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DNA METHYLATION BY DNMT1 AND DNMT3b METHYLTRANSFERASES IS DRIVEN BY THE MUC1-C ONCOPROTEIN IN HUMAN CARCINOMA CELLS

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Abstract

Aberrant expression of the DNA methyltransferases (DNMTs) and disruption of DNA methylation patterns are associated with carcinogenesis and cancer cell survival. The oncogenic MUC1-C protein is aberrantly overexpressed in diverse carcinomas; however, there is no known link between MUC1-C and DNA methylation. Our results demonstrate that MUC1-C induces expression of DNMT1 and DNMT3b, but not DNMT3a, in breast and other carcinoma cell types. We show that MUC1-C occupies the *DNMT1* and *DNMT3b* promoters in complexes with NF-κB p65 and drives *DNMT1* and *DNMT3b* transcription. In this way, MUC1-C controls global DNA methylation as determined by analysis of LINE-1 repeat elements. The results further demonstrate that targeting MUC1-C downregulates DNA methylation of the *CDH1* tumor suppressor gene in association with induction of E-cadherin expression. These findings provide compelling evidence that MUC1-C is of functional importance to induction of DNMT1 and DNMT3b and, in turn, changes in DNA methylation patterns in cancer cells.

Keywords

MUC1-C; DNMT1; DNMT3b; DNA methylation; E-cadherin; epigenetic regulation

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Author Contributions: H.R. proposed the studies, generated the promoter-reporter vectors, and performed ChIP, DNA methylation ELISA and MeDIP assays. A.T., M.A., A.B. and Y.S. performed ChIP, promoter-reporter, and immunoblotting assays. S.P. performed bioinformatics analysis. D.K. designed the studies and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Introduction

DNA methylation plays an essential role in the epigenetic control of gene expression in cancer (1–3). The DNA methyltransferases (DNMTs) are responsible for methylation of DNA through transfer of a methyl group to the cytosine base in CpG dinucleotides (1). DNMT1 localizes to replication foci and is primarily responsible for maintaining DNA methylation patterns from template strands to newly synthesized DNA strands (4). DNMT1 can function with DNMT3a and DNMT3b in de novo methyltransferase activity during development (5; 6). Moreover, DNMT3a and DNMT3b establish de novo postreplicative DNA methylation patterns (7; 8). DNMT1, DNMT3a and DNMT3b are often overexpressed in cancer cells (9–11). Transcriptional activation of the *DNMT1, DNMT3a* and *DNMT3b* genes has been linked to activation of the RAS-JUN pathway (12; 13) and to binding of the Sp1 and Sp3 zinc finger proteins to their promoters (14; 15). In addition, DNMT1, DNMT3a and DNMT3b expression is repressed by (i) p53, Rb and FOXO3a, and (ii) diverse miRNAs (16). Notably, DNMT1 and DNMT3b are both needed to silence genes in human cancer cells (17; 18). However, signaling pathways that selectively and coordinately induce DNMT1 and DNMT3b are not known.

Mucin 1 (MUC1) is a heterodimeric protein that is aberrantly overexpressed by breast, lung and diverse other carcinomas (19; 20). The oncogenic MUC1 C-terminal transmembrane subunit (MUC1-C) associates with receptor tyrosine kinases at the cell membrane and promotes their downstream signaling pathways (19; 20). MUC1-C also localizes to the nucleus, where it interacts with certain transcription factors and contributes to their transactivation function (19; 20). In this manner, MUC1-C has been linked to the NF-κB pathway through interactions with TAK1 (21) and the IKK complex (22). MUC1-C also binds directly to NF-κB p65 and thereby contributes to activation of NF-κB target genes, including *MUC1* itself, in an autoinductive loop (23). Involvement of MUC1-C in the TAK1→IKK→NF-κB p65 pathway in cancer cells has further supported a direct role for MUC1-C in induction of (i) inflammatory cytokines, including IL-8, (ii) the epithelial-mesenchymal transition (EMT) with downregulation of the *CDH1* gene that encodes E-cadherin (ECAD) and (iii) self-renewal capacity (21–25). These findings have supported the notion that MUC1-C contributes to cancer progression by multiple mechanisms.

