Dnmt1 deficiency leads to enhanced microsatellite instability in mouse embryonic stem cells

Myungjin Kim, Binh N. Trinh, Tiffany I. Long, Shirley Oghamian and Peter W. Laird*

Department of Surgery and Department of Biochemistry and Molecular Biology, University of Southern California, Keck School of Medicine, Norris Comprehensive Cancer Center, Los Angeles, CA 90089-9176, USA

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ABSTRACT

DNA hypomethylation is frequently seen in cancer and imparts genomic instability in mouse models and some tissue culture systems. However, the effects of genomic DNA hypomethylation on mutation rates are still elusive. We have developed a model system to analyze the effects of DNA methyltransferase 1 (Dnmt1) deficiency on DNA mismatch repair (MMR) in mouse embryonic stem (ES) cells. We generated sibling ES cell clones with and without functional Dnmt1 expression, containing a stable reporter gene that allowed us to measure the slippage rate at a mononucleotide repeat. We found that Dnmt1 deficiency led to a 7-fold increase in the microsatellite slippage rate. Interestingly, the region flanking the mononucleotide repeat was unmethylated regardless of Dnmt1 status, suggesting that it is not the local levels of DNA methylation that direct the increase in microsatellite instability (MSI). The enhanced MSI was associated with higher levels of histone H3 acetylation and lower MeCP2 binding at regions near the assayed microsatellite, suggesting that Dnmt1 loss may decrease MMR efficiency by modifying chromatin structure.

INTRODUCTION

Establishment and maintenance of proper DNA methylation are important in mammalian development and genomic stability (1). Global DNA hypomethylation is frequently observed in tumors (2,3). DNA hypomethylation can induce B- and T-cell lymphomas and sarcomas in mice (4–6). However, the effects of genomic DNA hypomethylation on mutation rates are still elusive. We have developed a model system to analyze the effects of DNA methyltransferase 1 (Dnmt1) deficiency on DNA mismatch repair (MMR) in mouse embryonic stem (ES) cells. We generated sibling ES cell clones with and without functional Dnmt1 expression, containing a stable reporter gene that allowed us to measure the slippage rate at a mononucleotide repeat. We found that Dnmt1 deficiency led to a 7-fold increase in the microsatellite slippage rate. Interestingly, the region flanking the mononucleotide repeat was unmethylated regardless of Dnmt1 status, suggesting that it is not the local levels of DNA methylation that direct the increase in microsatellite instability (MSI). The enhanced MSI was associated with higher levels of histone H3 acetylation and lower MeCP2 binding at regions near the assayed microsatellite, suggesting that Dnmt1 loss may decrease MMR efficiency by modifying chromatin structure.

Mismatch repair (MMR) is a genome-surveillance system that maintains the genomic integrity of mammalian organisms. The proteins encoded by MMR genes recognize mismatched nucleotides that arise during DNA replication, homologous recombination, or other forms of DNA damage (9,10). Impaired MMR can give rise to malignancies exhibiting microsatellite instability (MSI), which manifests itself as alterations in the length of simple, repetitive DNA sequences (11). Several lines of evidence suggest an association between DNA methylation and MMR. The properties of the methyl binding protein MBD4 provide the strongest link thus far between these two processes. MBD4 has a methyl binding domain as well as G/T and G/U glycosylase repair activities and interacts with the MMR protein MLH1 (12,13). Both Dnmt1 and MLH1 have a binding site for PCNA (Proliferating cell nuclear antigen), a processivity factor that is involved in DNA replication (14–16). Both proteins are active in S phase. Human MLH1 is often silenced by the promoter CpG island hypermethylation in about 15–20% of colorectal cancer that exhibits MSI (17). We showed that the reduction of Dnmt1 activity modifies tumor formation in MMR-deficient Mlh1<sup>−/−</sup> mice (4). Dam methylation plays an important role in MMR strand discrimination in Escherichia coli and is mediated by MutH (18). However, CpG methylation is not thought to be the strand discrimination signal in mammalian cells, but utilization of strand discontinuities such as nicks may be the signal for MMR (19). Furthermore, no MutH homolog has been identified in eukaryotes.

