# Inhibition of Histone Deacetylases Promotes Ubiquitin-Dependent Proteasomal Degradation of DNA Methyltransferase 1 in Human Breast Cancer Cells

**Qun Zhou,[1] Agoston T. Agoston,[1] Peter Atadja,[2] William G. Nelson,[1] and Nancy E. Davidson[1]**

*[1]The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, Maryland; and [2]Novartis Pharmaceuticals, Inc., Cambridge, Massachusetts*

## Abstract

**Histone deacetylases (HDAC) play a critical role in chromatin modification and gene expression. Recent evidence indicates that HDACs can also regulate functions of nonhistone proteins by catalyzing the removal of acetylated lysine residues. Here, we show that the HDAC inhibitor LBH589 down-regulates DNA methyltransferase 1 (DNMT1) protein expression in the nucleus of human breast cancer cells. Cotreatment with the proteasomal inhibitor MG-132 abolishes the ability of LBH589 to reduce DNMT1, suggesting that the proteasomal pathway mediates DNMT1 degradation on HDAC inhibition. Deletion of the $NH_2$-terminal 120 amino acids of DNMT1 diminishes LBH589-induced ubiquitination, indicating that this domain is essential for its proteasomal degradation. DNMT1 recruits the molecular chaperone heat shock protein 90 (Hsp90) to form a chaperone complex. Treatment with LBH589 induces hyperacetylation of Hsp90, thereby inhibiting the association of DNMT1 with Hsp90 and promoting ubiquitination of DNMT1. In addition, inactivation of HDAC1 activity by small interfering RNA and MS-275 is associated with Hsp90 acetylation in conjunction with reduction of DNMT1 protein expression. We conclude that the stability of DNMT1 is maintained in part through its association with Hsp90. Disruption of Hsp90 function by HDAC inhibition is a unique mechanism that mediates the ubiquitin-proteasome pathway for DNMT1 degradation. Our studies suggest a new role for HDAC1 and identify a novel mechanism of action for the HDAC inhibitors as down-regulators of DNMT1. (Mol Cancer Res 2008;6(5):873–83)**

Received 7/16/07; revised 1/18/08; accepted 1/23/08.
**Grant support:** National Cancer Institute grants CA88843 and CA58236, Department of Defense grant DAMD 17-03-1-0376, and Avon Foundation.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
**Requests for reprints:** Nancy E. Davidson, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, 1650 Orleans Street, CRB Room 144, Baltimore, MD 21231. Phone: 410-955-8489; Fax: 410-614-4073. E-mail: davidna@jhmi.edu

doi:10.1158/1541-7786.MCR-07-0330

## Introduction

Considerable evidence has been accumulated in the elucidation of the molecular mechanisms by which DNA methylation is involved in tumor suppressor gene silencing (1). Methylation of CpG dinucleotides is catalyzed by DNA methyltransferases (DNMT), which transfer the methyl moiety from the methyl donor *S*-adenosylmethionine to the 5′ position of the cytosine ring. DNMT1 has a preference for methylating hemimethylated DNA and is referred to as the primary maintenance DNMT (2, 3). We have previously reported that the *estrogen receptor-α* gene can be epigenetically silenced in human breast cancer cells (4, 5). Hypermethylation of the 5′ CpG island at the silenced estrogen receptor-α promoter is linked with aberrant association of DNMT1 (6, 7). Inhibition of DNMT1 by either pharmacologic or genetic approaches activates estrogen receptor-α gene expression and restores tamoxifen sensitivity in estrogen receptor-α–negative human breast cancer cells (6-10), suggesting a role for DNMT1 in maintenance of the gene silencing at this locus.

The 195-kDa DNMT1 is a nuclear protein that harbors the DNMT catalytic domain in its C terminus and several regulatory domains in its N terminus (3). Increased protein levels of DNMT1 are noted in MCF-7 human breast cancer cells compared with normal human mammary epithelial cells (11). Elevated DNMT1 protein levels in MCF-7 cells apparently reflect a dysfunctional $NH_2$-terminal regulatory domain, which is essential for its proper ubiquitination and degradation (11). That the proteasomal pathway plays a key role in the stability of DNMT1 is suggested by a recent study using HeLa and Cos-7 cells showing that DNMT1 can be selectively degraded in response to the DNMT inhibitor decitabine [5-aza-2′-deoxycytidine (Az) ref. 12]. Here, Aza acts on the $NH_2$-terminal nuclear localization signal and the bromo-adjacent homology domains of DNMT1, which are important for its nuclear localization and ubiquitin-mediated degradation. This effect is independent of DNA replication as well as DNMT enzyme activity (12).

Histone deacetylases (HDAC) and DNMT1 cooperatively initiate and sustain epigenetic gene silencing (13). *In vivo* studies have shown that DNMT1 associated with HDAC1 deacetylates chromatin and silences gene transcription (14, 15). However, HDAC actions are not restricted to modifications of histones, as some members of the HDAC family modulate acetylation status of nonhistone proteins, thereby regulating stability and subcellular localization (16-18). A prominent

example is the microtubule-associated deacetylase, HDAC6, which is localized largely in the cytoplasm and seems to be important in microtubule acetylation and chemotactic cell motility (19-21). HDAC6 has been characterized as a heat shock protein 90 (Hsp90) deacetylase as it dynamically regulates the ATP-dependent activity of the molecular chaperone Hsp90 protein (22). It has been shown that HDAC6 promotes Hsp90-assisted maturation, stability, and activity of client proteins, including dynein motors, glucocorticoid receptor, and breast cancer metastasis suppressor 1 (22-24). These proteins are critical for cell signaling pathways. Similarly, pharmacologic HDAC inhibitors induce hyperacetylation of Hsp90 and dissociate client proteins from the chaperone, leading to their degradation by the ubiquitin-dependent proteasomal pathway (25, 26). These studies highlight the fact that both general inhibition of HDACs and specific knockdown of HDAC6 can alter cytoplasmic-based processes (19, 25). Whether and how inhibition of HDACs regulates the stability of the nuclear protein DNMT1 in human breast cancer cells is poorly understood. Here, we present evidence, for the first time, that inhibition of HDACs is associated with interruption of the interaction between Hsp90 and DNMT1 and degradation of DNMT1 via the ubiquitin-proteasomal pathway. Our results show that multiple pathways are activated by HDAC inhibitors during epigenetic therapy.

## Results

### *The HDAC Inhibitor LBH589 Depletes DNMT1 Protein Expression without Altering DNMT1 mRNA Expression*

Our previous study showed that the HDAC inhibitor trichostatin A (TSA) down-regulated DNMT1 protein expression in human breast cancer cells (27). To understand the molecular mechanisms by which inhibition of HDACs reduces DNMT1 protein expression in human breast cancer cells, two cell lines, MDA-MB-231 and MDA-MB-435, were treated with 100 nmol/L LBH589, a clinically relevant HDAC inhibitor, for 12 to 48 h. Western blot analysis of whole-cell lysates showed that the DNMT1 protein level was decreased by ~50% after 24 h of LBH589 treatment and almost completely inhibited by 48 h (Fig. 1A). To address the question of whether reduction of DNMT1 by LBH589 results from down-regulation of DNMT1 mRNA, MDA-MB-231 cells were treated with LBH589 for up to 48 h. Reverse transcription-PCR showed that mRNA levels of DNMT1 were unaffected by LBH589 treatment (Fig. 1B). These results were confirmed by a quantitative real-time PCR assay (Fig. 1C). Thus, inhibition of DNMT1 protein by LBH589 is not due to decline in the steady-state mRNA level but may be through posttranscriptional modification.

### *Depletion of DNMT1 by LBH589 Results from Proteasomal Pathway-Mediated Degradation*

As DNMT1 localizes in the nucleus and is tightly associated with chromatin (3, 12), we determined whether the down-regulation of DNMT1 in cancer cells treated with HDAC inhibitors occurs in the nucleus. To address this issue, MDA-MB-435 cells were treated with 100 nmol/L LBH589 or 330 nmol/L TSA. As a positive control, cells were treated with Aza, an inducer of DNMT1 degradation (12). Nuclear extracts from these cells were subjected to Western blot analysis and Fig. 2A showed that a significant decrease in DNMT1 protein was observed after treatment of cells with Aza; both HDAC inhibitors LBH589 and TSA suppressed DNMT1 protein level to a lesser extent. DNMT1 level was virtually undetectable after the treatment sequence of Aza for 96 h with LBH589 for the last 24 h. These results support the notion that endogenous DNMT1 can be degraded within the nucleus in response to HDAC inhibitors.