Based on the premise that MUC1-C is of importance to the regulation of gene expression in cancer, the present studies investigated whether MUC1-C induces DNMT expression and alterations in DNA methylation patterns. The results demonstrate that MUC1-C selectively and coordinately activates the *DNMT1* and *DNMT3b* genes in cancer cells and thereby global and gene-specific changes in DNA methylation.

Results and Discussion

Stable silencing of MUC1-C in BT-549 breast cancer cells was associated with suppression of DNMT1 mRNA and protein (Fig. 1A). A similar response to MUC1-C silencing was observed in MDA-MB-231 breast cancer cells (Fig. 1B), indicating that MUC1-C promotes DNMT1 expression. To confirm this notion, we overexpressed MUC1-C in MCF-7 breast cancer cells (24) and observed an increase in DNMT1 mRNA and protein (Fig. 1C),

indicating that MUC1-C, and not the MUC1 N-terminal subunit (MUC1-N), is sufficient for this response. In extending these observations to other types of carcinomas, we found that silencing MUC1-C in KRAS mutant A549 (Fig. S1A) and H460 (Fig. S1B) non-small cell lung cancer (NSCLC) cells similarly results in decreased DNMT1 expression. In addition, MUC1-C conferred DNMT1 expression in SK-CO-1 colon cancer cells (Fig. S1C), indicating that this association is found in diverse types of carcinomas. In further studies, we found that silencing MUC1-C in BT-549 cells suppressed DNMT3b, but not DNMT3a, expression (Fig. 1D). The same pattern of response was observed in MDA-MB-231 (Fig. 1E), A549 NSCLC (Fig. S2A), H460 NSCLC cells (Fig. S2B), and SK-CO-1 colon cancer cells (Fig. S2C). We also found that overexpression of MUC1-C in MCF-7 cells increases DNMT3b and not DNMT3a (Fig. 1F). To provide additional support for these findings, we used other approaches for targeting MUC1-C expression. In this way, silencing MUC1 in H460 cells with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 editing resulted in the downregulation of DNMT1 and DNMT3b (Fig. S3A). Moreover, targeting MUC1-C function in BT-549 and A549 cells with the GO-203 inhibitor (26) decreased DNMT1 and DNMT3b levels (Figs. S3B and S3C). These results demonstrate that MUC1-C selectively drives DNMT1 and DNMT3b, but not DNMT3a, expression in breast and other carcinoma cells.

Based on these findings, we searched the *DNMT1* and *DNMT3b* promoters for the presence of cis-binding elements not shared with *DNMT3a*. Notably, an NF-κB binding site had been reported in the *DNMT1* promoter (Fig. 2A) (27) and putative consensus NF-κB sites were identified in the *DNMT3b*, but not in the *DNMT3a,* promoter (Fig. 2B). Accordingly and to investigate a transcriptional mechanism, we generated a DNMT1 promoter-luciferase reporter (pDNMT1-Luc) (Fig. S4A) and showed that silencing MUC1-C in MDA-MB-231 (Fig. 2A, left) and A549 (Fig. S4B) cells suppresses its activity. A similar experiment using a DNMT3b promoter-luciferase reporter (pDNMT3b-Luc) (Fig. S4C) demonstrated that silencing MUC1-C is also associated with decreased activation (Fig. 2B, left and Fig. S4D). In addition, mutation of the putative NF-κB p65 binding sites in the pDNMT1-Luc (Fig. 2A, right) and pDNMT3b-Luc (Fig. 2B, right) vectors decreased their activation. To extend this line of investigation, we found that silencing NF-κB p65 in BT-549 and MDA-MB-231 cells decreases DNMT1 and DNMT3b, but not DNMT3a, expression (Figs. 2C and 2D). Similar results were obtained in A549 cells (Fig. S4E). Moreover, treatment with the NF-κB inhibitor BAY11-7085 (28) decreased expression of DNMT1 and DNMT3b, and not DNMT3a (Fig. 2E). MUC1-C constitutively activates the NF-κB p65 pathway in mesenchymal MDA-MB-231 and BT-549, but not in luminal MCF-7, cells. However, the enforced overexpression of MUC1-C in MCF-7 cells induces NF-κB p65 activity (25), indicating that cell context is of importance for MUC1-C-induced activation of NF-κB p65 signaling. Indeed, in studies of MCF-7/MUC1-C cells, we also found that BAY11-7085 (i) attenuates activation of the DNMT1 promoter (Fig. 2F, left), and (ii) suppresses DNMT1 and DNMT3b expression (Fig. 2F, right), providing further evidence that the MUC1-C→NF-κB p65 pathway (21–23) confers *DNMT1* and *DNMT3b* activation.