Guo et al. (20) recently reported that Dnmt1-deficient ES cells showed MSI. This report appeared as we were preparing our work for publication. Remarkably, using different approaches and initial goals, both studies arrived at the same conclusion: Dnmt1 deficiency leads to reduced MMR efficiency. In the present study, we measured the slippage rate at a mononucleotide repeat tract in Dnmt1-deficient ES cells. In addition, we extended the findings of Guo et al. (20) by investigating potential mechanisms of enhanced slippage in Dnmt1-deficient ES cells. Our results indicate that Dnmt1 deficiency does not increase MSI through local changes in DNA methylation levels. This suggests that efficient MMR in mammals may involve participation by the Dnmt1 protein itself, either directly, or indirectly through modification of chromatin structure.
MATERIALS AND METHODS

Plasmids

The puromycin marker and the hygromycin marker-containing Dmnt1-targeting vectors, pMC-PURO and pMC-HYG, respectively (4), were used for Dmnt1 gene targeting. The slippage construct pZCTN25A was constructed as follows. A zeocin selectable cassette from pZeoSylaczZ (Invitrogen) was ligated into the 3' end of the artificially constructed in-frame triple fusion gene, bacterial chloramphenicol acetyltransferase (CAT [from pCAT-Blank]), herpes simplex virus thymidine kinase (HSV–TK), and neomycin phosphotransferase II (Neo) [from pPGKTKNeo (8)]. A 25 adenine (25A) mononucleotide repeat was inserted into the MluI site at the 5' end of TK, resulting in an out-of-frame TKNeo.

ES cell culture and transfection

Mouse ES cells were maintained in HEPES-buffered (20 mM, pH 7.3) DMEM (USC tissue culture core facility) supplemented with 15% fetal calf serum (Hyclone Labs), 0.1 mM non-essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and penicillin–streptomycin (Irvine Scientific). ES cells were grown on feeder layers of gamma-irradiated mouse embryonic fibroblast cells and supplemented with leukemia inhibitory factor (LIF; Chemicon) at 106 U/ml to prevent ES cell differentiation. Cells were electroporated in a mixture of 20 mM HEPES (pH 7.0), 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose, and 0.1 mM β-mercaptoethanol, with 30 μg of linearized DNA at a set voltage 400 V and a capacitance of 75 μF, in a 0.4 cm-diameter cuvette with a Bio-Rad GenePulser II. Antibiotic selection was initiated on the following day and continued for 8–21 days before picking. Puromycin (Invitrogen) was used at a concentration of 2 μg/ml. G418 (Invitrogen) was used at an active concentration of 250 μg/ml, and then increased to 500 μg/ml. Hygromycin B (Roche) was used at a concentration of 100 μg/ml. Zeocin (Invitrogen) was used at a concentration of 2 μg/ml. Cells were expanded and frozen two days after picking. A parallel plate was used for DNA isolation as described previously (21).

Luria–Delbrück slippage assay

Before fluctuation analysis, multiple parallel vials of cells were generated for each clone. Initial mutants present in the original samples were removed by 6 μM 1-(2-deoxy-2-fluoro-β-d-arabinofuranosyl)-5-iodouracil (FIAU) counter-selection. For fluctuation analysis, one of the parallel vials of 1000 cells per well in a six-well plate was plated onto feeders in normal medium without selection for the expansion phase of the fluctuation analysis. Counting the day of plating as day 0, medium was changed every 2 days. On day 10, the cells were trypsinized, and the number of live and dead cells was determined with a hemacytometer. For G418 selection, all of the trypsinized cells were transferred to a new six-well plate without selection. On the following day, each parallel culture was replaced with 250 μg/ml of G418-containing ES media. G418 concentration was raised to 500 μg/ml after 2 days of initial selection and maintained for the rest of selection phase. The number of colonies on the selection plates was determined by a systematic microscopic scan at a 100× magnification and the counting was performed on the same day for the different genotypes. About 1–2 colonies per well were picked for DNA analysis and the rest of the clones were stained with methylene blue. The Luria–Delbrück method of the means equation was used to calculate mutation rates (22,23). The equation \( r = aN\ln(N/c) \) was used, where \( r \) is the number of resistant clones per parallel expansion corrected for both plating efficiency and the fraction of live cells seeded for selection, \( a \) is the mutation rate, \( N \) is number of live cells seeded for selection, and \( C \) is the number of parallel cultures. The rates calculated from this transcendental equation were derived using a Microsoft Excel spreadsheet-assisted approximation method.

