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Gun E. Lee
University of Central Florida

Joo Hee Kim
University of Central Florida

Michael Taylor
University of Central Florida

Mark T. Muller
University of Central Florida

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DNA Methyltransferase 1-associated Protein (DMAP1) Is a Co-repressor That Stimulates DNA Methylation Globally and Locally at Sites of Double Strand Break Repair*

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Gun E. Lee, Joo Hee Kim, Michael Taylor, and Mark T. Muller

From the Department of Molecular Biology and Microbiology, College of Medicine, University of Central Florida, Orlando, Florida 32826

Correction of double strand DNA breaks proceeds in an error-free pathway of homologous recombination (HR), which can result in gene silencing of half of the DNA molecules caused by action of DNA methyltransferase 1 (DNMT1) (Cuozzo, C., Porcellini, A., Angrisano, T., Morano, A., Lee, B., Di Pardo, A., Messina, S., Iuliano, R., Fusco, A., Santillo, M. R., Muller, M. T., Chiariotti, L., Gottesman, M. E., and Avvedimento, E. V. (2007) PLoS Genet. 3, e110). To explore the mechanism that leads to HR-induced silencing, a genetic screen was carried out based on the silencing of a GFP reporter to identify potential partners. DMAP1, a DNMT1 interacting protein, was identified as a mediator of this process. DMAP1 is a potent activator of DNMT1 methylation in vitro, suggesting that DMAP1 is a co-repressor that supports the maintenance and de novo action of DNMT1. To examine critical roles for DMAP1 in vivo, lentiviral shRNA was used to conditionally reduce cellular DMAP1 levels. The shRNA transduced cells grew poorly and eventually ceased their growth. Analysis of the tumor suppressor gene p16 methylation status revealed a clear reduction in methylated CpGs in the shRNA cells, suggesting that reactivation of a tumor suppressor gene pathway caused the slow growth phenotype. Analysis of HR, using a fluorescence-based reporter, revealed that knocking down DMAP1 also caused hypomethylation of the DNA repair products following gene conversion. DMAP1 was selectively enriched in recombinant GFP chromatin based on chromatin immunoprecipitation analysis. The picture that emerges is that DMAP1 activates DNMT1 preferentially at sites of HR repair. Because DMAP1 depleted cells display enhanced HR, we conclude that it has additional roles in genomic stability.

DNA methylation is a post-replicative, covalent modification of genomic DNA that is a stable epigenetic mark implicated in growth homeostasis (1). Epigenetics is also important in a wide variety of processes such as differentiation (1), X chromosome inactivation (2), and genomic imprinting (3). Miscues in DNA methylation may cause growth defects, genomic instability (4), and cancer (5). In mammalian cells, DNA methyltransferases primarily target CpG dinucleotides leading to transcriptional repression most likely by generating chromatin that cannot engage basal transcription factors, thereby silencing linked genes. DNA methyltransferases are distinguished as either maintenance or de novo methyltransferases, depending on their preference for hemimethylated or fully methylated DNA, respectively (6). DNA methyltransferase 1 (DNMT1), for example, is generally considered to maintain DNA methylation patterns associated with DNA replication (7), and it has a strong preference for hemimethylated DNA; however, this biochemical feature is not absolute because DNMT1 also acts on unmethylated targets (8, 9). Additional evidence for de novo activity of DNMT1 in chromatin at sites of homologous recombination has recently been proposed (10).

Defects in DNA methylation may lead to a growth advantage in somatic cells when a tumor suppressor gene is silenced or when an oncogene becomes activated, thereby selecting for a malignant growth phenotype (11, 12). A complete understanding of somatic cells is an important yet challenging topic in cancer. The problem is complex because it is most likely a rare event that happens to single cells followed by selective outgrowth. Processes that reset methylation patterns may alter gene expression leading to unregulated cell growth behavior. Epigenetic reprogramming in somatic cells is not as well documented as it is during development when a genome wide era of epigenetic imprints takes place (13–17). It was recently demonstrated that DNA repair proteins may mediate the process (18). Indeed, somatic cell reprogramming leading to pluripotency involves base excision repair pathways downstream of the cytosine deaminases (19). These collective findings, in concert with the idea that methylation is linked to genome stability, strongly implicate DNA damage pathways in epigenetic reprogramming. A number of prior reports suggest a relationship between DNA repair, recombination, and gene silencing. Specifically, DNMT1 and proliferating cell nuclear antigen are binding partners (20) that provide a means to recruit DNMT1 to DNA repair sites (21). Moreover, DNMT1 mutant mouse cells are genetically unstable (22, 23), and in human cells, global reductions in Cpg methylation contribute to genome instability (24). Recently, demethylation pathways have been demonstrated that involve activities normally associated with base excision repair (25). In addition, we reported a relationship between DS DNA damage, homologous recombination (HR)

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2 The abbreviations used are: DNMT, DNA methyltransferase; ICM, in vivo complex of methylase; HM, hemimethylated; FM, fully methylated; HR, homologous recombination; aza-dC, 2’-aza-deoxycytidine; PMSF, phenylmethylsulfonyl fluoride; SPR, surface resonance plasmon; rec, recombinant; unrec, nonrecombinant; DS, double strand.

1 To whom correspondence should be addressed: 12722 Research Pkwy., Orlando, FL 32826. Fax: 407-384-2816; E-mail: mtmuller@mail.ucf.edu.

