Retinoblastoma Pathway Dysregulation Causes DNA Methyltransferase 1 Overexpression in Cancer via MAD2-Mediated Inhibition of the Anaphase-Promoting Complex

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We have examined the mechanism of normal DNA methyltransferase 1 (DNMT1) degradation as well as its mechanism of dysregulation in cancer. We have previously reported that DNMT1 protein levels were elevated and abnormally stabilized because of defective degradation through its N-terminal destruction domain. Here, we report that DNMT1 was abnormally stabilized in several cancer cell lines and that, in cells with normal DNMT1 destruction, depletion of CDC20 or FZR1 (two substrate recognition adaptor components of the anaphase-promoting complex) resulted in stabilization of DNMT1 that was partially dependent on the N-terminal destruction domain, thus implicating this cell cycle regulator in the destruction of DNMT1. MAD2, an inhibitor of CDC20, was shown to stabilize DNMT1 levels, and overexpression of MAD2, a consequence of retinoblastoma (RB) pathway dysregulation, was shown to correlate with impaired G₁ phase DNMT1 destruction and RB inactivation by hyperphosphorylation in several normal and cancer cell lines. Furthermore, in a series of 85 cases of human breast cancer, a moderately strong, but highly significant, correlation between MAD2 and DNMT1 immunohistochemical staining was observed, yielding a Spearman rank order correlation coefficient of 0.37 ($P < 0.001$). This suggests that RB pathway inactivation, a common dysfunction in cancer cells, may be the underlying cause of DNMT1 dysregulation. (*Am J Pathol* 2007, 170:1585–1593; DOI: 10.2353/ajpath.2007.060779)

Overexpression of DNA methyltransferase 1 (DNMT1) has been implicated in the process of tumorigenesis in

several studies,^{1–15} but the mechanism of DNMT1 dysregulation has been controversial. Early studies focused on *DNMT1* mRNA overexpression and its correlation with tumor progression,^{2,3} but subsequent investigations demonstrated that after controlling for S phase fraction, those differences were insignificant.^{16,17} We have previously shown that in breast cancer tissues and MCF-7 breast cancer cells, DNMT1 protein levels are discordant with cell proliferation and are dysregulated and expressed throughout the cell cycle.¹⁸ Although the N-terminal 118-amino acid domain of DNMT1 is clearly involved in its destruction,^{19,20} the mediators of this pathway have not been defined.

One possible mechanism of degradation is through the anaphase-promoting complex (APC), a multicomponent ubiquitin ligase complex consisting of 12 core proteins along with substrate recognition adaptors CDC20 and FZR1.²¹ The APC subunits APC2 and APC11 interact with the family of E2 enzymes that are known to function as ubiquitin carriers.²¹ The subcomplex comprised of APC1, APC4, and APC5 may interact with APC2/APC11 and connect these subunits to the APC3 and APC7 subunits. These APC3/APC7 subunits recruit the substrate recognition adaptors CDC20 and FZR1 to the APC, along with the substrates to be ubiquitinated. Together, this complex allows the APC to function as an E3 ubiquitin ligase complex, and the APC is involved in the ubiquitination and proteolysis of many cell cycle-regulated proteins including securin, M phase cyclins, and G₁ phase cyclins and is thus required for proper regulation of the cell cycle.²²

Some evidence exists for dysfunction of the APC in human cancers. Loss of APC7 has been reported in an

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immunohistochemical analysis of human breast cancer tissues, and this loss was more common in cancers with poor prognostic parameters or malignant characteristics.¹⁹ However, more studies have investigated the role of MAD2, a mitotic checkpoint mediator and inhibitor of CDC20. MAD2 is known to be activated by a conformational change that is induced by MAD1 at the site of unattached chromosome kinetochores.²³ This mitotic checkpoint-inducing conformation of MAD2 can bind to CDC20 and inhibit its ability to recruit substrates to the APC that are required for progression through mitosis.²³ Although defects in MAD2 function have been reported to cause aneuploidy in cancer cells by abrogation of the mitotic checkpoint, resulting in improper chromosome segregation,²⁴ overexpression of MAD2 has also been implicated in carcinogenesis. Specifically, in one study MAD2 was found to be a direct transcriptional target of E2F, and RB pathway inactivation was reported to cause overexpression of MAD2. This increased MAD2 activity caused overactivation of the mitotic cell cycle checkpoint and resulted in a prolonged mitosis, which was eventually completed but with irregular chromosome segregation and frequent tetraploidy.²⁵ The oncogenic properties of MAD2 and its ability to stabilize and dysregulate cell cycle-regulated components clearly warrant further investigation.