Mutation analysis

DNA slippages at the 25A mononucleotide repeat in G418 reversion mutants from a Luria–Delbrück fluctuation analysis was verified by radio–PCR with primers spanning mononucleotide repeat 5'-GCC AGC GCC TTG TAG AAG C-3' (OL510/sense) and 5'-CAG TAG CGT GGG CAT TTT CTG-3' (OL343/anti-sense). The [α-32P]-dCTP-labelled PCR product was fractionated on a 9% acrylamide gel and visualized with the phosphorImager (Molecular Dynamics).

Southern blot analysis

Ten micrograms of genomic DNA was digested with 50 U of the appropriate restriction endonuclease (New England Biolabs). Digested DNA was electrophoresed through 0.7% SeaKem ME agarose gels, and blotted onto Zetabind nylon membranes (Cuno Laboratory Products). Southern blot hybridization was performed as described previously (24,25). Blots were washed at 65°C with 0.5× SSC/1% SDS and exposed to X-Omat film (Kodak). The following probes were used: HV is a 592 bp HindIII–EcoRV fragment of genomic DNA that includes the second intron of Dmnt1; the minor satellite centromeric repeat probe is amplified from pMR150 (26) using the following primers 5'-GGATCGTGTTTACCAACACATATTGG-3' (OL361/sense) and 5'-GGA GTGGTGGTTTTCACTTTTG-3' (OL362/anti-sense); the Zeo probe is a 1 kb Smal fragment derived from pZCTN.

RT–PCR expression analysis

Real-time fluorescence-based RT–PCR (TaqMan) was performed as described previously (4,27). Total RNA was isolated from ES cells and lysed by a single-step guanidinium isothiocyanate method (Total RNA, Ambion). RNA samples were then treated twice with DNaseI to get rid of DNA contamination (DNA-free, Ambion). Two micrograms of total RNA were reverse transcribed with random hexamers, deoxynucleoside triphosphates, and SuperScript II reverse transcriptase (Invitrogen), as specified by the manufacturer. The TaqMan Primer and probe sequences are listed below. In all cases, the first primer listed is the forward PCR primer, second is the TaqMan probe, and the third is the reverse PCR primer. All probes utilize a 6FAM fluorophore and a TAMRA quencher. Dmnt1 (CCA TGG TGC TGA AGC TCA CA, 6FAM-5'-TAG CCC ATG CGG ACC AGG CAG-3'TAMRA, AGC ACA CCA AAG GTG CAC TG), TkNeo (ATA CCG ACG ATA TGC GAC CTG, 6FAM-5'-CGA CGA CTT TGG CCC GAT-3'TAMRA, CCA TCT TGT TCA ATC ATG CGA A)
Gadph (TTG TCA AGC TCA TTT CCT GGT ATG, 6FAM5′-CCA CCC TGT TGC TGT AGC GTG ATT CAT T-3′TAMRA, GCC ATG TAG GCC ATG AGC TG) and HistoneH4 (TCT CCG GCC TCA TCT ACG AG, 6FAM5′-CAC CTT CAC ACC ACC GCG GGT-3′TAMRA, CGG ATC ACG TTC TCC AGG A).