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and gene silencing in somatic mammalian cells using an HR reporter system developed by Jasin and co-workers (26) by analysis of methylation patterns of repaired DNA (10). Evidence was presented that short regions flanking the DS DNA break were subject to epigenetic resets following HR repair where one strand was completely demethylated and the opposite strand was overlaid with new methylation patterns. The result was twin populations of cells (in an approximate 1:1 ratio) where GFP was either silenced (hypermethylated) or expressed (hypomethylated). In the current work, an HR model GFP reporter was used to examine other factors that may be important in epigenetic reprogramming associated with repair in human cells. The DNMT1 binding partner DMAP1 was identified as a key participant in the silencing of HR repair products.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cell Culture, and Transient Transfections**—Lentiviral vectors (shDMAP1-1 and shDMAP1-2) were purchased from Open Biosystems (clone numbers TRCN0000021744 and TRCN0000021745) (Huntsville, AL). pCHA-DMAP1 expressing HA-tagged full-length DMAP1 was provided by Dr. Keith D. Robertson (Medical College of Georgia, Augusta, GA). Wild type HCT116 colon cancer cells were provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and grown in McCoy’s 5A with 10% fetal bovine serum. HeLa and HO1 cells (HeLa cells containing a DR-GFP (10) stably integrated) were grown in RPMI 1640 supplemented with 10% fetal bovine and periodically maintained in puromycin to ensure retention of the transgene. The transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Analysis of Homologous Recombination**—HR assays were performed as previously described (10, 26). Briefly, HO-1 cells transduced with lentivirus expressing shRNAs against human DMAP1 or control shRNA were transfected with pCBAsC expressing I-SceI endonuclease as well as pSVβGal (Promega, Madison, WI). Typically, cells with 50% of confluency in 60-mm dishes were transfected with 2 µg of pCBAsC and 0.5 µg of pSVβGal. Transfection efficiencies were over 60%. After transfection, the cells were incubated for 4 days, and GFP-positive cells were analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA). Transfection efficiency was normalized by the β-galactosidase assay system (Promega, Madison, WI).

**Protein Extraction and Western Blot Analysis**—To analyze the protein expression, nuclear proteins were extracted and prepared as follows. The cells were harvested and washed twice with a hypotonic buffer (10 mM Hepes, pH 7.6, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT) followed by a 10-min incubation with hypotonic buffer on ice. The cells were homogenized by 10 strokes with a tight fitting Dounce homogenizer, and the nuclei were collected with centrifugation at 13,000 rpm, 4 °C for 30 s. The nuclei were then suspended in a salt extraction buffer (20 mM Hepes, pH 7.6, 1.5 mM MgCl₂, 10% glycerol, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 420 mM NaCl) and incubated for 30 min on ice. The nuclear fraction was collected by centrifugation at 13,000 rpm, 4 °C for 30 min, and protein concentration was measured with Bio-Rad protein assay reagent. Typically, 5–10 µg of nuclear protein were run on SDS-PAGE, and after electrotransfer onto nitrocellulose, the membrane was probed with the following antibodies: anti-DMAP1 (ab2848; Abcam, Cambridge, MA), anti-DNMT1 (N-16; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-lamin A/C (50–714; Millipore, Billerica, MA). The signal was developed using SuperSignal West Dura kit (Thermo Fisher Scientific, Rockford, IL). The images were captured using the ChemiGenius documentation system (SynGene, Cambridge, UK).

**In Vivo Activity of DNMT1**—Assays to analyze in vivo activity of DNMT1 were performed as described by Liu et al. (27). Briefly, cells treated with 10 µM of aza-dC for 1 h were lysed with 1% sarkosyl in TE (10 mM Tris/HCl, pH 7.5, and 1 mM EDTA). The viscous lysates were layered onto a step CsCl gradient followed by centrifugation at 35,000 rpm, 22 °C with SW50.1 rotor for 20 h. The gradient was fractionated into 0.5-ml aliquots, and DNA fractions were pooled. DNA concentrations were determined by UV spectroscopy. Typically, three DNA concentrations (0.5, 1, and 2 µg) were placed on a slot blot device, and the membrane was probed with the anti-DNMT1 antibody. Immune complexes were detected with SuperSignal West Dura kit (Thermo Fisher Scientific), and images were captured using a ChemiGenius documentation system (SynGene, Cambridge, UK).

**In Vitro DNA Methylation Assays**—In vivo DNA methylation assay was performed as previously described (28). Briefly, the methylation activity of DNMT1 was measured by the incorporation of tritiated methyl group from radiolabeled S-adenosyl-L-[methyl-³H]methionine (PerkinElmer Life Sciences) into a double strand oligonucleotide substrate containing a single CpG site with or without a methyl group on the top strand (5′-GAAGCTGGGACTTCCCGAGGAGATGCAGAA-3′, where the underlining denotes a single CpG site). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Double strand oligonucleotides were annealed by mixing equal amounts of complimentary oligonucleotides, heating to 95 °C for 5 min, and cooling down to room temperature. Fully duplexed substrate was verified by agarose gel electrophoresis. The methylation reaction was carried out at a concentration of 0.5 µM of DNA, 0.5 µCi of S-adenosyl-L-[methyl-³H]methionine and variable amounts of purified DNMT1 (Methylation, Ltd., Port Orange, FL) in methylation reaction buffer (20 mM Tris/HCl, pH 7.5, 5 mM EDTA, 5 mM DTT, 1 mM PMSF, and 10% glycerol) at 37 °C for 1 h. Purified DMAP1 or ETS1 (purified by overexpression of his tagged proteins in E. coli) was added as indicated in the figure legends. The reactions were terminated by adding phenol/chloroform, and DNA was precipitated by adding the same volume of isopropyl alcohol. The DNA pellet was dissolved in TE buffer and spotted on glass fiber filter (Whatman, Kent, UK). Radioactivity was determined using a LS6500 scintillation counter (Beckman Coulter, Brea, CA).