MUC1-C binds directly to NF-κB p65 and promotes activation of NF-κB target genes, including *MUC1* itself in an autoinductive loop (23). To investigate whether MUC1-C occupies the *DNMT1* and *DNMT3b* promoters, we performed chromatin

immunoprecipitation (ChIP) studies on MDA-MB-231 cells. Analysis of the *DNMT1* promoter clearly demonstrated MUC1-C occupancy (Fig. 3A, left) and the presence of NF-κB p65 (Fig. 3A, right). Additionally, in re-ChIP experiments, we found that NF-κB p65 occupies the *DNMT1* promoter in a complex with MUC1-C (Fig. 3B), consistent with the above observation that *DNMT1* activation is dependent on both MUC1-C and NF-κB p65. We also found that silencing MUC1-C decreases occupancy of NF-κB p65 on the *DNMT1* promoter (Fig. 3C). Similar results were obtained from studies of the *DNMT3b* promoter; that is MUC1-C and NF-κB p65 occupancy (Fig. 3D, left and right) and the detection of MUC1-C/NF-κB p65 complexes (Fig. 3E). The results also demonstrate that MUC1-C promotes NF-κB p65 occupancy of the *DNMT3b* promoter (Fig. 3F).

The above findings invoked the possibility that MUC1-C also functions in regulating DNA methylation in cancer cells. Indeed, silencing MUC1-C in BT-549 (Fig. 4A) and MDA-MB-231 (Fig. S5A) cells was associated with significant decreases in the percentage of 5-mC in Long Interspersed Nucleotide Element-1 (LINE-1) repeats, a surrogate marker of global DNA methylation (29–31). These effects of targeting MUC1-C were confirmed using bisulfite sequencing of LINE-1 repeats (Fig. 4B), further supporting a role for MUC1-C in inducing global DNA methylation. Based on these results and previous work demonstrating that MUC1-C promotes EMT and downregulation of ECAD expression (24; 25), we investigated the involvement of MUC1-C in DNA methylation of the *CDH1* promoter. Using immunoprecipitation of methylated DNA (MeDIP) followed by qPCR of the precipitated DNA fragments, we found that silencing MUC1-C in MDA-MB-231 cells is associated with marked decreases in methylation of the *CDH1* promoter (Fig. S5B). In addition and in concert with this response, MUC1-C silencing resulted in significant induction of ECAD mRNA and protein (Fig. S5C). Similarly, silencing MUC1-C in BT-549 cells was associated with decreased methylation of the *CDH1* promoter and induction of ECAD expression (Fig. 4C). Additionally, bisulfite conversion of genomic DNA and pyrosequencing of the CpG region of the *CDH1* promoter (positions −169 to −116) demonstrated that MUC1-C silencing is associated with significant decreases in DNA methylation of multiple CpG sites (Fig. 4D).