The two adaptor proteins CDC20 and FZR1 are crucial in conferring different substrate specificities to the APC. CDC20 is the substrate recognition adaptor for the B-type mitotic cyclins and is required for progression through mitosis.²⁶ The substrate specificities of this APC adaptor protein include proteins that contain RXXL destruction boxes. FZR1 is the adaptor protein that can recognize D-type cyclins, thus allowing maintenance of G₁ phase of the cell cycle.²⁶ Its substrate specificities include proteins that contain either RXXL or KEN destruction boxes.²⁶ FZR1 is regulated by phosphorylation, which may be catalyzed by cyclin-dependent kinases,²⁷ and it can bind CDC20, which contains a KEN box, and recruit it to the APC for ubiquitination and degradation. This allows for sequential utilization of these adaptor proteins and, therefore, a substrate specificity switch for the APC between mitosis and early G₁ phase. Interestingly, DNMT1 has two RXXL domains within its N-terminal 118-amino acid domain, similar to cyclin D1, and it also has a KEN box 644 amino acids from the N terminus. Although one study has implicated FZR1 in the destruction of DNMT1 after 5-aza-2'-deoxycytidine treatment through this KEN box,²⁸ the normal physiological destruction pathway of DNMT1 and the mechanism behind its dysfunction in cancer cells is still unknown.

Here, we report that both CDC20 and FZR1 are required for normal destruction of DNMT1, and that this pathway is impaired in MCF-7 breast cancer cells. Furthermore, we show that overexpression of MAD2, which is an inhibitor of the APC through the binding and inactivation of its adaptor protein CDC20,²³ causes stabilization of DNMT1. MAD2, a mediator of the mitotic spindle checkpoint during metaphase, has been shown to be a transcriptional target of E2F, and retinoblastoma protein (RB) pathway inactivation can cause constitutive overex-

pression of MAD2 and uncoupling of cell cycle progression from mitotic checkpoint regulation.²⁵ Here, we show that impaired DNMT1 destruction correlates with MAD2 overexpression and RB inactivation in several normal and cancer cell lines and that MAD2 up-regulation correlates with increased DNMT1 levels by immunohistochemistry in a series of 85 human breast cancer tissues. This suggests that RB pathway inactivation itself may lead to DNMT1 dysregulation through MAD2 overexpression.

Materials and Methods

Cell Culture and Cell Cycle Synchronization

Human mammary epithelial cells (HMECs) and prostate epithelial cells (PRECs) were obtained from Cambrex (East Rutherford, NJ) and grown in mammary epithelial growth medium and prostate epithelial growth medium, respectively. U2OS osteosarcoma cells, MCF-7 breast cancer cells, and HCT116 colon cancer cells were obtained from American Type Culture Collection (Manassas, VA) and grown with 10% fetal bovine serum in McCoy's 5A, minimal essential medium, and McCoy's 5A media, respectively (Invitrogen, Carlsbad, CA). MCF-10A immortalized HMECs were obtained from American Type Culture Collection and grown in mammary epithelial growth medium serum-free media (Cambrex). For G₁ phase synchronization, cells were treated with 20 μ mol/L lovastatin for 24 hours (Sigma, St. Louis, MO) or with 0.1% (v/v) dimethyl sulfoxide as a vehicle control.

FLAG-Tagged DNMT1 and MAD2 Constructs

Full-length DNMT1 (nucleotides from 238 to 5088, National Center for Biotechnology Information (NCBI) RefSeq NM_001379) and N-terminal deletions were polymerase chain reaction (PCR) amplified with a *Sal*I site extension on the reverse primer (5'-GTCGACGCGG-TACCTTGGCAAAGCA-3') and the following forward primers: full-length 5'-ATGCCGGCGCGTACC-3' and 120-amino acid N-terminal deletion 5'-ATGGCAGATGCCAACAGCC-3'. These PCR products were cloned into the pCR2.1-TOPO plasmid of the TOPO TA cloning kit (Invitrogen). The *Eco*RI/*Sal*I fragment was then subcloned into the *Eco*RI/*Sal*I sites of the pEGFP-N1 Vector (Clontech, BD Biosciences, Palo Alto, CA). To generate the FLAG-tagged version, the *Age*I/*Not*I fragment containing green fluorescent protein (GFP) was removed and replaced by a linker with the eight-amino acid FLAG sequence (DYKDDDDK-STOP) flanked by *Age*I/*Not*I sites.