**RNA Extraction and Quantitative Real Time RT-PCR**—Total RNA was extracted with TRIZol reagent (Invitrogen) according to the manufacturer’s instructions. Extracted RNA was quantified using a NanoDrop (Thermo Fisher Scientific). The cDNAs were generated from 2 µg of total RNA in a 25-µl reaction volume using Moloney murine leukemia virus reverse tran-
DMAP1 in Repair-induced Silencing

scriptase and 0.5 μg of random hexamer (Promega, Madison, WI). Quantitative real time PCR was performed using Fast SYBR Green Master Mix and a 7500 Fast Real time PCR system (Applied Biosystems, Foster City, CA). The cDNA was amplified with the following primers: DMAP1-A, 5'-ACGGA-GCAATGTTCTTCCCAC-3'; DMAP1-B, 5'-CAGGCACCTG- CACAGCTTTA-3'; DNMT1-A, 5'-CAGCTCGAGGACCTG- GATA-3'; DNMT1-B, 5'-ACCAACTCTGATGAGATGC- 3'; GADPH-A, 5'-CTCTGCTCCCTCTGGTAC-3'; and GADPH-B, 5'-ACGACAAATCCGTTGACT-3'.

Genomic DNA Extraction and Bisulfite Sequencing—Genomic DNA was isolated from cells infected with lentivirus expressing shRNA against DMAP1 using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Genomic DNA (2 μg) was used for sodium bisulfite modification with EpitiTect bisulfite kit (Qiagen) and carried out using the commercially supplied kit protocol. Bisulfite-modified DNA (2 μl) was amplified with primers for p16 promoter: BS2-p16A, 5'-GAGGGGTTTGTTGATATTAGA-3'; and BS2-p16B, 5'-TACAAACCCTCTACCCACCTAAT-3'. Amplified DNA was subcloned into pGEM-T Easy Vector (Promega, Madison, WI), and 10 independent clones from lentivirus-infected cells were sequenced. Sequencing was performed by GENEWIZ, Inc. (South Plainfield, NJ). Sequence analysis and alignment were performed with BiQ Analyzer software (29).

Chromatin Immunoprecipitation—HeLa cells carrying DR-GFP were transfected with either empty vector or pCBASce in the 100-mm dishes. After 24 h, the cells were transferred into 150-mm dishes and incubated for further 72 h. After incubation for total 4 days, the cells were fixed with 1% formaldehyde in 1× PBS. Chromatin was prepared by sonication, and ChIP assays were performed using a ChiP-IT Express kit (Active Motif, Carlsbad, CA). For the recovery of chromatin DNA, the following antibodies were used: anti-DNMT1 (N-16; Santa Cruz Biotechnology), anti-DMAP1 (ab2848; Abcam), and anti-LexA (2-12; Santa Cruz Biotechnology). Anti-LexA antibody was used as a negative control. After chromatin immunoprecipitation, purified DNA fragments were used for PCR. The primers used for PCR were: rec, 5'-GAGGGCGAGGGCGAT-GCC-3'; unre, 5'-GCTAGGGATAACAGGGTAAT-3'; and reverse common, 5'-TGCACCGCTCCGCTCTCG-3'.

Purification of DMAP1—Human cDNA of DMAP1 was subcloned into pET-28a (EMD/Novagen, EMD Chemicals, Gibbstown, NJ). BL21 (DE3) Escherichia coli strain was used to produce His-tagged DMAP1 protein. Because DMAP1 was found to form the inclusion body in the pilot experiment, denaturation condition was used for the purification. BL21 (DE3) transformed with pET-DMAP1 was induced with 0.5 mM of isopropyl β-D-thiogalactopyranoside for 4 h at 37 °C. After harvest, bacterial pellet was resuspended in 10 ml of 1× PBS and sonicated. Inclusion body was collected by centrifugation and resuspended in the binding buffer containing 50 mM Na2HPO4, pH 8.0, 0.3 M NaCl, 1 mM PMSF, and 7 μM urea. After solubilization at room temperature for 30 min, the solution containing inclusion body was centrifuged at 15,000 rpm, 4 °C for 30 min. The supernatant containing His-tagged DMAP1 was removed and incubated with nickel-nitrilotriacetic acid-agarose beads (Qiagen) at 4 °C overnight. The protein/bead mixture was washed with wash buffer containing 50 mM Na2HPO4, pH 8.0, 0.3 M NaCl, 1 mM PMSF, 7 μM urea, and 10 mM imidazole. His-tagged DMAP1 was eluted with elution buffer containing 50 mM Na2HPO4, pH 8.0, 0.3 M NaCl, 1 mM PMSF, 7 μM urea, and 250 mM imidazole. The peak fractions were pooled and diluted into a solution with a concentration of 1 μg/μl. The protein solution was dialyzed against 1× PBS.

Surface Plasmon Resonance (SPR) Analysis—The association constants for DMAP1 and oligonucleotide DNAs were analyzed with a SR7000 SPR refractometer (Reichert, Depew, NY) in binding buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.01% Tween 20). All of the experiments were performed at a flow of 10 μl/min at 37 °C. Each experiment was repeated four times, and the standard errors were determined. Protein-DNA interaction was analyzed after oligonucleotide DNAs (hemimethylated, fully methylated, or unmethylated) were coupled on the neutravidin-coated gold sensor slides. DNA (25 μl of 100 nM) was injected for the immobilization, and the same amount of purified DMAP1 protein with concentrations of 1, 10, 25, 50, and 100 nM was used for the kinetic analysis. Data analysis was carried out using the SCRUBBER-2 software (University of Utah).