Aberrant expression of DNMTs and changes in DNA methylation have been linked to cancer cell progression and survival (2; 9; 11; 32; 33). Interestingly, genetic disruption of both *DNMT1* and *DNMT3b* substantially reduces DNMT activity and DNA methylation, indicating that these DNMTs cooperate to silence genes in human cancer cells (18). The present findings demonstrate that the MUC1-C oncoprotein is unique in that it selectively and coordinately induces DNMT1 and DNMT3b, but not DNMT3a, expression and thereby upregulates DNA methylation in diverse types of carcinoma cells (Fig. 4E). MUC1-C activates the TAK1→IKK→NF-κB pathway in an autoinductive loop that includes a direct interaction with NF-κB p65 and the induction of *MUC1* and other NF-κB target genes (19–23)(Fig. 4E). The present results thus provide support for a model in which MUC1-C→NF-κB p65 signaling links this inflammatory response to the induction of DNMTs (Fig. 4E). Based on these findings, we investigated the effects of targeting MUC1-C on DNA methylation patterns. LINE-1 retrotransposons constitute approximately 17–18% of the human genome, are aberrantly activated in cancer and may contribute to genome instability (34; 35). Based on the demonstration that MUC1-C regulates LINE-1 DNA methylation,

experiments will be needed to assess whether MUC1-C has an effect on LINE-1 retrotransposon activity in cancer cells. In this regard and although global hypomethylation promotes LINE-1 activation in cancer cells, individual LINE-1 elements are activated only when hypomethylation occurs in the promoter region of that element (36; 37).

MUC1-C has been linked to induction of EMT and the associated downregulation of ECAD expression in cancer cells (24; 25). The present results extend the importance of MUC1-C in EMT by demonstrating that MUC1-C drives DNA methylation of the *CDH1* promoter and thereby suppression of ECAD expression. These findings do not exclude the possibility that MUC1-C suppresses ECAD expression by additional mechanisms; for example, by promoting SNAIL-mediated repression of the *CDH1* promoter (38). The finding that targeting MUC1-C reverses EMT also raises the possibility that MUC1-C may epigenetically regulate other genes in the EMT program (24; 25). In that line of reasoning, the *MUC1* promoter itself is regulated by DNA methylation (39–41). Accordingly, studies of interest will be to assess the effects of downregulating DNMT1 and/or DNMT3b on DNA methylation of the *MUC1* promoter and MUC1-C expression. In summary, the present findings support the premise that targeting MUC1-C could represent a therapeutic approach capable of influencing global and gene-specific epigenetic changes, and in turn the reprogramming of cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DNMT	DNA methyltransferase
MUC1	mucin 1
EMT	epithelial-mesenchymal transition
ECAD	E-cadherin
NSCLC	non-small cell lung cancer
CRISPR	clustered regularly interspaced short palindromic repeats
ChIP	chromatin immunoprecipitation
LINE-1	Long Interspersed Nucleotide Element-1
MeDIP	immunoprecipitation of methylated DNA

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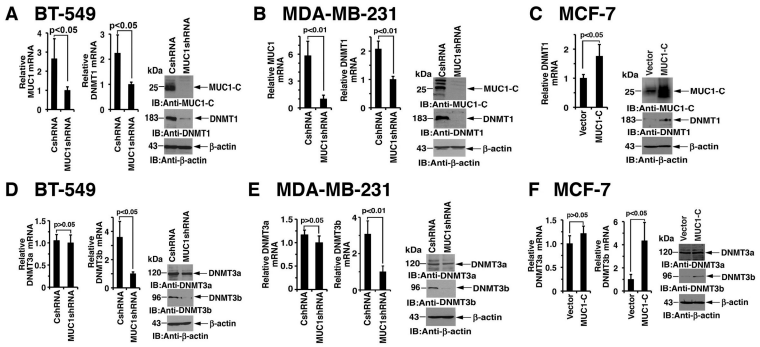