To further ascertain whether LBH589 depletes DNMT through the proteasomal pathway within the nucleus, nuclear and cytoplasmic extracts from LBH589-treated MDA-MB-231 cells were subjected to Western blot analysis. DNMT1 protein was undetectable in the cytoplasmic fractions. A reduction of DNMT1 protein was observed in the nucleus after 24 h of LBH589 treatment but not after 6, 8, or 12 h (Fig. 2B; data not shown). LBH589-mediated inhibition of DNMT1 was blocked in the presence of the proteasomal inhibitor MG-132 (Fig. 2B) as well as a second proteasome inhibitor, lactacystin (data not shown). To confirm absence of contamination between nuclear and cytoplasmic extracts, blots were stripped and reprobed with anti-Ku-86 (a nuclear protein marker) or anti-actin (a cytoplasmic protein marker) antibody. Ku-86 expression was detected only in the nuclear fraction in conjunction with DNMT1 protein. There was no detectable actin protein in nuclear extracts, attesting to their purity. Neither DNMT1 nor any low-molecular weight degradation product was observed in the cytoplasmic fractions from LBH589-treated cells, suggesting that it is unlikely that DNMT1 is translocated to the cytoplasm before degradation in response to LBH589 treatment. To assess whether LBH589 treatment is linked to DNMT1 ubiquitination, nuclear extracts from LBH589-treated cells were immunoprecipitated with anti-DNMT1 antibody followed by immunoblotting with anti-polyubiquitin antibody. LBH589 treatment of MDA-MB-231 cells led to marked accumulation of polyubiquitinated DNMT1 in the nuclear extracts (Fig. 2C). Similar results were observed with treatment with a second HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA; 10 μmol/L). These data suggest that LBH589-related degradation of DNMT1 results from the activity of the ubiquitin-dependent proteasomal pathway.

### *The 120–Amino Acid $NH_2$-Terminal Domain of DNMT1 Is Essential for LBH589-Induced Ubiquitination*

The $NH_2$-terminal domain of DNMT1 regulates multiple functions, including protein stability, protein-protein interaction, and nuclear localization (3, 11, 12, 28). Our previous domain deletion studies showed that the proliferating cell nuclear antigen–binding and the nuclear localization signal domains have little effect on DNMT1 protein stability, whereas the 120–amino acid $NH_2$-terminal domain is involved in ubiquitination and degradation of DNMT1 in human breast cancer cells (11). To determine if this domain also plays a critical role in LBH589-induced DNMT1 ubiquitination, a FLAG-tagged full-length or 120–amino acid deletion DNMT1 construct (Fig. 3A) was cotransfected with hemagglutinin (HA)-tagged ubiquitin plasmid into MDA-MB-231 cells. Twenty-four hours after transfection, cells were treated with

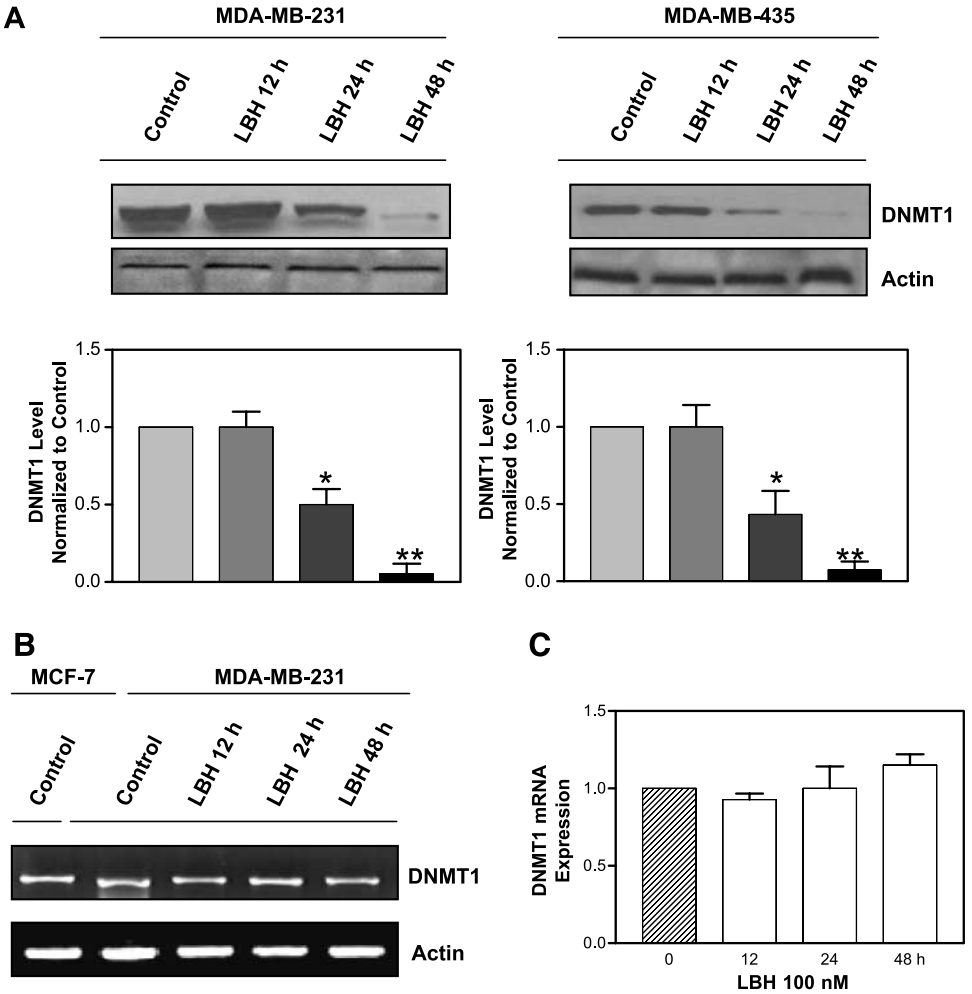


**FIGURE 1.** Inhibition of HDACs suppresses endogenous DNMT1 expression in human breast cancer cells. **A.** Inhibition of HDACs decreases protein levels of DNMT1. MDA-MB-231 and MDA-MB-435 cells were treated with 100 nmol/L LBH589 (*LBH*) for 12 to 48 h and protein levels of DNMT1 were determined by Western blot analyses using total cell lysates. After immunoblotting, the membranes were stripped and reprobed with β-actin antibody to assess the loading. The immunoblots were scanned and subjected to densitometric analysis by the Stratagene EagleSight software, and DNMT1 expression values were normalized to that obtained in the untreated cells (control). A representative Western blot is shown. Columns, mean densitometric quantification of the DNMT1 of three independent experiments; bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with untreated cells (control). **B** and **C.** Expression of DNMT1 mRNA is not altered by LBH589 treatment. MDA-MB-231 cells were treated with 100 nmol/L LBH589 for 24 h and mRNA levels of DNMT1 were determined by reverse transcription-PCR (**B**) or real-time PCR (**C**). Columns, mean real-time PCR results of four independent experiments showing no difference between treated and untreated cells; bars, SE.

100 or 200 nmol/L of LBH589 for an additional 24 h. Whole-cell lysates were immunoprecipitated with anti-FLAG antibody, and immunoprecipitated proteins were subjected to Western blot with anti-HA antibody to examine the ubiquitin modifications. As shown in Fig. 3B, LBH589 enhanced ubiquitination of the full-length DNMT1 compared with the deleted DNMT1 in a dose-dependent fashion. These data suggest that LBH589 directs ubiquitination of DNMT1 through its $NH_2$-terminal 120–amino acid domain.