RESULTS

Knockdown of DMAP1 Reduces HR Repair-dependent DNA Methylation—The DR-GFP reporter system is composed of two mutated GFP repeat elements, separated by the drug selection marker (puromycin N-acetyltransferase gene) (26). The first cassette (GFP-I) contains a unique I-SceI site derived from the BcgI recognition site, creating two in-frame stop codons, thereby inactivating expression. The second cassette contains partial internal coding sequence of wild type GFP spanning the BcgI site that serves as donor sequence for HR. The DR-GFP (Fig. 1A) provides a useful readout to evaluate the expression status of the GFP following transfection with I-SceI plasmid. The reporter is stably integrated into HeLa cells (HO-1 cells) as a single integrant, and gene conversion produces two GFP expression classes (10). The HR-L corresponds to cells that express lower levels of GFP because of DNMT1 activity that maps to I-SceI flanking sites. The DR-GFP system was used to identify candidate genes that participate in post HR repair silencing. DMAP1 is reported to be involved in genome stability, is a DNMT1 binding partner, and interacts with proliferating cell nuclear antigen/Caf-1 in DNA repair (30); therefore, we asked whether DMAP1 is involved in HR-dependent DNA methylation. Because DMAP1 is abundant in somatic cells (30), we tested the effects of DMAP1 knockdown using lentiviral shRNA. Two different constructs were evaluated, and both showed reductions in DMAP1 mRNA levels (Fig. 1B). DMAP1 protein levels were similarly affected (see also Fig. 4B). The two independent shRNA targeting sequences confirm that the down-regulation of DMAP1 is not likely due to nonspecific silencing associated with off target genes by the shRNAs selected. Because DMAP1 physically interacts with DNMT1, we were concerned that reducing DMAP1 might also affect its cognate partner; however, RT-PCR and Western blotting con-
firms that DNMT1 expression was not significantly altered (data for HeLa cells shown in Fig. 2C, and for colon cancer cells see Fig. 4, A and B). We performed fluorescence-based HR assays on I-SceI transfected and lentivirus-transduced cells. In the controls, GFP-expressing cells showed the typical 1:1 ratio between high (HR-H) and low expressing (HR-L) cells, which differ in DNA methylation states (10). These data (Fig. 1C) demonstrate that knocking down DMAP1 significantly increases the HR-H and decreases the fraction of HR-L (low expressors) without affecting the frequency of HR (Fig. 1D). Moreover, expression of DNMT1 was not affected in the DMAP1 knockdown cells (noted above); therefore, alterations in expression cannot be due to variable amounts of total endog-
DMAP1 in Repair-induced Silencing

Endogenous DNMT1 protein. We previously found that that HR-L (low expression class) was due to DNMT1 activity directed at I-SceI flanking sequences after gene conversion (10). An enrichment of HR-H would be expected to occur in the DMAP1 knockdown cells if DNMT1 action was inhibited.

Reduction in DMAP1 Reduces DNA Methylation by Endogenous DNMT1—We next considered the possibility that endogenous DNMT1 activity was influenced by alterations in DMAP1 in our experiments. To assess global or genome wide activity of DNMT1, we performed a catalytic trapping assay to measure the total amount of endogenous DNMT1 methylation using the ICM method (27). In this experiment, the cells are pulsed with aza-dC (which does not alter HR frequency) (10), and the resulting covalent DNA-DNMT1 complexes were purified by CsCl gradient centrifugation. Fixed amounts of genomic DNA are probed with anti-DNMT1 antibody to determine the amount of DNMT1 bound on a per genome basis (27). In the control cells, covalent binding of DNMT1 was dependent upon treatment with aza-dC as shown previously (27) (Fig. 2A). In the knockdown cells, we observed a large reduction (40–60% in different experiments) in the activity of the endogenous DNMT1 on genomic DNA (Fig. 2A, compare rows 2, 4, and 6). Also, we observed a gradual reduction in the growth rate of the cells (Fig. 2B), and by day 4 the cells essentially stopped growing; therefore, DMAP1 influenced growth-related gene expression circuits possibly through global methylation. This is consistent with the in vivo DNMT1 trapping data showing overall reductions in cellular methylation by DNMT1 in the transduced cells (Fig. 2A). As noted elsewhere (Figs. 1B and 4B) reduced DNMT1 catalytic activity is not due to alterations in total cellular DNMT1 protein in these experiments. Even as late as 18 days post-transduction, expression of DNMT1 was not diminished (based on RT-PCR) under conditions where DMAP1 transcription was significantly repressed (Fig. 2C). Taken together, the data suggest that DNMT1 activity in chromatin depends on DMAP1, and the slow growth phenotype is caused by alterations in expression status of genes involved in growth control circuits (for example hypomethylation of tumor suppressor genes; see Fig. 4).