Full-length MAD2 (nucleotides from 46 to 689, NCBI RefSeq NM_002358) was reverse transcriptase-PCR amplified from HMEC mRNA with an *Age*I site extension on the reverse primer (forward primer: 5'-GCGTGCTTTT-GTTTGTGTCC-3'; reverse primer: 5'-ACCGGTCCGT-CATTGACAGGAATTTGTAGGC-3'). This PCR product was cloned into the pCR2.1-TOPO plasmid of the TOPO TA cloning kit (Invitrogen). The *Eco*RI/*Age*I fragment was then subcloned into the *Eco*RI/*Age*I-digested DNMT1-FLAG plasmid described above, replacing the DNMT1

nucleotide sequence with MAD2. These constructs were transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Small Interfering RNA Transfection and Immunoblotting

RNAi depletion of CDC20, FZR1, and lamin A/C was performed by transfecting the following RNA duplexes obtained from Integrated DNA Technologies (Coralville, IA) with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols: CDC20, 5'-C-GGCAGGACUCCGGGCCGATT-3', 5'-UCGGCCCCGG-AGUCCUGCCGTT-3'; and FZR1, 5'-UGAGAAGUCUC-CCAGUCAGTT-3', 5'-CUGACUGGGAGACUUCUCAT-T-3'. Immunoblotting for protein levels was performed with the following antibodies: FLAG epitope (Stratagene, La Jolla, CA); CDC20, FZR1, cyclin B1, phospho-RB (Santa Cruz Biotechnology, Santa Cruz, CA); actin, cyclin D1 (Sigma); and MAD2 (Bethyl Laboratories, Montgomery, TX). For DNMT1 immunoblotting, we used an affinity-purified IgG preparation isolated from rat antiserum raised against a polypeptide (N-MADANSPPKPLSKPRTPRRS-C) derived from DNMT1.¹¹

Immunohistochemistry of DNMT1 and MAD2 in Breast Cancer Tissues

Using an affinity-purified IgG preparation isolated from rat antiserum raised against a polypeptide (N-MADANSPPKPLSKPRTPRRS-C) derived from DNMT1,¹¹ and an anti-MAD2 antibody obtained from Bethyl Laboratories, we performed immunohistochemistry on formalin-fixed, paraffin-embedded tissue from three breast cancer tissue microarrays representing a total of 85 human breast cancer cases (61 invasive ductal carcinomas, seven invasive lobular carcinomas, and 17 carcinomas with features of both) from the surgical pathology archives of the Johns Hopkins Hospital. The tissue microarrays were constructed as previously described.²⁹ The intensity of nuclear expression of DNMT1 and perinuclear/cytoplasmic MAD2 were visually scored by an independent pathologist (P.A.) on a scale of 1 to 4, and the Spearman rank order correlation coefficient was calculated. In cases with tumor tissues from multiple anatomical sites or duplications on the array, the scores were averaged. Estrogen receptor, progesterone receptor, DNA ploidy, and S phase fraction were quantified by the Roche Biomedical Laboratories, Indianapolis, IN, and correlation with MAD2 and DNMT1 immunostaining was determined by the Spearman rank order correlation coefficient as well. Furthermore, differences in MAD2 and DNMT1 immunostaining in tumor type (ductal versus lobular), degree of differentiation (poor versus moderate), and lymph node metastasis status (positive versus negative) were analyzed by the nonparametric Mann-Whitney *U*-test.

For DNMT1 and MAD2 immunostaining, we used a modification of the EnVision Plus detection system from DAKO (Carpinteria, CA), including all blocking steps.

Briefly, antigen retrieval for DNMT1 and MAD2 was achieved by steam heating in target retrieval buffer (DAKO) for 10 minutes. The primary anti-DNMT1 antibody (1:1000) or anti-MAD2 antibody (1:2400) was incubated for 45 minutes at room temperature. The Envision Plus horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was applied for 30 minutes at room temperature, and this was followed by application of the diaminobenzidine (brown staining) as the chromogen. Slides were counterstained with hematoxylin.