### *Inhibition of HDACs Acetylates Hsp90 and Disrupts Hsp90 Association with DNMT1*

Reversible histone acetylation has been characteristically linked to chromatin-dependent gene expression. However, acetylation of nonhistone proteins apparently regulates a much broader range of biological processes and may be a critical step for deciphering LBH589-induced DNMT1 degradation. Several studies have shown that HDAC inhibitors can mediate protein degradation through acetylation of the Hsp90 chaperone protein in human cancer cells (29-32). It is possible, based on the above observations, that DNMT1 is also processed in a similar way. We thus asked if endogenous Hsp90 is associated with DNMT1 in MDA-MB-231 cells and if this association could be altered by HDAC inhibition. Cells were treated with LBH589 for 12 to 48 h, and nuclear extracts were immunoprecipitated with anti-Hsp90 antibody followed by Western blotting using anti-acetylated lysine or anti-DNMT1 antibody. Figure 4A shows that LBH589 increased the lysine acetylation of Hsp90 by 12 h, and DNMT1 was simultaneously dissociated from the Hsp90 complexes after 24 h of LBH589 treatment. Thus, hyperacetylation of Hsp90 by inhibition of HDACs is associated with disruption of the Hsp90-DNMT1 complexes. Induction of Hsp90 acetylation by LBH589 was also observed in two other human breast cancer cell lines studied, MCF-7 and T47D (Fig. 4B). This effect is associated with reduction of DNMT1 protein (Fig. 4C), consistent with the hypothesis that Hsp90 acetylation is correlated with reduction of DNMT1. These data indicate that induction of Hsp90 acetylation and disruption of Hsp90 association with DNMT1 may be a general characteristic in LBH589-treated human breast cancer cells.

To further confirm if disruption of Hsp90-DNMT1 complexes is associated with DNMT1 degradation via the proteasomal pathway, MDA-MB-231 cells were treated with LBH589, MG-132, or a combination of both for 24 h, and nuclear fractions were immunoprecipitated with an anti-Hsp90 antibody followed by immunoblotting using anti-Hsp90 or

anti-DNMT1 antibody. As shown in Fig. 4D, MG-132 alone did not alter association of DNMT1 with Hsp90 compared with vehicle treatment (control). Consistent with the results from Fig. 4A, LBH589 treatment caused a clear decrease in association of DNMT1 with Hsp90, and this effect can be blocked by cotreatment with MG-132. In parallel, Fig. 4E shows that, in MDA-MB-435 cells, SAHA-induced dissociation of DNMT1 from Hsp90 was apparent as early as 12 h, but greater effects were observed after 24 h. In the presence of a second proteasome inhibitor, lactacystin, the dissociation of DNMT1 from Hsp90 complexes was not altered after 12 h of SAHA treatment but was completely blocked in cells treated with SAHA for 24 h, suggesting that DNMT1 association with Hsp90 is rescued by lactacystin in a time-dependent manner. Thus, it seems that the stability of DNMT1 is maintained through an interaction with Hsp90 in two different cancer cell lines and treatment with a HDAC inhibitor interrupts this interaction.

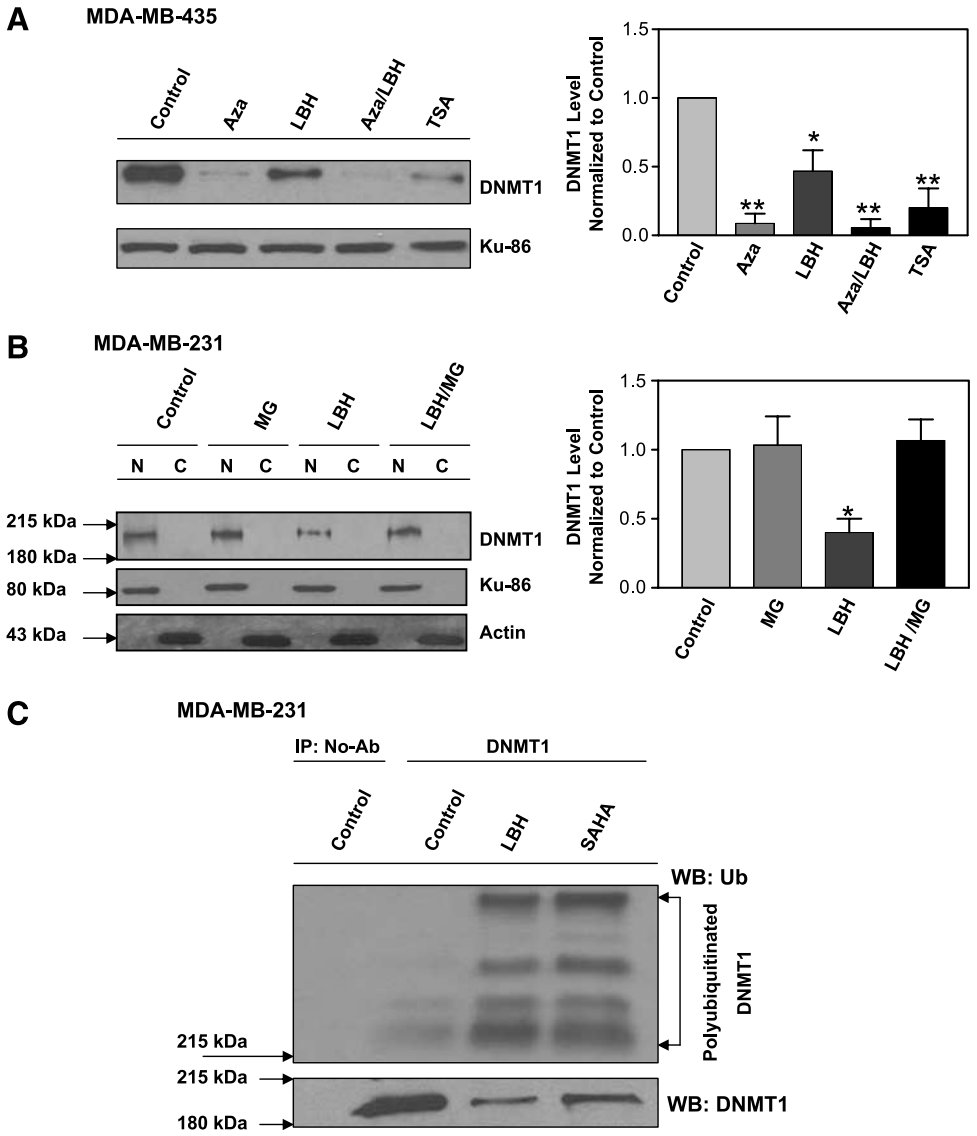


**FIGURE 2.** LBH589 induces DNMT1 degradation through the proteasomal pathway. **A.** Effects of inhibitors of DNMTs and HDACs on soluble DNMT1 expression in MDA-MB-435 cells. Cells were treated with 2.5 μmol/L Aza for 96 h or 100 nmol/L LBH589 for 24 h or the sequence of Aza for 96 h with LBH589 for the last 24 h or 330 nmol/L TSA for 48 h. Equal amounts of nuclear extracts (50 μg/lane) were subjected to Western blot analysis with anti-DNMT1 antibody. The blot was reprobed with anti-Ku-86 antibody as a protein loading control. A representative immunoblot is shown. Columns, mean densitometric quantification of the DNMT1 protein expression of three independent experiments; bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with the control cells. **B.** LBH589 induces proteasomal degradation of DNMT1 in the nucleus. MDA-MB-231 cells were treated with 100 nmol/L LBH589 or 10 μmol/L MG-132 (*MG*) for 24 h or the sequence of 10 μmol/L MG-132 for 6 h followed by addition of LBH589 for 24 h (*LBH/MG*). Cytoplasmic (*C*) and nuclear (*N*) extracts were subjected to Western blot with anti-DNMT1 antibody. The membrane was stripped and reprobed with Ku-86 and β-actin antibodies to show equal loading of proteins from nuclear and cytoplasmic fractions, respectively. A representative immunoblot is shown. Columns, mean densitometric quantification of the nuclear DNMT1 of four independent experiments; bars, SE. *, $P < 0.05$, compared with the control cells (nuclear extract). **C.** Inhibition of HDACs promotes ubiquitination of DNMT1. The nuclear proteins from MDA-MB-231 cells treated with 100 nmol/L LBH589 or 10 μmol/L SAHA for 24 h were immunoprecipitated by anti-DNMT1 antibody, and the immunocomplexes were analyzed by Western blot with anti-ubiquitin or anti-DNMT1 antibody. IP, immunoprecipitation; WB, Western blot. A representative Western blot from three independent experiments giving similar results is shown.