DMAP1 Enhances DNMT1 Activity in Vitro—The data in Fig. 2A are based on short pulses with aza-dC to trap endogenous DNMT1 on genomic DNA in cells; however, we were concerned that the slower growth of transduced cells (Fig. 2B) might alter the incorporation of the hypomethylating drug into the DMAP1 knockdowns. Others have demonstrated DMAP1-DNMT1 complex with HDAC2 at replication foci (31–33); therefore, to determine whether DMAP1 has the ability to directly modulate DNMT1 action, independent of other factors, we performed in vitro DNA methylation assays with purified DNMT1 and a target oligonucleotide containing a single CpG. Although DNMT1 has a strong preference for hemimethylated targets, it also performs de novo, as indicated with HR silencing (10). In this experiment, both hemi- and unmethylated DNA substrates were examined. The data show that DNMT1 activity is enhanced 2.5-fold by DMAP1 on hemimethylated DNA and ~1.5-fold on unmethylated DNA (Fig. 3). Human ETS1 protein did not alter DNMT1 activity on either hemi- or unmethylated targets (ETS1 is not a DNMT1 interactive factor, and the oligonucleotide substrate does not contain ETS1 binding sites). Because the primary amino sequence of DMAP1 does not show any homology with catalytic domains found in DNA methyltransferases, the elevation of DNA methylation should be attributed to the activity of DNMT1 (DMAP1 alone does not methylate DNA) (Fig. 3). The in vitro data are consistent with the in vivo results (aza-dC trapping; Fig. 2A) and confirm that DMAP1 stimulates DNMT1 activity and that this stimulatory action on DNMT1 proceeds independently of other cellular components; however, it is likely that other factors may acutely impact events in a chromatin context.

Reduced DMAP1 and Demethylation of the p16 Promoter—The data thus far suggest that DMAP1 plays a supportive role in DNMT1 action globally (Fig. 2A) and specifically in HR repair chromatin locally. This result suggests that DMAP1 represents a central component in DNMT1 regulation and can influence
methylation in a specific fashion. The effects of DMAP1 shRNA on inhibition of cell growth (Fig. 2) may be a consequence of DNMT1 inhibition, resulting in hypomethylation of unknown gene targets culminating in a slow growth phenotype. To test this, we examined methylation of the p16 promoter, which is silenced in HCT-116 (34). HCT-116 cells were transduced with the shRNA lentivirus, and expression of DMAP1 was evaluated by real time PCR. At 10 days post-transduction, the two independent shRNA constructs reduced DMAP1 expression by $\sim 60-80\%$ (Fig. 4A), and Western blots confirm down-regulation at the polypeptide level (Fig. 4B). As noted with the HO-1 cell line, knocking down DMAP1 expression did not alter DNMT1 expression (see also Fig. 2C). The methylation status of the $\sim 500$-bp region in p16 promoter was also analyzed (Fig. 4C) after infecting HCT116 cells with lentivirus expressing shRNA, at days 10 and 18. Mock infected cells showed $\sim 50\%$ of the methylation level consistently at days 10 and 18. The shDMAP1-1 knockdown cells displayed $\sim 30\%$ of reduction in methylation level ($\sim 50\%$ to $\sim 20\%$). Interestingly, shDMAP1-2 showed little if any change at day 10; however, by day 18, methylation levels decreased to $\sim 30\%$ as cells slowed or stopped growing. As a control, the cells were treated with 5 $\mu M$ aza-dC for 7 days, which strongly affected cell growth. The region was completely demethylated by aza-dC, and we noted that cell division was strongly inhibited. These results suggest that DMAP1 knockdowns ultimately cause hypomethylation of the p16 promoter region over time. To examine the generality of demethylation, we checked several DNA sequences that are known to be highly methylated in HCT116 cells: AluSx, AluJ, LINE1, TIMP-3, and VEGFR1 (data for LINE-1 and TIMP-3 shown in Fig. 4D). These highly methylated genomic DNA regions did not show any change in DNA methylation pattern. Among the genomic DNA sequences examined, only the p16 promoter was hypomethylated.

**DMAP1 Is a DNA-binding Protein That Preferentially Binds Hemimethylated DNA**—DMAP1 is a co-repressor that forms a complex with DNMT1 and targets replication foci in the S phase (31). Mobility shift experiments demonstrated that DMAP1 binds DNA in a non-sequence-specific manner (data not shown), and we next asked whether DNA methylation status was important in DNA binding. For this analysis, we quantified DMAP1/DNA binding using SPR analysis to derive rate constants, $k_a$ (association) and $k_d$ (dissociation), and from this relationship ($k_d/k_a$), we determined the equilibrium binding constant ($K_D$). The $K_D$ value is the affinity binding constant that represents complex life span; specifically, a lower $K_D$ value corresponds with a higher affinity (35, 36). These SPR experiments were conducted with purified DMAP1 and unmethylated, hemimethylated, or fully methylated 30-bp oligonucleotides containing a single, centrally located CpG site. The $K_D$ values therefore reflect the importance of a single or double methyl group on opposing strands. The relative affinity of DMAP1 for hemimethylated (HM) DNA (Table 1 and Fig. 5A) was $280\%$ greater than unmethylated DNA and $32\%$ greater than fully methylated DNA targets. The binding preference was therefore hemimethylated $> \text{fully methylated} > \text{unmethylated}$. A $K_D$ value in the low nanomolar range (HM DNA) corresponds to a relatively long half-life for the complex. The prediction is that under such conditions, the majority of endogenous DMAP1 should be bound at HM sites in association with DNMT1. We considered that in this case, ectopic addition of DMAP1 should not influence endogenous DNMT1 methylation of genomic DNA, if all of the HM DNA sites are already bound. To test this, we transfected HO-1 cells with DMAP1 plasmid and tested the activity of DNMT1 on genomic DNA using ICM. The cell

![FIGURE 4. Methylation of p16 in HCT116 cells.](image-url)
growth behavior was not affected by ectopic addition of DMAP1 (not shown). The data show that the addition of excess DMAP1 did not significantly alter global methylation by DNMT1 (Fig. 5B). This suggests that endogenous DNMT1 is sequestered with the endogenous DMAP1 and that additional protein is not able to further impact cellular methylation profiles.