The anti-DNMT1 antibody dilution (1:1000) was optimized on formalin-fixed, paraffin-embedded cell lines, and the HCT116 colon cancer cell line was used as a positive control with a derivative of this same cell line HCT116 DNMT1^{-/-} (a generous gift from Dr. Bert Vogelstein, Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University, Baltimore, MD) used as a negative control. The HCT116 DNMT1^{-/-} cells were derived from HCT116 parental cells after targeted disruption of both endogenous DNMT1 alleles. As an additional control, DNMT1 staining was completely abolished after preincubation with 100-fold excess cognate peptide, but not with an unrelated peptide. Likewise, the anti-MAD2 antibody dilution (1:2400) was optimized on formalin-fixed, paraffin-embedded cell lines U2OS and MCF-7, which had low and high levels of MAD2 protein, respectively, as determined by immunoblotting.

Results

DNMT1 Was Selectively Stabilized in MCF-7 Cells versus U2OS Cells

U2OS osteosarcoma cells were used as a normal comparison for MCF-7 cells because of their intact DNMT1 destruction phenotype and their amenability to transfection experiments. cDNA encoding FLAG-tagged full-length DNMT1 (DNMT1-FLAG) and its stabilized 120-amino acid N-terminal deletion mutant (Δ N-DNMT1-FLAG) were transfected into U2OS and MCF-7 cells, and protein levels were determined 24 hours later by immunoblotting with an anti-FLAG antibody (Figure 1, A and B). Both isoforms were transcribed from the same constitutively active cytomegalovirus promoter and transfected in the same manner to control for transcriptional variation. Therefore, although the absolute protein levels in U2OS and MCF-7 cells may be affected by differential transfection efficiency, the variation in the ratio of these two isoforms by immunoblotting likely reflected differences in N-terminal-dependent posttranscriptional regulation. As previously reported, in MCF-7 cells the ratio of DNMT1-FLAG relative to Δ N-DNMT1-FLAG was greater than 0.6.¹⁸ In contrast, U2OS cells showed significantly lower DNMT1-FLAG protein levels relative to Δ N-DNMT1-FLAG with a ratio less than 0.3 despite similar cell cycle kinetics (data not shown), a phenotype similar to that previously reported in primary cells such as HMECs (ratio <0.10).¹⁸

Figure 1. DNMT1-FLAG protein expression in U2OS and MCF-7 cells. **A:** FLAG-tagged DNMT1 full-length and 120-amino acid N-terminal deletion mutants were transfected into U2OS and MCF-7 cells, and protein levels were determined after 24 hours by anti-FLAG immunoblotting and densitometry (**B**). Total protein staining by Ponceau S was performed as a loading control. Error bars are $\pm$ SD of duplicate lanes ($n = 2$).

DNMT1 Protein Destruction Was Mediated by the Anaphase-Promoting Complex and Its Adaptor Proteins CDC20 and FZR1 in U2OS Cells and Was Impaired in MCF-7 Cells

The APC adaptor proteins CDC20 and FZR1 were depleted by RNA interference in MCF-7 and U2OS cells for 36 hours, with depletion of lamin A/C as a control, and cDNA encoding DNMT1-FLAG or Δ N-DNMT1-FLAG were then transfected. Protein levels were determined by immunoblotting 24 hours later (Figure 2, A and B). RNAi depletion of either APC adaptor protein CDC20 and FZR1 in U2OS resulted in significant increases in DNMT1-FLAG, both in the absolute levels and relative to Δ N-DNMT1-FLAG. Furthermore, the ratio of DNMT1-FLAG: Δ N-DNMT1-FLAG increased from ~ 0.2 to ~ 0.6 , similar to the baseline ratio in MCF-7 cells. Knockdown of the adaptor proteins CDC20 and FZR1 in MCF-7, on the other hand, did not cause any significant increase in DNMT1-FLAG levels. These data suggest that this pathway of normal DNMT1 destruction through the APC is impaired in MCF-7 cells.

APC Components APC3, APC7, CDC20, and FZR1 Were Not Mutated in MCF-7 Cells

The cDNA sequences of the critical APC adaptors CDC20 and FZR1 as well as their core APC subunit binding partners APC3 and APC7 were reverse transcriptase-PCR amplified from HMEC mRNA, cloned, and sequenced for mutations. There were no sequence differences or mutations in any of these genes between any of the cell lines, and no discrepancies were found between these sequences and those deposited at the National Center for Biotechnology Information (data not shown).