**Bisulfite genomic sequencing**

Bisulfite genomic sequencing of the TK locus containing the 25A mononucleotide repeat was performed as described previously (28). Primers 5′-GTA GAA TGT TTA ATG AAT TAT AAT AG-3′ (OL741/sense) and 5′-CAA AAA CCA CCA ACA ATT A-3′ (OL742/anti-sense) were used to amplify a 364 bp PCR product from the top strand of bisulfite converted genomic DNA. The PCR product was cloned into pCR2.1–TOPO (Invitrogen) and sequenced with the M13 forward primer (5′-CGG AGC GGC CGG A-3′). In subsequent steps, the fraction of immunoprecipitated protein–DNA complex was washed, eluted, and the crosslink was reversed by aldehyde at 37°C for 15 min and washed twice with ice-cold HEPES-buffered saline solution (HBSS). In subsequent steps, a protease inhibitor cocktail (CompleteMini, Roche) was added to block protein degradation. After the cells were scraped and spun down, the cell pellet was lysed in lysis buffer (1% SDS, 10 mM EDTA, 500 mM Tris–HCl pH 8.1, and 2× protease inhibitor) then precleared with protein G-agarose beads (SantaCruz Biotechnology), 2 µg of sheared salmon sperm DNA (Invitrogen), and 1 µg/µl of BSA (Roche) for 1 h at 4°C with gentle agitation. The precleared chromatin preparation was then precipitated with antibodies (Upstate) against MeCP2 and acetylated Histone H3 (Lys9/Lys14). Normal rabbit IgG (SantaCruz Biotechnology) was used as a no antibody control (NAC). Immunoprecipitated protein–DNA complex was washed, eluted, and the crosslink was reversed by incubation at 65°C for 4 h. DNA was recovered by phenol–chloroform extraction and ethanol precipitation. TK and Neo genes were were analyzed by real-time PCR (TaqMan) with the following TaqMan primers: TK (Forward 5′-ATA CCG ACG ATA TGC GAC CTG-3′; Probe 6FAM5′-CGC GCA CGT TTG CCC GAT C-3′TAMRA; Reverse 5′-CCA TCT TGT TCA ATC ATG CGA A-3′), Neo (Forward 5′-GAT GGA TTG CAC GCA GGT T-3′; Probe 6FAM5′-CTC CAC CCA AGC GGC CGG A-3′TAMRA, Reverse 5′-GCC CAG TCA TAG CCG AAT AGC-3′. The fraction of immunoprecipitated DNA was calculated as [amount of input DNA (10%) – amount of NAC]/[amount of immunoprecipitated sample with antibody – amount of NAC]/[amount of input DNA (10%)] – amount of NAC].

**RESULTS**

To study the contribution of Dnmt1 to DNA MMR, a sequential Dnmt1 gene targeting technique combined with a microsatellite slippage detection assay was employed. The slippage construct consists of three open reading frames (ORF) (CAT, HSV–TK and Neo) fused together in-frame. Tk and Neo were used in a double selection scheme (30). A 25A mononucleotide repeat was inserted 5′ of the TK ORF, bringing both the TK and Neo coding sequences out of frame. This slippage construct allows for the direct elimination of any initial in-frame mutants by FIAU counterselection and the enrichment of reversion mutants by G418 selection (Figure 1).