To examine the molecular association in chromatin, we carried out ChIP experiments using PCR primer pairs that detect either recombinant (rec) or nonrecombinant (unrec) GFP molecules (see Fig. 1 map); therefore, we could examine chromatin

| DNA               | $k_a$ ($s^{-1}$) | $k_d$ ($s^{-1}$) | $K_D$ ($\mu M$) |
|-------------------|------------------|------------------|-----------------|
| Unmethylated      | $1.75 \times 10^4$ | $2.02 \times 10^3$ | $1.16 \mu M (\pm 5 \mu M)$ |
| Hemimethylated    | $1.86 \times 10^4$ | $7.77 \times 10^2$ | $4.15 nm (\pm 20 nm)$ |
| Fully methylated  | $1.51 \times 10^4$ | $2.0 \times 10^1$ | $131.7 nm (\pm 68 nm)$ |

**FIGURE 5. DNA binding activity and localization of DMAP1 in recombinant HR chromatin.** A, DNA binding activity of DMAP1 was analyzed using SPR assay. Biotinylated hemimethylated, unmethylated, or fully methylated double strand oligonucleotide DNA were immobilized on the gold chip coated with NeutrAvidin. Purified DMAP1 protein was applied to the chip at a concentration of 1, 10, 25, 50, and 100 nM (flow rate of 10 μL/min). The $K_D$ values shown were derived from kinetic parameters based on the relationship $K_D = k_d/k_a$, where $k_d$ is the dissociation rate constant, and $k_a$ is the association rate constant. The $k_a$ and $k_d$ rate constants (Table 1) were derived by nonlinear curve fitting of sensogram data at the concentrations indicated above. A representative SPR trace is shown for each DNA target, and the analysis was repeated four times with different oligonucleotide and protein preparations. B, effects of overexpression of DMAP1 on endogenous DNMT1 activity. The ICM method, which measures the total amount of endogenous DNMT1 trapped on genomic DNA (34), was used to examine the influence of high level expression of DMAP1 on global methylase activity mediated by DNMT1. HCT-116 cells were transfected with vector (mock) or DMAP1 plasmid (2.5 μg), and 48 h later, exponentially growing cells were incubated with 10 μM aza-dC for 1 h and immediately lysed with sarkosyl. The indicated amounts of genomic DNA were spotted on a slot blot and probed with anti-DNMT1 antibody. C, ChIP analysis. The molecular association between recombinant GFP, DMAP1, and DNMT1 in a chromatin context was analyzed by chromatin immunoprecipitation assay. After transfection of either mock or I-SceI plasmid, the cells were formaldehyde-fixed and harvested. The sonicated DNAs were mixed with antibodies as indicated. Final immunoprecipitated DNAs were amplified with Rec primers, which detects only the recombinant GFP DNA. Anti-LexA antibody was used as a negative control. D, band intensity ratios of DMAP1:DNMT1 before HR (– I-SceI) and after HR (+ I-SceI) in unrecombined chromatin (unRec primers) and recombinant chromatin (Rec primers). The analysis was repeated in three independent experiments (see Fig. 1A for details on primer construction).
structures that exist before and after HR repair/gene conversion. The 5’ unrec primer will only amplify the nonrecombinant GFP molecules from Cassette I because the 3’ primer is missing from Cassette II. The 5’ rec primer is based on the converted BglI sequence and only amplifies recombinant GFP molecules (Fig. 1A). The data confirm that in the absence of I-SceI, we did not detect any PCR products in the various ChIPs in the absence of HR (Fig. 5C). We detected DNMT1 and DMAP1 in the unrecombined GFP chromatin. There is evidence for transgene silencing (37), suggesting that that integrated DR-GFP is methylated by DNMT1 (which explains the presence of the co-repressor, DMAP1). After HR (+ I-SceI) both DNMT1 and DMAP1 were in a complex specific to recombinant chromatin. Following HR, the unrecombined GFP chromatin contained lower levels of DNMT1 and DMAP1; however, analysis of DMAP1/DNMT1 ratios (from three independent ChIP experiments) revealed that unrecombined templates contained approximately half as much DMAP1 compared with DNMT1 (Fig. 5D); therefore, DMAP1 was preferentially enriched in post-HR GFP chromatin. A negative control with the LexA antibody did not recover any DNA products. Collectively, these data suggest a specific association between DNMT1, DMAP1, and recombinant GFP chromatin generated by HR repair.

DMAP1 Depletion and p16 Hypomethylation—An important question from these studies relates to the basis for selective hypomethylation of the p16 gene (Fig. 4) given our evidence suggesting a role in HR-specific events (Fig. 5). In other words, why would DMAP1 depletion incite hypomethylation at a gene that is not specifically damaged by I-Sce1? One possibility we considered is that DMAP1, in addition to promoting DNMT1 silencing at sites of HR repair, might also be important in genome stability. To address this, we examined DMAP1 in HR repair using the HO-1 cell reporter system. In this experiment, DMAP1 was depleted using shRNA (or in mock RNA controls) in HR reporter cells before and after I-Sce1 transfection. The mock infected cells displayed the expected levels of GFP expression before and after I-Sce1 expression (10); however, in the DMAP1 knockdown cells, significant levels of WT GFP were detected in the absence and presence of I-Sce1 (Fig. 6, top panel). This was also seen in the shDMAP1-2-treated cells (not shown). This striking increase in the percentage of GFP suggests that depletion of DMAP1 significantly activates HR pathways in these cells, and because we detected a clear increase in GFP positive cells even in the absence of I-Sce1 (Fig. 6, top panel), we conclude that DMAP1 depletion stimulates HR as a result of DS DNA breaks and genome instability. The HR-H (high expression) fraction was elevated in recombinant cells because of low DNMT1 activity (see Fig. 2A) in cells lacking DMAP1 co-repressor activity (Fig. 6, bottom panel). In support of this, a recent report showed that DMAP1 depletion leads to genome instability in mouse cells, in particular in a p53 minus background (HO1 cells are p53 minus) (30). Moreover, DMAP1 knockdowns accumulate DS DNA breaks, are aneuploid, and are highly tumorigenic in mice (30). These findings help explain why the p16 gene is altered in DMAP1 knockdown cells. In this case, DMAP1 depletion causes genome instability leading to damage (and repair) of genes such as p16. The resulting DNA repair-based silencing is inhibited in DMAP1-depleted cells leading to hypomethylation of HR repair sites (along with an increase in HR-H expressor class). Because p16 is only partially methylated in these cells (Fig. 4C), any DNA damage and repair without the support of DMAP1 co-repressor could potentially induce hypomethylation and p16 expression. Note that Line-1 and TIMP-3 are much less likely to suffer genetic instability in the DMAP1 knockdown cells because of repressed chromatin associated with hypermethylated domains (Fig. 4D).