MAD2 Overexpression Inhibited the Destruction of DNMT1 in U2OS Cells and Recapitulated the Impaired Destruction Phenotype of MCF-7 Cells

cDNA encoding pCMV-MAD2 or pCMV-GFP (control) plasmids were co-transfected with DNMT1-FLAG or Δ N-DNMT1-FLAG into U2OS cells, and protein levels were determined by immunoblotting 24 hours later (Figure 3A)

Figure 2. DNMT1-FLAG protein expression after small interfering RNA (siRNA) depletion of CDC20 and FZR1 in U2OS and MCF-7 cells. **A:** CDC20, FZR1, and lamin A/C (negative control) were depleted by siRNA transfection for 36 hours, and FLAG-tagged DNMT1 full-length and 120-amino acid N-terminal deletion mutants were transfected into U2OS and MCF-7 cells. **B:** Protein levels were determined after 24 hours by anti-FLAG immunoblotting and densitometry. RNAi depletion of proteins was confirmed by immunoblotting for CDC20, FZR1, and lamin A/C, and actin immunoblotting was performed as a loading control. Error bars are $\pm$ SD of duplicate lanes ($n = 2$).

Figure 3. MAD2 overexpression and stabilization of DNMT1-FLAG in U2OS cells. MAD2 or GFP control was co-transfected into U2OS cells with full-length DNMT1 or its 120-amino acid N-terminal deletion mutant, and protein levels were determined after 24 hours by anti-FLAG immunoblotting (**A**) and densitometry (**C**). **D:** The ratio of DNMT1-FLAG to ΔN-DNMT1-FLAG was calculated as well. Immunoblotting for cyclin B1 was performed to control for M phase fraction, and Ponceau S staining was performed as a loading control. Endogenous DNMT1 levels were also determined after MAD2 or GFP overexpression by immunoblotting (**B**) and densitometry (**C**) with β-actin as a loading control. Error bars are ±SD of duplicate lanes (*n* = 2).

followed by densitometry (Figure 3C). MAD2 co-transfection significantly increased DNMT1-FLAG levels compared with co-transfection of GFP protein, and the stabilized protein levels were as high as ΔN-DNMT1-FLAG with the GFP control (Figure 3C). Furthermore, although MAD2 also increased ΔN-DNMT1-FLAG levels as well, the ratio of DNMT1-FLAG:ΔN-DNMT1-FLAG was increased from ~0.2 (similar to U2OS baseline ratio) to ~0.6 (Figure 3D), which recapitulated the baseline ratio seen in MCF-7 cells. In addition, cyclin B1 levels were not affected by MAD2 overexpression, thus excluding the confounding factor of redistribution of cells into M phase. This suggests that overexpression of MAD2, as well as knockdown of CDC20 or FZR1, can stabilize DNMT1 protein levels and recapitulate the impaired DNMT1-FLAG destruction phenotype of MCF-7 cells.

Endogenous DNMT1 levels were also examined by immunoblotting after MAD2 overexpression, and a modest but reproducible increase was found compared with GFP transfection control in both U2OS and MCF-7 cells (Figure 3, B and C). We suspect that this is because of the limited transfection efficiency of the MAD2 expression plasmid, thus resulting in a large background of endogenous DNMT1 in untransfected cells that cannot be stabilized by MAD2. For this reason, we prefer to examine co-transfected FLAG-tagged DNMT1 isoforms, which are presumably expressed only in those cells that also have MAD2 overexpression.

Dysregulation of Endogenous DNMT1

Correlated with MAD2 Overexpression and RB Pathway Inactivation in U2OS, MCF-7, HMECs, MCF-10A, PRECs, and HCT116 Cells

To correlate MAD2 expression with the impaired DNMT1 destruction phenotype, various normal and cancer cells were treated with lovastatin, a drug that causes early G₁ phase arrest.³⁰ As previously described, 24 hours of lovastatin treatment of a cell type with intact DNMT1 protein destruction causes a large reduction of protein levels, usually less than 30% of control. However, in cells with impaired DNMT1 destruction, this reduction is minimal, usually more than 60% of control. The normal cells used were HMECs and PRECs, and the cancer cells used were MCF-7 breast adenocarcinoma cells, HCT116 colonic adenocarcinoma cells, and the U2OS osteosarcoma cells. In addition, we examined MCF-10A cells, which are a spontaneously immortalized cell line derived from HMECs. MCF-10A cells are known to have a homozygous deletion of p16^{INK4A} locus, which is one of the best characterized forms of dysregulation of the RB protein pathway.³¹