Figure 2 shows a pedigree of cell lines used in this study along with a Southern blot analysis of the Dnmt1 locus. First, one Dnmt1 allele was disrupted in wild-type J1 cells (7 kb of KpnI fragment) (31) using a puromycin-resistant insertion-type targeting vector (P). The resulting heterozygous Dnmt1+/P BA cell line (8) contains a wild-type allele (7 kb) as well as a mutant P allele (22 kb). Second, the slippage construct was randomly integrated into BA cells giving rise to a Dnmt1+ P DKB6 cell line. A single-copy integration of this slippage construct was verified by Southern blot analysis using a 1 kb SmaI fragment containing the Zeocin gene as a probe (data not shown). Since this slippage construct was integrated before the second round of Dnmt1 allele targeting, any potential positional effects would be similar in the sister cell lines, allowing us to accurately compare the MSI rate. Third, the second Dnmt1 allele was disrupted in DKB6 cells using a hygromycin-resistant insertion-type targeting vector (H). The resulting sibling ES clones were either Dnmt1P+/H (DLC1, DLC4) or Dnmt1+/PH (DLC2, DLC3), depending on whether the hygromycin vector had recombined at the wild-type allele or at the previously targeted puromycin allele. Southern blot analysis was performed to verify targeting events as shown in Figure 2B. The H allele could be identified by the 21 kb KpnI fragment whereas the PH allele generated a 36 kb KpnI fragment. The absence of wild-type 7 kb KpnI fragment distinguished Dnmt1P/H from Dnmt1+/PH ES cells.
Dnmt1 mRNA levels were greatly reduced in the homozygous knockout cell lines (Dnmt1<sup>P/H</sup>), whereas the heterozygous cell lines (Dnmt1<sup>+/P/H</sup>) gave approximately half the expression levels seen in the wild-type cells (Figure 3A). This decrease of Dnmt1 mRNA was also associated with a concomitant decrease of methylation at the minor satellite centromeric repeat (26), which suggests a drastic reduction in global methylation content (Figure 3B).

To quantitate the effects of Dnmt1 deficiency on DNA slippage rates, we performed Luria–Delbrück fluctuation analyses using the method of means equation (22,23). In each experiment, Dnmt1<sup>P/H</sup> (DLC1, DLC4) and Dnmt1<sup>+/P/H</sup> (DLC2, DLC3) cells were tested side by side under identical experimental conditions. We performed six independent fluctuation analyses of sibling pairs, with each analysis consisting of six parallel expansions. After G418 selection, resistant colonies were visualized by methylene blue staining and counted. There were more G418 resistant colonies in the Dnmt1<sup>P/H</sup> cells than in Dnmt1<sup>+/P/H</sup> cells (Figure 4A). The average reversion rate in Dnmt1<sup>P/H</sup> cells was 4.2 × 10<sup>-5</sup>, whereas the reversion rate was 2.9 × 10<sup>-5</sup> in Dnmt1<sup>+/P/H</sup> cells. Thus, the MSI rate was consistently higher in Dnmt1-deficient cells as compared to Dnmt1-proficient cells by an average of 6.8-fold (Figure 4B). This difference was statistically significant (two-tailed \( P = 0.0152 \)) by a Mann–Whitney test.

To examine whether the G418 mutation mutants were generated by expansion or contraction of the mononucleotide tract, we used PCR to amplify a 204 bp fragment of TK gene using flanking primers as depicted in Figure 1. Figure 4C shows an example of seven G418 mutation mutants isolated from a Luria–Delbrück fluctuation experiment. When amplified PCR products were resolved on an acrylamide gel, 6 out of 7 mutant PCR products showed size reductions as compared to pre-expansion controls, indicating that the mononucleotide repeat had undergone contraction. Slippage was not detected in the R1 mutant, suggesting the possibility of a mutational event occurring outside of the PCR amplified region or the limitation of the assay to detect small deletions.

To exclude the possibility that the observed increase in G418 resistant colonies was due to the differences in the growth rate, we investigated whether there was indeed a difference in growth rate between Dnmt1-proficient cells by an average of 6.8-fold (Figure 4B). To examine whether the G418 reversion mutants were generated by expansion or contraction of the mononucleotide tract, we used PCR to amplify a 204 bp fragment of TK gene using flanking primers as depicted in Figure 1. Figure 4C shows an example of seven G418 reversion mutants isolated from a Luria–Delbrück fluctuation experiment. When amplified PCR products were resolved on an acrylamide gel, 6 out of 7 mutant PCR products showed size reductions as compared to pre-expansion controls, indicating that the mononucleotide repeat had undergone contraction. Slippage was not detected in the R1 mutant, suggesting the possibility of a mutational event occurring outside of the PCR amplified region or the limitation of the assay to detect small deletions.