DISCUSSION

Here we demonstrate that DMAP1 participates in the epigenetic reprogramming that was previously shown to attend homology-directed DNA repair (10). This means that DMAP1 acts a co-repressor in global maintenance methylation (31), as well as cooperating with DNMT1 in epigenetic alterations associated with repair of DS DNA breaks. Mechanistically, it
appears that DMAP1 has a strong binding preference for hemi-methylated DNA and stimulates DNA methylation mediated by DNMT1. DMAP1 stimulates maintenance as well as de novo DNMT1 activity in vitro. The silencing of recombinant GFP following HR was previously shown to involve DNMT1, based on mouse ES cell DNMT1+/− mutants and on ChIP experiments performed in human cell lines (10). HR repaired DNA is hemi-methylated, and subsequent outgrowth of cells yielded two expression classes of GFP, a high (HR-H) and a low (HR-L) group of cells that differ in DNA methylation status at DNA sites flanking the I-SceI site. Depleting DMAP1 using shRNA increased expression of the HR-H class (undermethylated, high expression class), whereas the HR-L (low expressor) class was reduced; however, HR frequency was not similarly affected. This result is consistent with the biochemical data showing that DMAP1 stimulates DNMT1 activity on hemi- and unmethylated DNA targets. We propose that in a chromatin setting, DMAP1 provides support as a co-repressor for DNMT1, and in DMAP1 limited cells, HR repaired GFP is hypomethylated as a result of depletion of DMAP1. The end result is an overall increase in GFP attended by a decrease in the HR-L (methylated) expression class.

**DMAP1 in Repair Methylation**—DMAP1 is a DNMT1 binding partner (31), and in vitro biochemical data (Fig. 3) confirm a supportive role in maintenance and de novo methylation. To extend these findings in vivo, we examined whether eliminating DMAP1 would alter the ability of DNMT1 to act globally on the genome, using a brief aza-dC pulse to trap the methylase on genomic DNA (Fig. 2A) (27). This method gives an overview of the genome wide activity of a specific methylase (DNMT1 in this case). The results are consistent with the biochemical data and show that DMAP1 promotes DNMT1-DNA covalent complexes. In the absence of DMAP1, the genome is hypomethylated, thereby leading to a growth reduction arising from reactivation of genes that negatively modulate growth (such as tumor suppressor genes) (34). This was confirmed in two ways. First, we demonstrated that DMAP1 knockdowns initially displayed a slow growth phenotype and eventually ceased growing (Fig. 2B). Second, we found that the p16 gene was hypomethylated in the DMAP1 knockdown cells (Fig. 4C). Reactivation of p16 would explain the growth phenotype we observe. Note that we did not detect any changes in terminal restriction fragment lengths using a telomere-specific probe,3 which suggests that telomere erosion/repair was not involved. Moreover, hypomethylation cannot be attributed to a reduction in DNMT1 levels in the shRNA transduced cells, based on Western blots. The more likely interpretation is that DNMT1 activity is affected by limited DMAP1 availability, leading to less robust methylation in these cells. The data suggest that DMAP1 has multiple roles as a DNMT1 co-repressor because DNMT1 targets HR repair chromatin (10) and other repair sites most likely through its proliferating cell nuclear antigen-binding domain (21, 30).

