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Loss of maintenance DNA methylation results in abnormal DNA origin firing during DNA replication

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ABSTRACT

The mammalian maintenance methyltransferase DNMT1 [DNA (cytosine-5-)-methyltransferase 1] mediates the inheritance of the DNA methylation pattern during replication. Previous studies have shown that depletion of DNMT1 causes a severe growth defect and apoptosis in differentiated cells. However, the detailed mechanisms behind this phenomenon remain poorly understood. Here we show that conditional ablation of *Dnmt1* in murine embryonic fibroblasts (MEFs) resulted in an aberrant DNA replication program showing an accumulation of late-S phase replication and causing severely defective growth. Furthermore, we found that the catalytic activity and replication focus targeting sequence of DNMT1 are required for a proper DNA replication program. Taken together, our findings suggest that the maintenance of DNA methylation by DNMT1 plays a critical role in proper regulation of DNA replication in mammalian cells.

1. Introduction

DNA 5- cytosine methylation is a major epigenetic modification that regulates gene expression, genome imprinting, X-chromosome inactivation and silencing of retrotransposable elements in mammals [1] [2] [3] [4]. It has been reported that DNA methylation promotes the compaction and stabilization of the nucleosome by facilitating rigid wrapping of DNA around the histone core [5] [6].

DNMT1 [DNA (cytosine-5-)-methyltransferase 1] is the principal methyltransferase in mammalian cells required for maintenance of DNA methylation coupled with DNA replication [7] [8]. The DNMT1 protein is characterized by a C-terminal catalytic domain (CTD) and an N-terminal regulatory region [1]. The N-terminal region of DNMT1 contains several functionally important motifs including the replication focus targeting sequence (RFT), the CXXC domain that binds unmethylated CpG sites and the PCNA-binding region [9] [10]. Recently, it has been shown that recognition of unmethylated CpGs by the CXXC domain plays a role in autoinhibition of the catalytic activity by the CTD [11]. It has also been reported that without a substrate (i.e. hemimethylated DNA), the RFT domain may interact with the CTD to prevent aberrant DNA methylation [12] [13].

Specific recruitment of DNMT1 to hemimethylated DNA by NP95 (nuclear protein 95 kDa, also known as UHRF1 and ICBP90) ensures faithful inheritance of DNA methylation marks [14] [15]. NP95 possesses E3 ubiquitin ligase activity [16] and by using a *Xenopus* egg extract system we previously found that ubiquitination of histone H3 lysine 23 (uH3K23) mediated by NP95 plays a critical role in the recruitment of DNMT1 to the replication foci and subsequent maintenance DNA methylation [17].

Recently, zebra fish Uhrf1 mutant larvae were shown to exhibit up-regulation of many S-phase cyclins as well as DNA replication licensing and processivity factors [18]. It is also known that the timing of DNA replication correlates with the chromatin structure [19] and that aberrant timing of DNA replication leads to genomic instability [20]. Interestingly, it was reported that the DNA damage response is activated in *Dnmt1* knockout [21] [22] and *Dnmt1* knockdown [23]. *Dnmt1* depletion causes embryos to die. [7] [24] [25], indicating that DNMT1 has a potential role in maintaining genome stability *in vivo*. Surprisingly, murine embryonic stem cells (ESCs) deficient in all three DNA methyltransferase genes, namely *Dnmt1*, *Dnmt3a* and *Dnmt3b*, are viable and apparently do not show

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any defects related to cell growth [26]. However, genetic ablation of *DNMT1* in human ESCs caused cell death according to a recent study [27], indicating that the requirement for DNMT1 could be different, at least in terms of cell survival, between murine and human ESCs.

A number of studies have shown that DNMT1 is essential for survival and proliferation. However, the detailed mechanisms behind DNMT1-mediated regulation of cell growth and the cell cycle are not fully understood. In this study, employing a previously described *Dnmt1* allele [22], we have established a conditional DNMT1 MEF cell line in which the endogenous *Dnmt1* gene (flanked by LoxP sequences) is ablated by the addition of 4OH tamoxifen (4OHT). We found that MEFs exhibited impaired cell growth accompanied by a misregulated S phase upon deletion of *Dnmt1*. By carrying out rescue experiments with wild type (WT), mutant and truncated DNMT1 proteins, we revealed that both the catalytic activity and RFT domain are required for regulation of proper cell cycle progression and cell growth. Taken together, our study demonstrates that there is a mechanistic link between DNA methylation and proper cell growth and regulation of the DNA replication program.

2. Materials and methods

2.1. Cell culture and transfection

Dnmt1$^{Flox/Flox}$ MEFs were prepared from heterozygous intercrosses of mice provided by Dr.R.Jaenisch (Jackson-Grusby et al., 2001). All genotypes were confirmed by PCR. The cells were immortalized by means of the 3T3 method. 3T3 Dnmt1$^{Flox/Flox}$ MEFs were infected with retrovirus carrying MSCV CreERT2 (addgene #22776). The cells underwent loss of Dnmt1 by the addition of (Z)-4-Hydroxytamoxifen (4OHT) (Sigma H7904). G0 synchronization of MEFs was carried out for 55 h under serum starvation (2.5% FBS) Cells were maintained in DMEM containing 10% FBS (Gibco) and 1% Pen-Strep (Invitrogen) All cells were cultured at 37 °C under 5% CO_2.

