ij/>) was used to quantify the McrBC-resistant fractions from digital images. The McrBC-sensitive fraction shown in Fig. 3 D was calculated as follows: (resistant fraction – mock-resistant fraction)/mock-resistant fraction. For HPLC analysis, DNA samples were further treated with RNase A and T1, phenol extracted, dialyzed extensively against 10 mM Tris, pH 7.2, and 0.1 mM EDTA, and hydrolyzed to nucleosides as described previously (Song et al., 2005) except that 5 U Antarctic phosphatase (New England Biolabs, Inc.) was used for dephosphorylation. HPLC was performed on the Alliance system (Waters) using a Nucleosil C-18 column at a flow rate of 0.5 ml/min with a linear increase of buffer A (0.1 M HNEt₃OAc) from 0 to 20% in buffer B (0.1 M HNEt₃OAc in 80% acetonitrile) in 30 min.

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