Figure 1. MUC1-C induces DNMT1 and DNMT3b, but not DNMT3a, in human carcinoma cells

(A–B) BT-549 (A) and MDA-MB-231 (B) breast cancer cells stably expressing a Control shRNA (CshRNA) or a MUC1 shRNA (MUC1shRNA) were analyzed for MUC1 and DNMT1 mRNA levels by qRT-PCR with pairs of primers listed in Table S1. The results (mean±SD of 3 determinations) are expressed as relative mRNA levels compared to that obtained with cells expressing MUC1shRNA (assigned a value of 1) (left and middle panels). Lysates were immunoblotted with the indicated antibodies (right panels). (C) MCF-7 breast cancer cells stably expressing an empty vector (MCF-7/vector) or MUC1-C (MCF-7/MUC1-C) were analyzed for DNMT1 mRNA levels by qRT-PCR. The results (mean±SD of 3 determinations) are expressed as relative mRNA levels compared to that obtained for vector expressing cells (assigned a value of 1) (left panel). Lysates from the indicated cells were analyzed by immunoblotting (right panel). (D–E) The indicated BT-549 (D) and MDA-MB-231 (E) cells were analyzed for (i) DNMT3a (left panels) and DNMT3b mRNA (middle panels). The results (mean±SD of 3 determinations) are expressed as relative mRNA levels compared to that obtained with cells expressing MUC1shRNA (assigned a value of 1). Lysates were analyzed by immunoblotting with the indicated antibodies (right panels). (F) MCF-7/vector and MCF-7/MUC1-C cells were analyzed for DNMT3a (left panel) and DNMT3b (middle panel) mRNA levels by qRT-PCR (mean±SD of 3 determinations) as compared to that obtained for vector expressing cells (assigned a value of 1). Lysates from the indicated cells were analyzed by immunoblotting (right panel).