**DMAP1 Depletion, p16 Hypomethylation**—The data suggest that DMAP1 participates in HR repair silencing (based on DR-GFP reporter cells) and possibly in global methylation (based on ICM (27) data); however, we propose that p16 hypomethylation in DMAP1 knock-outs (Fig. 4) is the collective result of genome instability and co-repressor activity by DMAP1. We show that depleting DMAP1 in HO-1 reporter cells containing the DR-GFP cassette (Fig. 1) generated WT GFP protein in the absence of I-SceI-induced cleavages. Initially, we considered this to be a background problem in our assay; however, the observation is highly reproducible and was detected with both shRNA-DMAP1 constructs but not in mock or scrambled RNA controls. Moreover, in I-SceI-transfected HO-1 cells, HR repair was significantly elevated as well. As expected, limiting DMAP1 resulted in hypomethylated GFP products (HR-H or high expressor class). Collectively, these data indicate that DMAP1 plays a key role in genome stability, either by directly promoting HR repair or by stimulating DNMT1 methylation events, which also promotes genome stability (21, 22). Our data do not allow us to discriminate between these related consequences of DMAP1 depletion. In our previous work we proposed that DS DNA breaks in general and HR repair sites in particular are marked by discontinuous hypermethylation in a segment 3′ of the DS break (10). Such hypermethylation marks would be enhanced by the combined action of DMAP1 and DNMT1. A striking hallmark of such hypermethylation events at breaks sites is that roughly 50% of the sequenced alleles are hypermethylated. This can be seen in the WT p16 gene of HCT-116 cells (Fig. 4C, Mock), where roughly half of the sequences display hypermethylation; therefore, the p16 gene appears to have the epigenetic features of a recombination product. We propose that in the absence of DMAP1, the methylation status is reprogrammed by two related phenomena: first, DNA instability leads to elevated damage at chromatin-accessible regions of the genome (p16 being one of these sites), and second, faithful HR repair ensues, but without DMAP1 support, the p16 gene loses methyl-CpG residues, and the gene reactivates, leading to the slow growth phenotype we observe. Possibly as a result of post-HR hypomethylation and low DMAP1, this cycle of chromosomal breakage, HR repair, and hypomethylation is repeated, leading to an amplified outcome at selective genetic loci. Thus, the p16 gene is particularly sensitive to DNA damage and epigenetic reprogramming associated with DNA repair. Although DNA damage may be random in the absence of DMAP1, certain genetic loci will be hypersensitive to damage (much in the way the DNase I hypersensitive regions allow nuclease access), and lacking co-repressor support, such regions are repaired to give WT gene function but will be epigenetically reprogrammed for expression. Indeed, our findings may explain the efficacy of hypomethylating drugs in cancer therapy that selectively reactivate tumor suppressor genes (38–41).

Combining these observations with affinity binding data for methylated DNA leads us to propose the model described in Fig. 7. The two situations described (Fig. 7C, DMAP1 knockdown, and Fig. 7B, WT DMAP1) are based on previous reports that DNMT1 may act as a de novo methylase to establish a hemimethylated repair intermediate (which does not segregate) leading to twin populations of expression class (HR-H and HR-L) in roughly equal proportions (10). This conclusion is based on evidence for new methylation events that did not exist

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3 G. E. Lee, J. H. Kim, M. Taylor, and M. T. Muller, unpublished observation.
prior to HR; however, the possibility that epigenetic patterns are overlaid onto the original pre-HR template was also proposed (10). This suggests that DNMT1 methylation events are an admixture of de novo and maintenance methylation. In this case, some degree of hemimethylation post HR exists (for instance 5′ or 3′ regions that flank the I-SceI region) to recruit DMAP1, which retains DNMT1 at the site of HR. Note that the $K_D$ values for DNMT1 for HM DNA are in the micromolar range; therefore, DMAP1 is likely to play a central role in assembly of the silencing complex during or after HR. The HR expressor classes are detected following cell division and outgrowth leading to silenced daughter cells (HR-L) and high expression (unmethylated) daughter cells (HR-H). DMAP1 also recruits or retains DNMT1 at I-SceI flanking DNA during subsequent S phase maintenance methylation schedules. DMAP1 knockdown cells display elevated genomic instability (Fig. 6A) and are also impaired in their ability to strand-specifically methylate HR repaired products, which leads to hypomethylation post-HR and an increase in the HR-H population in the daughter cells. These progeny cells would then display slow growth as p16 expression ramps up.

**DMAP1 Preferentially Interacts with HR Repair Chromatin**—To demonstrate a molecular association with recombinant GFP and DMAP1 in chromatin post HR, we carried out immunoprecipitation of sheared, cross-linked chromatin (ChIP). By using primer pairs that interrogate separately rec and unrec chromatin, we were able to determine pre- and post-HR chromatin affiliation with each protein. That the presence of DMAP1 in chromatin fragments is specific for recombinant chromatin comes from the following pieces of evidence. First, the PCR signal was specific for DMAP1 antibody but negative with heterologous control antisera. Second, DMAP1 antibody signals were clearly enriched using PCR primers that amplify the recombinant GFP products generated in I-SceI transfecants. Third, positive control anti-DNMT1 antibody yielded signals from I-SceI positive cells but nothing from the I-SceI negative counterparts. We conclude that DMAP1 is preferentially bound to HR chromatin and helps shape the final epigenetic...
expression status of the repaired template. In the ChiP experiments, the unrec primer without I-SceI represents silencing/chromatin assemblies that exist prior to HR repair in Cassette II (Fig. 1A). By comparing DMAP1 before and after I-SceI transfection, we could interrogate DMAP1 binding in the donor sequence (Cassette II) before and after HR (Fig. 5D). There was a slight but reproducible elevation in DMAP1 occupancy post HR. In chromatin from +1-SceI/rec primer ChiP, DMAP1 occupancy increased more than 2-fold, suggesting that DMAP1 is preferentially recruited to the HR repair complex. Because this result is highly reproducible, the most reasonable interpretation is that DMAP1 is involved in HR repair-based silencing outcomes. Given the rather extreme preference of DMAP1 for hemimethylated DNA and that DNMT1 methylation is stimulated preferentially on HM DNA, we propose that DMAP1 is important in events that occur after the primary process of HR (as the GFP-positive cells enter the cell cycle and replicate). Hemimethylated DNA, which appears downstream of the de novo event (10), should be an attractive substrate for DMAP1 binding and co-repressor activity, in a fashion similar to that proposed by Np95 (42, 43). Thus, our model would be that during or following HR repair, DNMT1 re-establishes methylation marks over a short range of flanking sequences near the I-SceI cleavage in a DMAP1-dependent fashion. Any resulting hemimethylation regions would be high avidity sites for DMAP1, which would then recruit and assist DNMT1 into these chromatin domains. Further studies will be important to identify key regulatory components that participate in HR silencing; however, this work establishes a tractable model for insight into rare epigenetic events associated with repair of double strand DNA breaks.

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