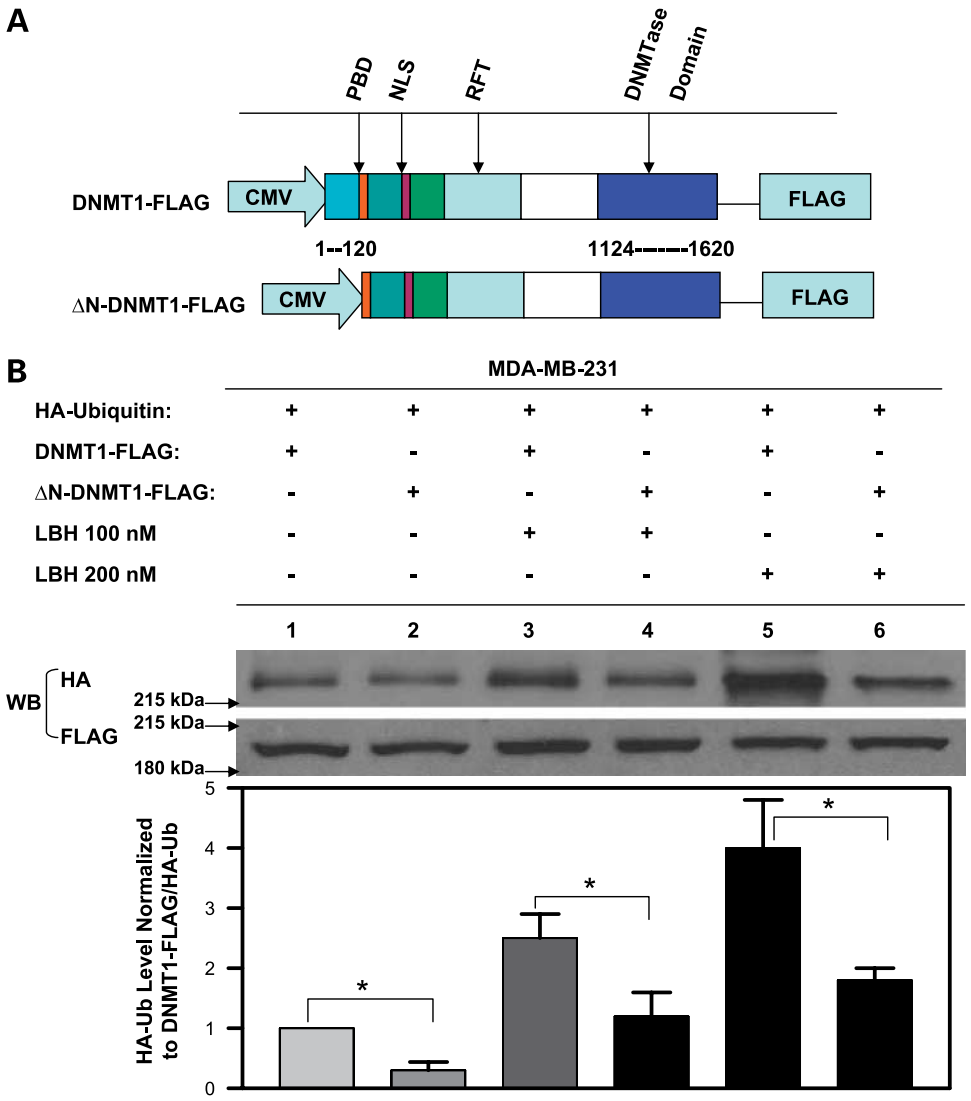


**FIGURE 3.** The 120–amino acid $NH_2$-terminal domain is critical for LBH589-induced ubiquitination of DNMT1. **A.** Schematic representation of FLAG-tagged DNMT1 full-length and the 120–amino acid $NH_2$-terminal deletion constructs. PBD, proliferating cell nuclear antigen–binding domain; NLS, nuclear localization signal; RFT, replication foci-targeting domain. **B.** The full-length (*DNMT1-FLAG*) or $NH_2$-terminal deletion (*ΔN-DNMT1-FLAG*) construct was cotransfected with HA-tagged ubiquitin plasmid into MDA-MB-231 cells. Twenty-four hours later, cells were treated with or without LBH589 for additional 24 h. Whole-cell lysates were immunoprecipitated with anti-FLAG antibody, and the precipitates were immunoblotted with anti-HA or anti-FLAG antibody. A representative Western blot from three independent experiments is shown. Lane 1, Western blot results were normalized against the densitometric reading for the DNMT1-FLAG cotransfected with HA-ubiquitin. Columns, mean densitometric quantification of the DNMT1 of three independent experiments; bars, SE. *, $P < 0.05$.

*HDAC1 Regulates Hsp90 Acetylation and Alters DNMT1 Protein Expression*

Because *in vitro* studies show that inhibition of HDACs acetylates Hsp90, we speculated that a specific member of the HDAC family may target Hsp90. Because HDAC1 has the ability to physically bind DNMT1 in the nucleus (14) and HDAC6 has been shown to deacetylate Hsp90 in cytoplasmic-based processes (19), we tested whether knockdown of HDAC1 or HDAC6 could alter Hsp90 acetylation. MDA-MB-231 cells were transfected with either short interfering RNA (siRNA) specific for HDAC1 or a scrambled siRNA as a negative control for 24 h. Quantification of HDAC1 mRNA by real-time PCR confirmed that HDAC1 siRNA depleted HDAC1 mRNA expression compared with scrambled siRNA treatment (data not shown). Consistent with this, HDAC1 protein was markedly reduced by HDAC1 siRNA (Fig. 5A) in whole-cell lysates subjected to immunoblotting using anti-HDAC1 antibody. In addition, knockdown of HDAC1 by siRNA resulted in down-regulation of DNMT1 protein expression by immunoblotting. In parallel studies with HDAC6 siRNA, the expected knockdown of HDAC6 protein was noted but HDAC6 siRNA failed to alter DNMT1 protein expression. These findings imply that HDAC1 but not HDAC6 plays a role in stability of DNMT1 protein.

We next determined if HDAC1 or HDAC6 modulates acetylation status of Hsp90. Nuclear fractions prepared from scrambled and siRNA knockdown MDA-MB-231 or MDA-MB-435 cells were immunoprecipitated with anti-Hsp90 antibody followed by immunoblotting using anti-acetylated lysine or anti-Hsp90 antibody (Fig. 5B). Hyperacetylation of endogenous Hsp90 was observed in HDAC1 siRNA-treated cells compared with scrambled control cells, indicating that HDAC1 can deacetylate Hsp90. HDAC6 siRNA was unable to modify Hsp90 in the nucleus of MDA-MB-231 cells (Fig. 5B).

To further test the role of HDAC1 or HDAC6 in Hsp90 acetylation, MDA-MB-231 cells were treated with the selective HDAC1 inhibitor MS-275 (33) or the selective HDAC6 inhibitor MS-344 (34) for 24 h (Fig. 5C). Immunoprecipitation of Hsp90 from the nuclear and cytoplasmic fractions followed by Western blotting analysis of lysine acetylation revealed that