These cells were treated with 20 μmol/L lovastatin for 24 hours, and the levels of DNMT1, MAD2, phospho-RB, RB, cyclin D1, and actin (loading control) were determined by immunoblotting (Figure 4, A and B). The cancer

Figure 4. Correlation of MAD2 overexpression with DNMT1 dysregulation and RB hyperphosphorylation. **A:** U2OS, MCF-7, HMEC, MCF-10A, PREC, and HCT116 cells were synchronized in G₁ phase for 24 hours with 20 μ mol/L lovastatin, and immunoblotting was performed for endogenous DNMT1, MAD2, phospho-RB, RB, and actin (loading control). The right lane in each set of two corresponds to lovastatin-treated, G₁-arrested cells (Lova), and the left lane represents untreated control (Ctrl). **B:** Protein levels were quantified by densitometry.

cell lines MCF-7 and HCT116 clearly showed increased DNMT1 levels with impaired destruction on G₁ synchronization with lovastatin as well as MAD2 overexpression compared with the normal DNMT1-degrading HMEC, PREC, and U2OS phenotypes. Interestingly, the RB pathway-dysregulated cell line MCF-10A, as predicted, showed overexpression of the MAD2 protein and concomitant impairment of DNMT1 destruction. Furthermore, as expected, the MAD2 protein overexpression phenotype correlated well with hyperphosphorylated RB levels, a marker of RB inactivation.

Increased DNMT1 Levels Correlated with MAD2 Up-Regulation by Immunohistochemistry in a Series of 85 Cases of Human Breast Cancer

MAD2 and DNMT1 levels were determined by immunohistochemistry in a series of 85 cases of human breast cancer on a tissue microarray. The staining intensities were visually scored and the Spearman rank order correlation coefficient was determined to be 0.37 ($P < 0.001$), a moderately strong but highly significant correlation considering the heterogeneity of the different human breast cancer tissues and slight variations in fixation. Selected cases are shown in Figure 5 with low MAD2 and DNMT1 staining (Figure 5, A and B) and high MAD2 and DNMT1 staining (Figure 5, C and D) as well as staining controls U2OS (MAD2-negative control, Figure 5E), MCF-7 (MAD2-positive control, Figure 5G), HCT116 DNMT1^{-/-} (DNMT1-negative control, Figure 5F), and HCT116 DNMT1^{+/+} (DNMT1-positive control, Figure 5H). A scatter plot of cases with MAD2 and DNMT1 immunoreactivity is shown in Figure 5I with the relative width of each scatter dot representing multiplicity.

MAD2 and DNMT1 immunostaining intensities were also correlated with several other clinicopathological factors including estrogen and progesterone receptor expression, DNA ploidy, and S phase fraction by Spearman rank order analysis. Furthermore, differences in MAD2

and DNMT1 immunostaining between breast cancer types (ductal versus lobular), degrees of differentiation (poor versus moderate), and status of lymph node metastasis (positive versus negative) were calculated by the nonparametric Mann-Whitney *U*-test. These results are shown in Table 1. MAD2 immunostaining exhibited a moderately strong but highly significant inverse correlation (-0.34 , $P = 0.002$) with progesterone receptor expression, a less significant positive trend with DNA ploidy (0.18 , $P = 0.11$), and higher immunostain scoring in ductal versus lobular carcinoma ($+0.5$, $P = 0.10$). DNMT1 immunostain scoring was statistically significantly higher in ductal carcinomas versus lobular ($+1.1$, $P = 0.002$) and poorly differentiated versus moderately differentiated tumors ($+1.0$, $P = 0.02$). It also showed a less significant negative trend with estrogen receptor expression (-0.19 , $P = 0.11$) and progesterone receptor expression (-0.17 , $P = 0.11$) as well as a positive trend with DNA ploidy (0.22 , $P = 0.06$) and S phase fraction (0.28 , $P = 0.07$).