Inactivation of Dnmt1 was examined by real-time fluorescence-based RT–PCR (TaqMan) analysis (8). Figure 3A shows the Dnmt1 expression analysis of wild-type, Dnmt1<sup>+/PH</sup>, and Dnmt1<sup>P/H</sup> cell lines. The P, H, and PH alleles were generated by the disruption of a ClaI site in Dnmt1 that leads to protein truncation. The RT–PCR primers were designed to span the wild-type sequence at this ClaI site, which allowed us to specifically detect expression from the wild-type endogenous Dnmt1 sequence in the knockout cell lines. Dnmt1 mRNA levels were greatly reduced in the homozygous knockout cell lines (Dnmt1<sup>P/H</sup>), whereas the heterozygous cell lines (Dnmt1<sup>+/PH</sup>) gave approximately half the expression levels seen in the wild-type cells (Figure 3A). This decrease of Dnmt1 mRNA was also associated with a concomitant decrease of methylation at the minor satellite centromeric repeat (26), which suggests a drastic reduction in global methylation content (Figure 3B).

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been reported to associate with histone remodeling proteins (32–34). Thus, a lack of Dnmt1 could perhaps lead to a permissive chromatin complex in which mismatched DNA is corrected at a lower efficiency. To investigate this possibility, we performed ChIP analyses of the slippage construct with antibodies against MeCP2 and acetylated H3-K9/14 in Dnmt1\(^{+/PH}\) and Dnmt1\(^{P/H}\) cells (Figure 6). MeCP2, known to directly bind to Dnmt1 as well as methylated CpGs (35), is a marker of closed chromatin that is not actively transcribed and alternatively, histone acetylation is correlated with active transcription (36–38). After chromatin immunoprecipitation, the quantitation of immunoprecipitated DNA was determined by real-time fluorescence-based PCR using primers located in the TK locus, downstream from the mononucleotide repeat as shown in Figure 6. No detectable MeCP2 recruitment was observed in Dnmt1\(^{P/H}\) cells. We found reduced MeCP2 occupancy in general at the TK locus (Figure 4), which is in agreement with the observed lack of DNA methylation in the region surrounding the mononucleotide repeat (Figure 5). We also observed higher levels of H3-K9/14 acetylation in Dnmt1\(^{P/H}\) cells as compared to Dnmt1\(^{+/PH}\) cells. In addition, the Neo locus downstream of TK showed a similar trend of decreased MeCP2 recruitment and increased histone H3 acetylation in Dnmt1\(^{P/H}\) cells (data not shown). This observation was found consistently in three independent ChIP experiments. We conclude that Dnmt1 deficiency may be associated with chromatin alterations. A further investigation is needed to address whether the chromatin alterations form the basis for the observed increase in MSI.

**DISCUSSION**

In the present study, we used mouse ES cells as a model system to study whether Dnmt1 affects MMR efficiency. Our results demonstrated that disrupting both copies of Dnmt1 caused an elevated MSI. The slippage rate was approximately 7-fold higher in homozygous cells and was predominantly due to the contraction of the mononucleotide repeat. While this manuscript was in preparation, Guo et al. reported quite similar findings (20). In that study, Dnmt1 was identified as a novel MMR gene in a genetic screen for MMR mutants in Bloom’s syndrome protein (Blm)-deficient ES cells. When Guo et al. assayed slippage at a (CA\(_{17}\)) dinucleotide repeat using a similar selection strategy to ours, they found a nearly 4-fold enhancement of MSI in homozygous Dnmt1 knockout cell line when compared with wild-type. Our findings are in agreement with those of Guo et al. Although Guo et al. reported that Dnmt1 deficiency does not result in alterations in the expression of MMR genes, they did not further investigate how Dnmt1 loss influences local methylation or chromatin modification at the assayed microsatellite (20). Luria–Delbrück fluctuation results are dependent on cellular growth rates and thus a difference in growth could skew mutation rate estimations (22). We investigated whether there was indeed a difference in growth rate between Dnmt1\(^{PH}\) and Dnmt1\(^{P/H}\) ES cells with and without G418 selection and found no apparent difference (data not shown). This is consistent with previous reports that Dnmt1-deficient ES cells retain their normal phenotype and growth characteristics (8,31).
There would be two distinct models by which the enhanced rate of MSI in Dnmt1-deficient ES cells can be explained. One model is that local changes in methylation pattern can alter MMR efficiency, and the other model is that the Dnmt1 protein itself can participate in MMR either directly or indirectly. We observed that the region flanking the 25A repeat was generally unmethylated regardless of Dnmt1 status, which is inconsistent with the idea that local methylation patterns affect MMR efficiency. However, we cannot rule out the possibility that sequences outside the region assayed may be differentially methylated, affecting MMR efficiency from a distance. Drummond et al. (19) have shown that MMR strand discrimination in artificially generated mismatched DNA does not require CpG methylation.