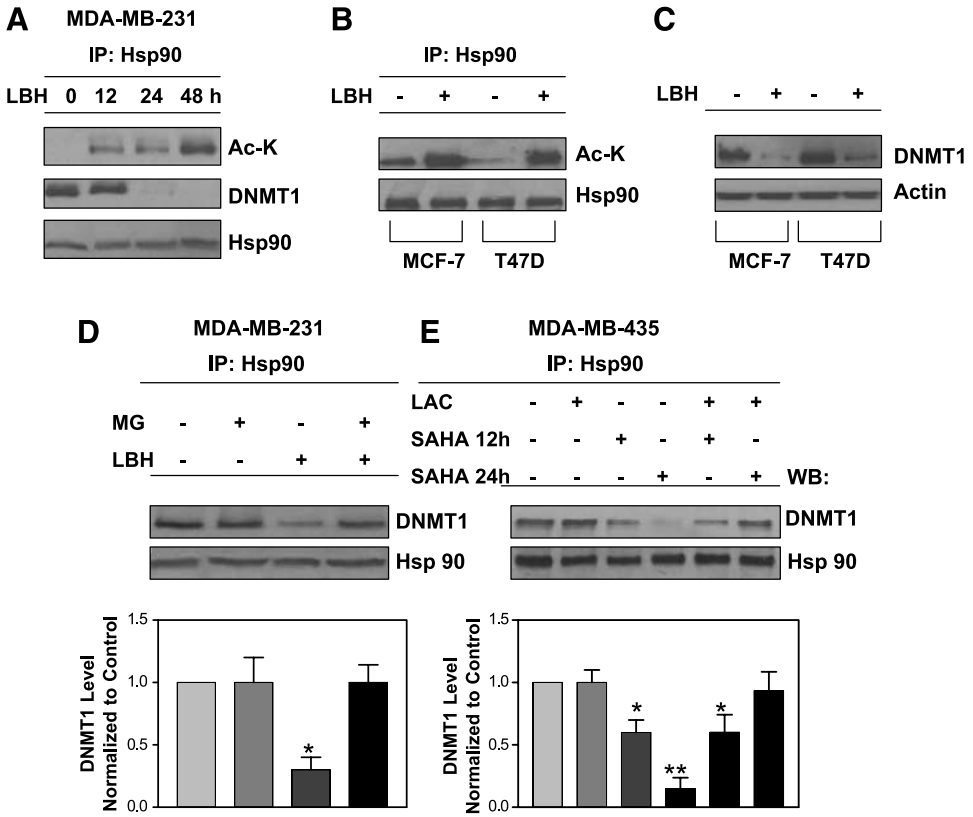


**FIGURE 4.** Inhibition of HDACs disrupts DNMT1 association with Hsp90. **A** to **C.** LBH589 increases acetylated lysine residues on Hsp90 and abolishes Hsp90 association with DNMT1. **A.** MDA-MB-231 cells were treated with 100 nmol/L LBH589 for 12 to 48 h. **B** and **C.** MCF-7 and T47D cells were treated with 100 nmol/L LBH589 for 24 h. **A** and **B.** Nuclear extracts were prepared and immunoprecipitated by anti-Hsp90 antibody, and immunocomplexes were analyzed by Western blot with anti-acetylated lysine (*Ac-K*), anti-DNMT1, or anti-Hsp90 antibody. **C.** DNMT1 protein levels from nuclear extracts were determined by Western blot analysis. A representative Western blot from three independent experiments is shown. **D** and **E.** Disruption of DNMT1 association with Hsp90 after HDAC inhibitor treatment can be rescued by proteasome inhibitors. **D.** MDA-MB-231 cells were treated with 10 μmol/L MG-132 or 100 nmol/L LBH589 for 24 h or the sequence of MG-132 for 6 h followed by exposure to LBH589 for 24 h. **E.** MDA-MB-435 cells were treated with 3 μmol/L lactacystin (*LAC*) for 24 h or SAHA for 12 or 24 h or the sequence of lactacystin for 6 h followed by exposure to SAHA for 12 or 24 h. Hsp90 was immunoprecipitated from nuclear extracts, and the blot was probed for Hsp90 and DNMT1. A representative Western blot is shown. Columns, mean densitometric quantification of the DNMT1 protein expression of three independent experiments; bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with the untreated cells.

MS-275 induces Hsp90 acetylation in the nucleus but not in the cytoplasm. In contrast, MS-344 treatment preferentially caused an accumulation of acetylated Hsp90 in the cytoplasm. To determine whether MS-275 treatment would induce DNMT1 degradation by the proteasome pathway within the nucleus, nuclear extracts from LBH589-treated MDA-MB-231 cells were subjected to Western blot analysis. A reduction of DNMT1 protein was observed in the nucleus after 24 h of MS-275 treatment, and cotreatment with the proteasome inhibitor MG-132 restored protein levels of DNMT1 in MS-275–treated cells. However, we did not observe any alteration in DNMT1 protein expression after 24 h of treatment with MS-344 or MG-132 alone or a combination of both, consistent with the fact that HDAC6 is a cytoplasmic deacetylase (19, 35). In summary, these results suggest that the specific inhibition of HDAC1 could result in proteasome-mediated degradation of DNMT1 in the nucleus.

### *Disruption of Hsp90 Leads to DNMT1 Ubiquitination*

Because knockdown of HDAC1 siRNA enhances detection of acetylated Hsp90 in the nucleus, the relationship between Hsp90 and ubiquitinated DNMT1 was examined. MDA-MB-231 cells were treated with siRNA HDAC1, and the nuclear fraction was subjected to precipitation with anti-DNMT1 antibody followed by Western blot analysis using an anti-polyubiquitination antibody (Fig. 6A). Polyubiquitinated DNMT1 was detected in the HDAC1 siRNA-treated cells, but no signal was obtained when the cells were treated with a scrambled siRNA control. The effects of Hsp90 on DNMT1 ubiquitination were assessed after transfection of Hsp90 expression vector into HDAC1 siRNA-treated MDA-MB-231 cells. Overexpression of Hsp90 by transient transfection was determined by immunoblotting (data not shown). Levels of ubiquitinated DNMT1 in HDAC1 siRNA-treated cells were diminished after transfection with increasing amounts of Hsp90 expression vector. In contrast, transfection with Hsp90 expression vector alone had no effect on DNMT1 ubiquitination. These results suggest that the observed DNMT1 ubiquitination was mediated by an interaction with functional Hsp90.

We next examined whether DNMT1 directly interacts with Hsp90. To verify this, green fluorescent protein (GFP)-Hsp90

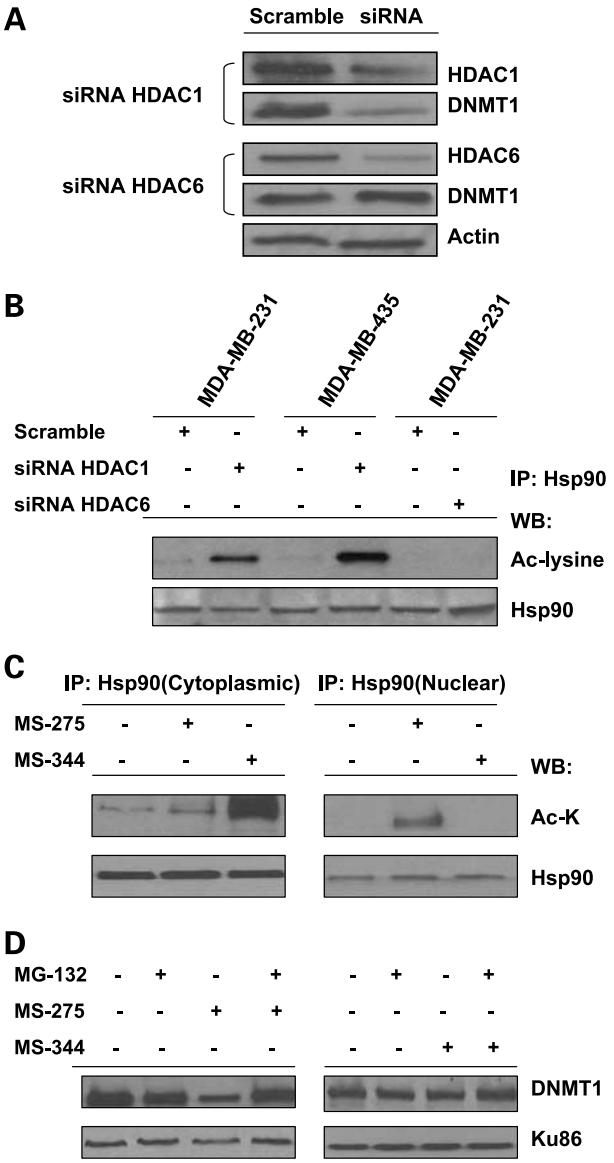


**FIGURE 5.** Knockdown of HDAC1 but not HDAC6 by siRNA causes hyperacetylation of Hsp90 and down-regulation of DNMT1 protein. **A.** HDAC1 siRNA reduced DNMT1 protein. HDAC1 siRNA, HDAC6 siRNA, or relevant scrambled siRNA controls were transiently transfected into MDA-MB-231 cells. Western blot analyses for HDAC1, HDAC6, DNMT1, and β-actin were done using whole-cell lysates after 24 h of transfection. A representative immunoblot from three independent experiments is shown. **B.** Knockdown of HDAC1 by siRNA induces hyperacetylation of Hsp90. Nuclear fractions were prepared after 24 h of siRNA transfection and then immunoprecipitated with anti-Hsp90 antibody followed by immunoblotting using anti-acetylated lysine or anti-Hsp90 antibody. A representative Western blot from three independent experiments that gave similar results is shown. **C.** Inhibition of HDAC1 by MS-275 enhances Hsp90 acetylation in MDA-MB-231 cells. Cytoplasmic and nuclear fractions were prepared after 24 h of 300 nmol/L MS-275 or 100 nmol/L MS-344 and then immunoprecipitated with anti-Hsp90 antibody followed by immunoblotting using anti-acetylated lysine or anti-Hsp90 antibody. A representative Western blot from two independent experiments is shown. **D.** The selective HDAC1 inhibitor MS-275 but not the HDAC6 inhibitor MS-344 promotes proteasome-mediated degradation of DNMT1 in MDA-MB-231 cells. Nuclear fractions were prepared after 24 h of 10 μmol/L MG-132, 300 nmol/L MS-275, 100 nmol/L MS-344, or combination of MG-132 with MS-275 or MS-344. DNMT1 protein was measured by immunoblotting. A representative Western blot from two independent experiments is shown.