Discussion

We have shown that DNMT1 degradation was impaired in MCF-7 cells relative to U2OS cells and that this degradation was mediated by the APC via its substrate recognition adaptor proteins CDC20 and FZR1, because RNAi depletion of CDC20 or FZR1 both stabilized DNMT1 levels. Although the Δ N-DNMT1-FLAG destruction domain deletion mutant was stabilized as well, the full-length DNMT1-FLAG was stabilized even more so, causing the DNMT1-FLAG: Δ N-DNMT1-FLAG ratio to rise threefold. This suggests that APC-mediated destruction was partially dependent on the N-terminal 120-amino acid destruction domain. Because depletion of the adaptor proteins in MCF-7 cells did not result in any significant increase in DNMT1 levels, we conclude that this pathway is dysfunctional in MCF-7 cells. Notably, baseline CDC20 levels were low in MCF-7 cells, similar to the level in

Figure 5. Correlation of increased DNMT1 levels with MAD2 up-regulation by immunohistochemistry in human breast cancer tissues. MAD2 and DNMT1 levels were determined by immunohistochemistry in a series of 85 human breast cancer cases. The staining intensities were visually scored, and the Spearman rank order correlation coefficient was determined to be 0.37 ($P < 0.001$), a moderately strong but highly significant correlation. One selected case (**A**, **B**) is shown with low MAD2 immunostaining (**A**), which correlates with low DNMT1 immunostaining (**B**). In another case (**C**, **D**), higher MAD2 immunostaining (**C**) correlates with higher DNMT1 levels (**D**). Staining controls are shown: U2OS (MAD2-negative control, **E**), MCF-7 (MAD2-positive control, **G**), HCT116 DNMT1^{-/-} (DNMT1-negative control, **F**), and HCT116 DNMT1^{+/+} (DNMT1-positive control, **H**). A scatter plot of cases with MAD2 and DNMT1 immunoreactivity is shown (**I**) with the relative width of each scatter dot representing multiplicity. Original magnifications, $\times 200$ (**A–D**).

U2OS cells after CDC20 depletion, and this may have been a major contribution to the phenotype of impaired DNMT1 destruction.

Overexpression of MAD2, an inhibitor of CDC20, was shown to cause stabilization of DNMT1 and recapitulated the effect of RNAi depletion of CDC20, namely a small

Table 1. Correlation of MAD2 and DNMT1 Immunostaining with Clinicopathologic Characteristics of Breast Cancer

	Mean staining intensity (difference by Mann-Whitney <i>U</i> test)			Correlation by Spearman rank order coefficient (95% confidence interval)			
	Type ductal/lobular	Differentiation poor/moderate	Lymph node positive/negative	Estrogen receptor	Progesterone receptor	DNA ploidy	S phase percent
MAD2	2.1/1.6 0.5 (<i>P</i> = 0.10)	2.2/1.9 0.2 (<i>P</i> = 0.60)	2.1/1.9 0.0 (<i>P</i> = 0.53)	0.10 (<i>P</i> = 0.37) CI: (−0.12, 0.32)	−0.34 (<i>P</i> = 0.002) CI: (−0.52, −0.13)	0.18 (<i>P</i> = 0.11) CI: (−0.04, 0.39)	0.12 (<i>P</i> = 0.44) CI: (−0.19, 0.41)
DNMT1	3.0/2.0 1.1 (<i>P</i> = 0.002)	3.3/2.5 1.0 (<i>P</i> = 0.02)	3.0/2.9 0.0 (<i>P</i> = 0.75)	−0.19 (<i>P</i> = 0.11) CI: (−0.39, 0.04)	−0.17 (<i>P</i> = 0.13) CI: (−0.38, 0.05)	0.22 (<i>P</i> = 0.06) CI: (−0.01, 0.42)	0.28 (<i>P</i> = 0.07) CI: (−0.02, 0.54)

MAD2 and DNMT1 immunostaining levels were correlated by Spearman rank order coefficient to estrogen receptor expression, progesterone receptor expression, DNA ploidy, and S phase fraction. The 95% confidence intervals are shown. Differences in MAD2 and DNMT1 immunostaining between tumor type (ductal versus lobular), degree of differentiation (poor versus moderate), and status of lymph node metastasis (positive versus negative) were calculated by the nonparametric Mann-Whitney *U*-test.