2.2. Retrovirus infection

Plat-E cells (1×10^6 cells) were seeded onto 60-mm dishes one day prior to infection. Transfection with retroviral vector by Lipofectamine® 2000 (Thermo) was performed according to the manufacturer's protocol. After 48 h, the retroviral supernatant was used for infection of the cells.

2.3. Immunofluorescence

Cells grown on coverslips were labeled for 30 min with 100 μM CldU, rinsed with PBS and fixed with ice-cold methanol/acetic acid for 30 min at −20 °C. Cellular DNA was denatured in 2 N HCl for 30 min. The cells were washed with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Next cells were incubated for more than 1 h at room temperature with anti-BrdU rat monoclonal antibody (1:400) (BU1/75: ab6326, Abcam) diluted with blocking buffer. They were then incubated with Fluor-conjugated secondary antibodies (1:500) (Life Technologies) for 1 h. DNA was counterstained with Hoechst 33342 (1:1000). Images were acquired using a DeltaVision Elite (Applied Precision).

2.4. Immunoblotting

Collected cells were suspended in sample buffer, boiled for 5 min and used as a total cell lysate. The chromatin fraction was prepared as described previously [28].

2.5. Antibodies

The following antibodies were used in this study: mouse anti-Flag (F3165; Sigma), mouse anti-Chk1 (C9358; Sigma) mouse anti-β-actin (ab6276; Abcam), rabbit anti-Dnmt1 (sc-20701; Santa Cruz), rabbit anti-Histone H2AX (ab11175; Abcam), mouse anti-phospho Histone H2AX (Ser139) (JBW301 05–636; Upstate), rabbit anti-phospho-Chk1 (Ser345) (#2348; Sigma), rabbit anti-Histone H3 (ab1791-100; Abcam), rat anti-BrdU (ab6326; Abcam) and anti-rat IgG conjugated with Alexa Fluor 594 (Life Technologies).

2.6. Plasmid construction

To generate lentivirus constructs, wild type and several mutants of mouse *Dnmt1* with *Not*I-3 × Flag and *Eco*RV (blunt end) sites were obtained by PCR amplification of the wild type, C1229S, H168R and a small deletion fragment of RFT (428–503) mouse *Dnmt1* DNA (a gift of M. Okano) and ligated into CS-IV-CMV-MCS-IRES2-Bsd vector (a gift of H. Miyoshi) cut by Eco47iii (blunt end) and *Not*I. The sequences used in PCR were as follows: *Dnmt1*-F (*Eco*RV + 3 × Flag) ACTCGGATATCCATGGATTA-CAAGGATGACGACGATAAGGACTATAAGGACGATGATGA-CAAGGACTACAAAGATGATGACGATAAAATGCCAGCGCGAACAG, *Dnmt1*-R (*Not*I) GAGGCTGCTACCAAGGACTAGGCGGCCGCACTC.

2.7. Lentiviral transduction

Lentiviral transduction was performed essentially as previously described [29].

2.8. FACS analysis

Cells were fixed with 70% ethanol and DNA was stained with 0.1 mg/ml propidium iodide containing RNase for 30 min at 37 °C. Flow cytometry was performed using a FACSVerse (BD Biosciences).

2.9. Real-time PCR

Total RNA was extracted as previously described [30]. Expression levels were normalized to GAPDH. Primers used in the real-time PCR were IAPez-gag (F) 5'-cacgctccggtagaatacttacaaat-3′, IAPez-gag (R) 5'-cctgtctaactgcaccaaggtaaaat-3′, GAPDH(F) 5'-aactttggcattgtggaagg-3′ and GAPDH(R) 5'-ggatgcagggatgatgttct-3'.

2.10. Micrococcal nuclease (MNase) assay

MNase assays were performed essentially as previously described [31]. The same amounts of MNase buffer containing MNase (1 units/ml) were used in this study.

3. Results

3.1. DNMT1 depletion results in an abnormal DNA replication program

In this study, we first established a *Dnmt1* conditional murine embryonic fibroblast cell line (immortalized MEFs) to elucidate the roles of DNMT1 in genome stability and cell growth. In these MEF cells, the endogenous *Dnmt1* exons (exon 4 and exon 5) were flanked by LoxP sequences and deletion of these exons was induced by infection with a retroviral vector expressing Cre-ER^{T2} and the addition of 4OH tamoxifen (4OHT) [22]. Given that DNMT1 has a role in regulating cell growth and survival, we investigated the cell cycle pattern in WT and *Dnmt1*cKO MEFs and confirmed that the