was cotransfected with Myc-DNMT1 into MDA-MB-231 cells for 24 h, and the cells were treated with LBH589, MG-132, lactacystin, or the Hsp90 inhibitor geldanamycin for an additional 24 h (Fig. 6B). Whole-cell lysates were immunoprecipitated with anti-GFP antibody followed by immunoblotting using anti-GFP or anti-Myc antibody. Hsp90 specifically interacted with DNMT1 because the precipitated complexes (Hsp90-DNMT1) were not detected when either DNMT1 or Hsp90 was transfected alone (data not shown). This interaction is unaffected by MG-132 or lactacystin alone, but LBH589 or the Hsp90 inhibitor geldanamycin could disrupt

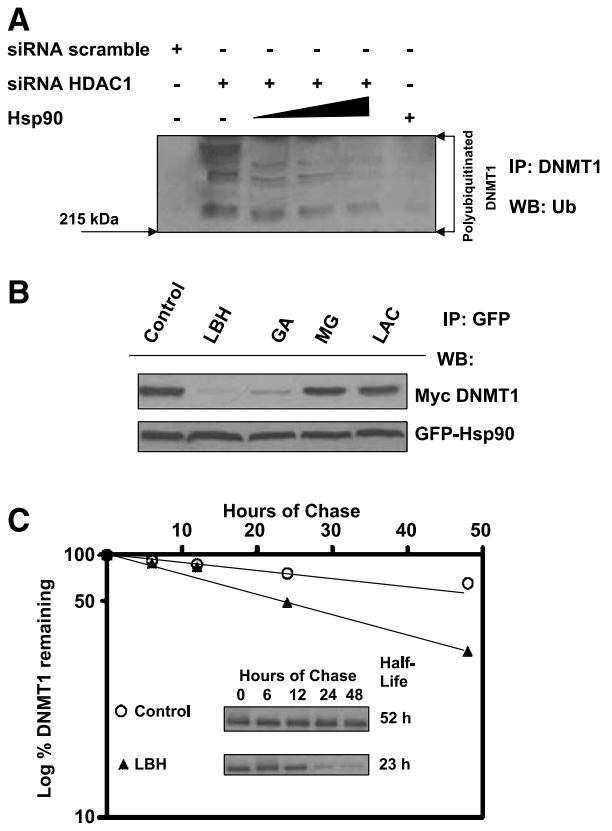


**FIGURE 6.** Inhibition of HDACs mediates DNMT1 protein stability. **A.** Functional Hsp90 mediates ubiquitination of DNMT1 in MDA-MB-231 cells. Nuclear fractions were prepared after 24 h of transfection of siRNA HDAC1 or Hsp90 expression vector (3 μg) alone and cotransfection of siRNA HDAC1 with increasing amounts of Hsp90 expression vector (1, 2, and 3 μg). Immunoprecipitation was done with anti-DNMT1 antibody followed by immunoblotting using anti-polyubiquitination antibody. A representative Western blot from three independent experiments is shown. **B.** Inhibition of HDACs blocks the interaction of DNMT1 with Hsp90. MDA-MB-231 cells were transiently cotransfected with indicated constructs and then treated with 100 nmol/L LBH589, 10 μmol/L MG-132, 3 μmol/L lactacystin, or 10 μmol/L geldanamycin (*GA*) for 24 h. Whole-cell lysates were immunoprecipitated with anti-GFP antibody followed by Western blot analysis using anti-Myc or anti-GFP antibody. A representative Western blot from three independent experiments is shown. **C.** Effect of LBH589 on half-life of DNMT1. MDA-MB-231 cells were metabolically radiolabeled with 100 μCi of [$^{35}$S]methionine for 8 h, washed thrice, and then incubated with unlabeled growth medium with or without 100 nmol/L LBH589 for the times indicated. Remaining incorporated radiolabel was determined by immunoprecipitation of DNMT1, SDS-PAGE, autoradiography, and quantification. Data represent the average of two independent experiments that gave similar results.

the Hsp90-DNMT1 complex. Together, results in Fig. 6A and B indicate that Hsp90 directly interacts with DNMT1 and this interaction helps to maintain DNMT1 stability.

### *Inhibition of HDAC by LBH589 Reduces DNMT1 Protein Half-life*

To determine whether the effects of LBH589 on DNMT1 protein levels reflect the rate of DNMT1 turnover, we used a pulse-chase analysis to study DNMT1 decay in MDA-MB-231 cells. After incubation of cells with growth medium containing [$^{35}$S]methionine, cells were washed and incubated for various times with unlabeled growth medium with or without 100 nmol/L LBH589. DNMT1 protein was immunoprecipitated, and the remaining incorporated radioactivity was used as a measure of protein stability as described in our previous studies (11). Consistent with our previous observation in MCF-7 cells (11), DNMT1 is very stable in untreated MDA-MB-231 cells, displaying a half-life of ~52 h (Fig. 6C). In contrast, DNMT1 from LBH589-treated cells is less stable than that of untreated cells, exhibiting a half-life of ~23 h. These data support the hypothesis that HDAC activity plays a critical role in DNMT1 stability in human breast cancer cells.

## Discussion

The understanding that epigenetic modifications in gene expression play a substantive role in malignant progression and that these modifications are potentially reversible has led to the development of epigenetically targeted therapies. Indeed, two DNMT inhibitors, 5-azacitidine and decitabine, as well as a HDAC inhibitor, vorinostat or SAHA, have entered the clinic for treatment of myelodysplastic syndrome and cutaneous T-cell lymphoma, respectively. LBH589 is a novel hydroxamic acid analogue HDAC inhibitor currently in early clinical trials (36, 37). *In vitro* studies show that this drug induces cell cycle arrest and apoptosis through both caspase-dependent and caspase-independent pathways in various tumor cell types at low nanomolar concentrations (38, 39). *In vivo* LBH589 inhibits tumor angiogenesis as evidenced by blocking new blood vessel formation in human prostate carcinoma cell PC-3 xenografts (40). Clinical data showed that LBH589 has shown promising antitumor activity with tolerable side effects (36, 37). These clinical developments have sharpened the focus in the laboratory on mechanisms of action of these inhibitors.

A key protein in these processes seems to be the maintenance DNMT1. Our recent work in human breast cell models showed that aberrant DNMT1 protein expression in MCF-7 cells stems from a dysfunctional $NH_2$-terminal regulatory domain that is essential for normal DNMT1 ubiquitination and degradation (11). Further, a role for this conserved $NH_2$-terminal regulatory domain of DNMT1 has been identified in DNMT inhibitor-mediated DNMT1 degradation (12). Finally, our previous work also documented the unexpected finding that the HDAC inhibitor TSA inhibited DNMT1 protein levels in MDA-MB-231 human breast cancer cells. These observations prompted us to explore whether inhibition of HDACs also targets DNMT1 degradation by the proteasomal pathway. Human breast cancer cell lines, including MDA-MB-231, MDA-MB-435, MCF-7, and T47D, were used to test this hypothesis. In this study, we observed that inhibition of HDACs promotes ubiquitin-proteasome–dependent degradation of DNMT1 in the nucleus.