rise in the Δ N-DNMT1 destruction domain deletion mutant coupled with a greater rise in the full-length protein resulting in a threefold increase in the ratio between the two isoforms. This overexpression of MAD2 also recapitulated the impaired DNMT1 destruction phenotype seen in MCF-7 cells. One possible confounding factor considered was the role of MAD2 as a mitotic spindle checkpoint mediator. If MAD2 were to cause arrest of cells in M phase, this would deceptively appear to stabilize DNMT1 while only preventing progression of the cell cycle to the phase in which another mediator degrades it. However, the lack of increase in cyclin B1 levels, a well-characterized mitotic cyclin, as determined by immunoblotting, argued against this artifact. Furthermore, the morphology of the cells overexpressing MAD2 did not have the characteristic rounded appearance that M phase-arrested cells have when treated with nocodazole, a M phase synchronizing agent (data not shown).

Furthermore, overexpression of endogenous MAD2 was correlated with dysregulation of endogenous DNMT1 in several normal primary cells (HMECs and PRECs), immortalized cells (MCF-10A), and cancer cell lines (U2OS, MCF-7, HCT116), as well as in a large series of 85 cases of human breast cancers, which resulted in a Spearman correlation coefficient of 0.37 (*P* < 0.001). Although the correlation coefficient was less than 1, it was highly statistically significant, and moreover, we did not expect MAD2 levels to be the only determinant of DNMT1 levels as variations in S phase fraction and APC dysfunction may have contributed as well in this heterogeneous group of human breast cancers.

MAD2 immunostaining was also shown to have a highly significant negative correlation with progesterone receptor expression and a less significant negative trend was observed between DNMT1 immunostaining and both estrogen and progesterone receptor expression. This was quite interesting considering previous studies in breast cancer have shown a correlation between lack of estrogen and progesterone receptor expression and methylation of their promoters.^{32,33} Furthermore, these tumors tend to be more poorly differentiated, and we also observed that DNMT1 immunostaining was significantly higher in poorly versus moderately differentiated tumors. The actual estrogen and progesterone methylation status in these tumors clearly warrants further investigation. In addition, the S phase fraction correlation with DNMT1 was only 0.28, consistent with our previous finding that S

phase fraction alone does not explain DNMT1 overexpression in cancer and that abnormal stabilization by MAD2 overexpression is a plausible mechanism.¹⁸ Finally, the correlation between both MAD2 and DNMT1 immunostaining with DNA ploidy is also consistent with previous studies reporting that RB pathway inactivation with subsequent MAD2 overexpression tends to result in higher rates of tetraploidy.²⁵

We have also found that the overexpression of MAD2 in several cell lines was correlated with dysregulation of the RB pathway, as measured by hyperphosphorylation of RB protein. The most closely related cells in these comparisons that differed in DNMT1 degradation phenotype were the HMECs and MCF-10A cells. MCF-10A cells are a spontaneously immortalized cell line derived from HMECs and are known to have a homozygous deletion of the p16^{INK4A} locus.³¹ Thus, MCF-10A cells conveniently represent a version of HMECs with the RB pathway dysregulated. The data showed an increase in hyperphosphorylated RB in MCF-10A relative to HMECs, although this difference was small because the logarithmically growing HMECs had almost identical growth kinetics and doubling times. However, the expression of MAD2 was unmistakably higher in MCF-10A cells, and these cells clearly exhibited the DNMT1 dysregulation phenotype. Furthermore, MCF-7 cells, which showed even greater impairment of DNMT1 destruction, had even higher levels of MAD2 and hyperphosphorylated RB. These data were consistent with previously published findings reporting that MAD2 is a direct transcriptional target of E2F-1,²⁵ and this suggests that DNMT1 dysregulation via MAD2 may be a result of RB pathway dysregulation, a very common phenotype of many cancers.³⁴

The possible existence of a CpG island methylator phenotype in cancer has been controversial. Several studies have observed clustering of hypermethylated genes in certain cancer types.^{35,36} Furthermore, there is growing evidence that many, if not all, types of cancers have some sort of dysregulation of the RB pathway, with different degrees of dysregulation resulting from RB inactivation itself or loss of cyclin-dependent kinase inhibitors such as p16, p21, or p27, for instance.^{37,38} If this is true, then our data linking the inactivation of the RB pathway with DNMT1 protein overexpression via MAD2 up-regulation would suggest a highly prevalent but variable CpG island hypermethylator phenotype in the vast majority of cancers.

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