As an alternative explanation, we hypothesize that MMR could have been perturbed by the absence of Dnmt1. Aside from its methylating property, the Dnmt1 protein itself could
directly participate in MMR. Interestingly, we observed that the disruption of Dnmt1 was associated with the modification of local chromatin structure (34). Dnmt1 deficiency may be associated with the formation of distinct chromatin structures that alter the fidelity of DNA replication and repair, leading to different repair efficiency. It has been reported that DNMT1 is required to maintain proper nuclear architecture (39). Thus, it is possible that the MMR machinery was affected by altered chromatin structure in the knockout cells. Our data is consistent with a previous study, which shows that histone H3 acetylation is increased in DNMT1-deficient human cancer cells (39). MeCP2 can interact with Dnmt1 directly (35), not just by binding to methylated CpGs. Hence, Dnmt1 deficiency may have an impact on MeCP2 recruitment and its coupled chromatin modification, although the regional methylation differences between Dnmt1<sup>+/PH</sup> and Dnmt1<sup>−/PH</sup> cells seen in Figure 5 were not obvious. Taken together, the participation of Dnmt1 protein in MMR either directly or indirectly through chromatin modification may be a mechanism by which the enhanced MSI in Dnmt1-deficient ES cells can be explained, although this needs to be addressed by further research.

DNA hypomethylation has been associated with genomic instability. DNA hypomethylation promotes tumorigenesis in some mouse models and is associated with the acquisition of additional chromosomal changes such as loss of heterozygosity (LOH) and chromosome 15 trisomy (5,6). We have demonstrated that Mlh1<sup>−/+</sup> mice are more susceptible to T and B-cell lymphoma in the Dnmt1 hypomorphic genetic background (4). Consistent with this, human ICF (immunodeficiency, centromere instability and facial anomalies) syndrome caused by mutations in DNMT3b is characterized by genomic hypomethylation and genomic instability (40). However, it is not clear whether the loss of genomic stability is a consequence of DNA methylation loss. We have shown that the rates of CpG mutagenesis and LOH are decreased in Dnmt1-deficient cells (8). Thus, the quantitative effects of DNA methylation on various mechanisms of genomic stability will require further investigation.

Although we found that Dnmt1 loss led to MSI in mouse ES cells, it is unlikely that this is a major mechanism by which MSI is induced in human cancers. Mutation of DNMT1 has not been demonstrated to be common in human cancers (41). Moreover, loss of Dnmt1 is lethal during embryonic development as well as in differentiated cells (31,42). The enhanced MSI reported in this study and by Guo et al. (20) was not caused by the same mechanism of MSI seen in colorectal cancer, which is mainly due to the promoter CpG island hypermethylation of MLH1 (17). The global hypomethylation in Dnmt1-deficient cells is much more severe than that which has been commonly observed in cancer cells (2,3,43).

In conclusion, our study extends the current understanding of the relationship between DNA methylation and genomic stability to MMR. Our results suggest that Dnmt1 may be closely linked to MMR to maintain genomic integrity.

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