Although the studies have shown that DNMT1 mRNA levels are S phase regulated throughout the cell cycle in human breast cancer cells (41), our results using a clinically relevant HDAC inhibitor, LBH589, show that it is unable to alter DNMT1 mRNA expression. We observed that LBH589 treatment promotes DNMT1 degradation in the nucleus through the ubiquitin-dependent proteasome pathway. This conclusion is supported by two important findings. First, immunoprecipitation of LBH589-treated cell lysates with anti-DNMT1 antibody followed by immunoblotting with anti-ubiquitin antibody reveals a polyubiquitination ladder in LBH589-treated but not control-treated cells; similar results are seen with a second clinically relevant HDAC inhibitor, vorinostat (SAHA). Second, MG-132, the 26S proteasome inhibitor (as well as a second proteasome inhibitor, lactacystin), blocks LBH589-induced DNMT1 degradation. The DNMT1 degradation occurs predominantly in the nucleus and does not seem to involve cytoplasmic translocation before degradation. To investigate the role of the $NH_2$-terminal regulatory domain for ubiquitination of DNMT1 in HDAC inhibitor-treated cells, FLAG-tagged full-length or deleted 120–amino acid $NH_2$-terminal DNMT1 expression vector was cotransfected with HA-tagged ubiquitin vector into MDA-MB-231 cells. These studies show that deletion of this $NH_2$-terminal domain inhibits LBH589-induced DNMT1 ubiquitination, indicating that this domain is necessary for LBH589-activated proteasomal degradation of DNMT1.

Although a major target for HDAC inhibitors has been believed to be alteration in transcription because of modulation of histone acetylation, it has become clear that there are multiple nonhistone effects as well. For example, recent evidence has been presented that inhibition of HDACs by pharmacologic HDAC inhibitors or specific siRNA acetylates lysine residues on Hsp90, which abolishes its ATP-dependent chaperone function with consequent accelerated degradation of client proteins in human cancer cells (29, 32). We therefore examined the possible involvement of Hsp90 in HDAC inhibitor-induced DNMT1 degradation. Coimmunoprecipitation analyses showed that the HDAC inhibitor LBH589 acetylates Hsp90, disrupts an association of DNMT1 with Hsp90, and directs polyubiquitination of DNMT1. We also found that LBH589 reduces DNMT1 protein half-life by pulse-chase assay. Collectively, these results support the notion that HDACs mediate the proteasomal pathway through the molecular chaperone Hsp90.

Although HDAC6, a microtubule-associated deacetylase, contains an ubiquitin binding zinc finger and associates with ubiquitinated proteins in the cytoplasm (42), selective inhibition of HDAC6 genetically by siRNA or pharmacologically by MS-344 did not alter acetylation status of Hsp90 in the nucleus or DNMT1 protein level. This is perhaps not surprising given the exclusively nuclear localization of DNMT1 and our findings that its degradation occurs without cytoplasmic translocation. Thus, HDAC6 is unlikely to play a role in

mediating LBH589 effects on DNMT1 in the MDA-MB-231 cell culture model studied here. In contrast, the accumulation of acetylated Hsp90 by the HDAC1-selective inhibitor MS-275 or HDAC1 siRNA implies that HDAC1 could function as a Hsp90 deacetylase. Inactivation of HDAC1 by pharmacologic or siRNA approaches results in hyperacetylation of Hsp90 in conjunction with ubiquitination of DNMT1, suggesting that the capacity of HDAC1 to associate with DNMT1 and Hsp90 contributes to maintenance of stability of DNMT1 protein. Further studies are necessary to elucidate precisely how acetylation of Hsp90 by HDAC1 regulates the process of DNMT1 ubiquitination.

In summary, our studies using pharmacologic HDAC inhibitors and siRNA knockdown suggest that inhibition of HDACs promotes ubiquitin-dependent degradation of DNMT1. The most likely mechanism seems to be that inhibition of HDACs, particularly HDAC1, acetylates Hsp90 and prevents the formation of a Hsp90 chaperone-DNMT1 complex, thus promoting DNMT1 ubiquitination. Because HDAC1 seems to play a role in regulation of chromatin modifications and the stability of DNMT1, specific HDAC1 inhibitors may be promising candidates for therapeutic applications. Finally, these results underscore the multiple mechanisms through which HDAC inhibitors can alter transcription and cell growth.

## Materials and Methods

### *Reagents and Antibodies*

LBH589 was provided by Novartis Pharmaceuticals, Inc. MG-132, Aza, MS-275, MS-344, and TSA were obtained from Sigma-Aldrich, and vorinostat (SAHA) was purchased from BioVision. Geldanamycin and lactacystin were obtained from Calbiochem. The generation of anti-DNMT1 antibody has been described (11). Polyclonal anti-ubiquitin antibody was from Sigma-Aldrich. Antibodies against HDAC1 and HDAC6 were obtained from Upstate Biotechnology. For tags, anti-HA 12CA5 (Roche), anti-FLAG M2 (Sigma-Aldrich), anti-GFP (Santa Cruz Biotechnology), and anti-Myc 9B11 mouse antibodies (Cell Signaling Technology) were used. Mouse anti-Hsp90 monoclonal antibody was purchased from Stressgen Biotechnology.

### *Plasmids, siRNA, and Transfections*

The HA-tagged ubiquitin, FLAG-tagged DNMT1 full-length, and 120–amino acid $NH_2$-terminal deletion constructs were generated as described by Agoston et al. (11). The Myc-tagged full-length DNMT1(Myc-DNMT1) was a gift from Dr. Tony Kouzarides (Wellcome/CRC Institute and Department of Pathology, Cambridge University, Cambridge, United Kingdom). The GFP-Hsp90 construct was provided by Dr. Joon Kim (Department of Biology, Mokpo National University, Mokpo, South Korea). siRNA was designed and constructed using the Silencer siRNA Construction kit (Ambion, Inc.) according to the manufacturer's recommendations. The sequences for the DNA oligonucleotides were as follows: HDAC1, AGATGTTCCAGCCTACTGCTT (sense) and AAGCAGTAGGCTGGAACATCT (antisense). siRNA for HDAC6 and a scrambled control were obtained from Santa Cruz Biotechnology.

MDA-MB-231, MDA-MB-435, MCF-7, and T47D cells were cultured in DMEM supplemented with 5% fetal bovine serum (Gemini Bio-Products) and 2 μmol/L L-glutamine (Invitrogen) at 37°C in a humidified $CO_2$ incubator. For transient transfection, MDA-MB-231 cells were plated at a density of $1 \times 10^6$ per 10-cm culture dish ~24 h before transfection. A final concentration of 20 nmol/L siRNA or 10 μg plasmids as indicated was transfected into the cells with Oligofectamine 2000 (Invitrogen). After 24 h of incubation, cells were then treated with or without the designated drugs.

### *RNA Isolation, Reverse Transcription-PCR, and Real-time PCR*

RNA was harvested from cells using the Trizol reagent (Invitrogen). cDNA was synthesized from 3 μg total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo(dT) primers (Invitrogen) at 37°C for 1 h (7). Conventional PCR was done in cDNA samples using the following primers: DNMT1, 5′-CATAAATGAATGGTGGATCACTGGCTTTGA-3′ (forward) and 5′-GCAGGCTTTACATTTCCCACACTCAGG-3′ (reverse); actin, 5′-ACCATGGATGATGATATCGC-3′ (forward) and 5′-ACATGGCTGGGGTGTTGAAG-3′ (reverse). PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Real-time PCR was done in cDNA samples using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). The data were normalized by expression of the *GAPDH* housekeeping gene (7).

### *Preparation of Whole-Cell Lysates and Cytoplasmic and Nuclear Extracts and Western Blotting*

To prepare whole-cell lysates, cells were rinsed twice with cold 1× PBS and lysed in 1% SDS cell lysis buffer [1% SDS, 10 mmol/L Tris-HCl (pH 7.4)] with freshly added 1× complete protease inhibitor tablets (Roche). Whole-cell lysates were centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 10 min) at 4°C, and then the supernatants were collected. Nuclear and cytoplasmic cell extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce) following the manufacturer's instruction. Equal amounts of proteins were denatured in 4× Laemmli's sample buffer and separated on 10% polyacrylamide gels (GeneMate, ISC Biotechnology). Separated proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 5% milk in TBS-Tween 20 overnight at 4°C with constant shaking and then probed with appropriate primary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham). The expression of β-actin was used as a control. Bands were analyzed by densitometric scan (Stratagene EagleSight).

### *Immunoprecipitation and Pulse-Chase Analysis*

Cell lysates were precleared to block nonspecific binding using 40 μL packed agarose A/G beads (Upstate Biotechnology) for 30 min with rotation. Primary antibody was added to precleared supernatants, and proteins were immunoprecipitated overnight at 4°C. Protein samples were then washed

extensively, denatured in 4× Laemmli's sample buffer, separated on a 10% polyacrylamide gel, and subjected to immunoblotting as described above.

The half-life of DNMT1 protein was measured by pulse-chase analysis. Newly translated proteins were metabolically radiolabeled with [$^{35}$S]methionine, and DNMT1 was immunoprecipitated using the anti-DNMT1 antibody. Immunoprecipitated DNMT1 was resolved by SDS-PAGE, and the radioactivity associated with DNMT1 was quantified by PhosphorImager analysis (11).

### *Statistical Analysis*

Data are presented as mean ± SE. One-way ANOVA followed by Bonferroni's *t* test was used to assess statistically significant differences between two groups. Statistical analysis was done using GraphPad Prism for Windows version 4.00 (GraphPad Software). $P < 0.05$ was considered statistically significant.

## Acknowledgments

We thank Dr. Saraswati Sukumar (The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University) for helpful discussion.

## References

1. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction? Nat Rev Cancer 2006;6:107–16.

2. Pradhan S, Bacolla A, Wells RD, Roberts RJ. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of *de novo* and maintenance methylation. J Biol Chem 1999;274:33002–10.

3. Bestor TH. The DNA methyltransferases of mammals. Hum Mol Genet 2000;9:2395–402.

4. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res 1994;54:2552–5.

5. Lapidus R, Ferguson AT, Ottaviano Y, et al. Methylation of estrogen and progesterone receptor 5′ CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res 1996;2:805–10.

6. Sharma D, Blum J, Yang X, Beaulieu N, Macleod AR, Davidson NE. Release of methyl CpG binding proteins and histone deacetylase 1 from the estrogen receptor α (ER) promoter upon reactivation in ER-negative human breast cancer cells. Mol Endocrinol 2005;19:1740–51.

7. Zhou Q, Atadja P, Davidson NE. Histone deacetylase inhibitor LBH589 reactivates silenced estrogen receptor α (ER) gene expression without loss of DNA hypermethylation. Cancer Biol Ther 2007;6:64–9.

8. Ferguson AT, Vertino PM, Spitzner JR, Baylin SB, Muller MT, Davidson NE. Role of estrogen receptor gene demethylation and DNA methyltransferase. DNA adduct formation in 5-aza-2′deoxycytidine-induced cytotoxicity in human breast cancer cells. J Biol Chem 1997;272:32260–6.

9. Yan L, Nass SJ, Smith D, Nelson WG, Herman JG, Davidson NE. Specific inhibition of DNMT1 by antisense oligonucleotides induces re-expression of estrogen receptor-α (ER) in ER-negative human breast cancer cell lines. Cancer Biol Ther 2003;2:552–6.

10. Sharma D, Saxena NK, Davidson NE, Vertino PM. Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes. Cancer Res 2006;66:6370–8.

11. Agoston AT, Argani P, Yegnasubramanian S, et al. Increased protein stability causes DNA methyltransferase 1 dysregulation in breast cancer. J Biol Chem 2005;280:18302–10.

12. Ghoshal K, Datta J, Majumder S, et al. 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. Mol Cell Biol 2005;25:4727–41.

13. Bird A. Molecular biology. Methylation talk between histones and DNA. Science 2001;294:2113–5.

14. Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat Genet 2000;24:88–91.

15. Fuks F. DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev 2005;15:490–538.

16. Yang XJ, Gregoire S. Class II histone deacetylases: from sequence to function, regulation, and clinical implication. Mol Cell Biol 2005;25:2873–84.

17. Kong X, Lin Z, Liang D, Fath D, Sang N, Caro J. Histone deacetylase inhibitors induce VHL and ubiquitin-independent proteasomal degradation of hypoxia-inducible factor 1α. Mol Cell Biol 2006;26:2019–28.

18. Lee H, Sengupta N, Villagra A, Rezai-Zadeh N, Seto E. Histone deacetylase 8 safeguards the human ever-shorter telomeres 1B (hEST1B) protein from ubiquitin-mediated degradation. Mol Cell Biol 2006;26:5259–69.

19. Hubbert C, Guardiola A, Shao R, et al. HDAC6 is a microtubule-associated deacetylase. Nature 2002;417:455–8.

20. Matsuyama A, Shimazu T, Sumida Y, et al. *In vivo* destabilization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J 2002;21:6820–31.

21. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A 2003;100:4389–94.

22. Kovacs JJ, Murphy PJ, Gaillard S, et al. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Mol Cell 2005;18:601–7.

23. Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell 2003;115:727–38.

24. Hurst DR, Mehta A, Moore BP, et al. Breast cancer metastasis suppressor 1 (BRMS1) is stabilized by the Hsp90 chaperone. Biochem Biophys Res Commun 2006;348:1429–35.

25. Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) 2003;228:111–33.

26. Pearl LH, Prodromou C, Workman P. The Hsp90 molecular chaperone: an open and shut case for treatment. Biochem J 2008;410:439–53.

27. Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, Davidson NE. Synergistic activation of functional estrogen receptor (ER)-α by DNA methyltransferase and histone deacetylase inhibition in human ER-α-negative breast cancer cells. Cancer Res 2001;61:7025–9.

28. Araujo FD, Croteau S, Slack AD, et al. The DNMT1 target recognition domain resides in the N terminus. J Biol Chem 2001;276:6930–6.

29. Nimmanapalli R, Fuino L, Bali P, et al. Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells. Cancer Res 2003;63:5126–35.

30. Castro JE, Prada CE, Loria O, et al. ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia. Blood 2005;106:2506–12.

31. George P, Bali P, Annavarapu S, et al. ZAP-70 is a novel conditional heat shock protein 90 (Hsp90)-client protein: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis and impaired signaling in chronic lymphocytic leukemia. Blood 2005;105:1768–76.

32. Murphy PJ, Morishima Y, Kovacs JJ, Yao TP, Pratt WB. Regulation of the dynamics of hsp90 action on the glucocorticoid receptor by acetylation/deacetylation of the chaperone. J Biol Chem 2005;280:33792–9.

33. Hu E, Dul E, Sung CM, et al. Identification of novel isoform-selective inhibitors within class I histone deacetylases. J Pharmacol Exp Ther 2006;307:720–8.

34. Heltweg B, Dequiedt F, Marshall BL, et al. Subtype selective substrates for histone deacetylases. J Med Chem 2004;47:5235–43.

35. Verdel A, Curtet S, Brocard MP, et al. Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm. Curr Biol 2000;10:747–9.

36. Giles F, Fischer T, Cortes J, et al. A phase I study of intravenous LBH589, a

novel cinnamic hydroxamic acid analogue histone deacetylase inhibitor, in patients with refractory hematologic malignancies. Clin Cancer Res 2006;12:4628–35.

37. Prince HM, George DJ, Johnstone R, et al. LBH589, a novel histone deacetylase inhibitor (HDAC), treatment of patients with cutaneous T-cell lymphoma (CTCL). Changes in skin gene expression profiles related clinical response following therapy [abstract 7501]. J Clin Oncol 2006;24:18–422s.

38. Maiso P, Carvajal-Vergara X, Ocio EM, et al. The histone deacetylase inhibitor LBH589 is a potent antimyeloma agent that overcomes drug resistance. Cancer Res 2006;66:5781–9.

39. George P, Bali P, Annavarapu S, et al. Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and AML cells with activating mutation of FLT-3. Blood 2005;105:1768–76.

40. Qian DZ, Kato Y, Shabbeer S, et al. Targeting tumor angiogenesis with histone deacetylase inhibitors: the hydroxamic acid derivative LBH589. Clin Cancer Res 2006;12:634–42.

41. Robertson KD, Keyomarsi K, Gonzales FA, Velicescu M, Jones PA. Differential mRNA expression of the human DNA methyltransferases (DNMTs) 1, 3a and 3b during the G(0)/G(1) to S phase transition in normal and tumor cells. Nucleic Acids Res 2000;28:2108–13.

42. Hook SS, Orian A, Cowley SM, Eisenman RN. Histone deacetylase 6 binds polyubiquitin through its zinc finger (PAZ domain) and copurifies with deubiquitinating enzymes. Proc Natl Acad Sci U S A 2002;99:13425–30.

Molecular Cancer Research

# Inhibition of Histone Deacetylases Promotes Ubiquitin-Dependent Proteasomal Degradation of DNA Methyltransferase 1 in Human Breast Cancer Cells

Qun Zhou, Agoston T. Agoston, Peter Atadja, et al.

*Mol Cancer Res* 2008;6:873-883.