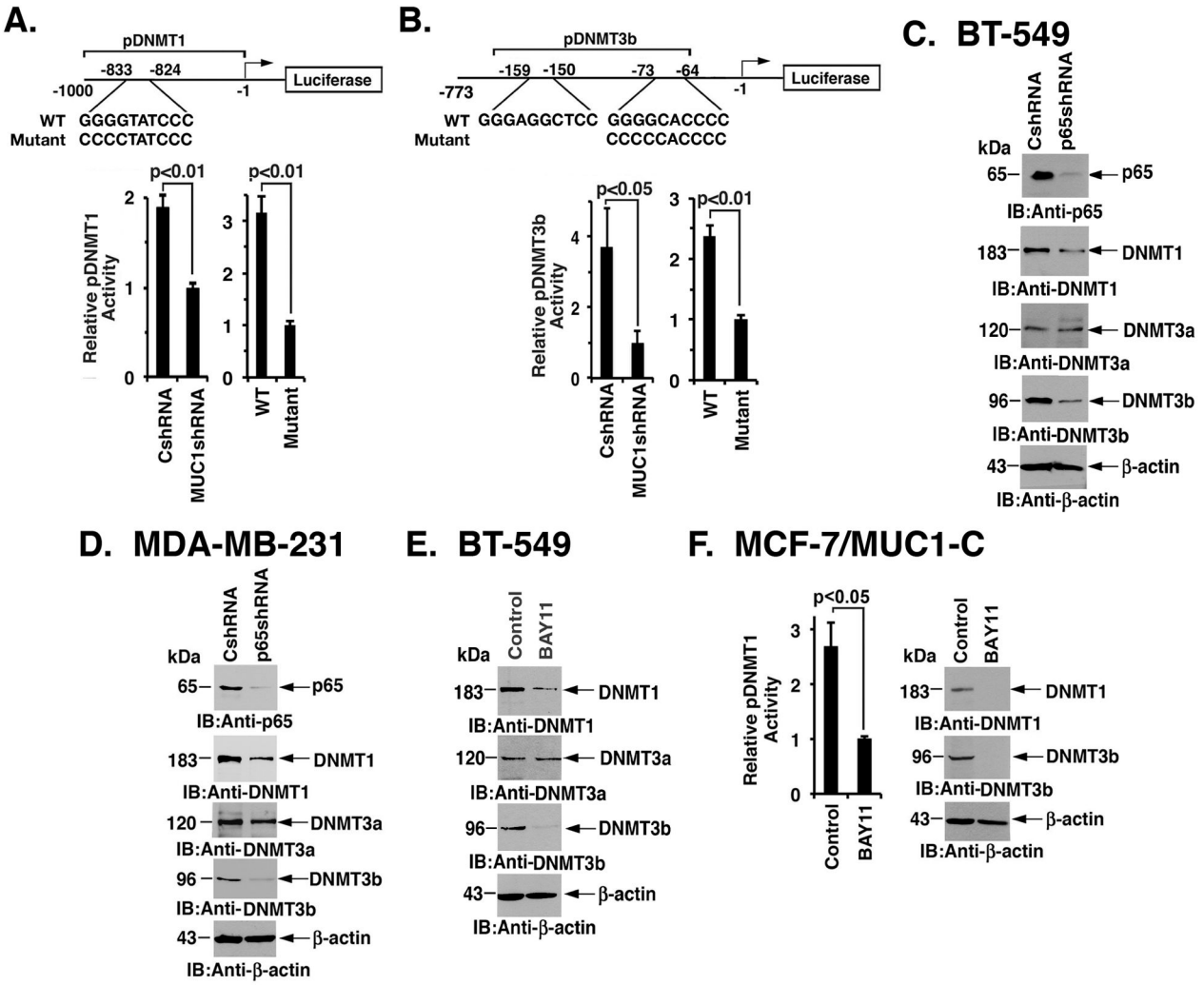

Figure 2. MUC1-C activates DNMT1 and DNMT3b expression by an NF-κB-mediated mechanism

(A) Schema of the DNMT1 promoter-luciferase (pDNMT1-Luc) reporter with an NF-κB binding motif at -833 to -824 upstream to the transcription start site. MDA-MB-231 cells were transfected with (i) the empty pGL3-Basic Luc vector or pGL3-pDNMT1-Luc, and the SV-40-*Renilla*-Luc plasmid as an internal control (left), and the empty pGL3-Basic Luc vector, wild-type (WT) pGL3-pDNMT1-Luc or mutant pGL3-pDNMT1-Luc and the SV-40-*Renilla*-Luc plasmid (right). Dual luciferase activity was measured at 48 h after transfection. The results (mean±SD of 3 determinations) are expressed as the relative luciferase activity compared to that obtained with (i) the MDA-MB-231/MUC1shRNA cells (assigned a value of 1)(left) and (ii) the mutant pGL3-pDNMT1-Luc (assigned a value of 1) (right). (B) Schema of the DNMT3b promoter-Luciferase (pDNMT3b-Luc) reporter with two putative consensus NF-κB binding sites at the indicated positions. MDA-MB-231 cells were transfected with the pGL3 vector, wild-type (WT) pGL3-pDNMT3b-Luc or mutant pGL3-pDNMT3b-Luc and SV-40-*Renilla*-Luc. The cells were then analyzed for luciferase activity as described above. (C–D) Lysates from the BT-549 (C) and MDA-MB-231 (D) cells

without and with p65 silencing were immunoblotted with the indicated antibodies. (E) Lysates from BT-549 cells treated with control vehicle (DMSO) or 10 μM BAY11-7085 for 24 h were immunoblotted with the indicated antibodies. (F) MCF-7/MUC1-C cells were transfected with the pGL3 or pGL3-pDNMT1-Luc, and the SV-40-*Renilla*-Luc plasmid. The cells were also treated with control vehicle (DMSO) or 10 μM BAY11-7085. Luciferase activity was measured at 48 h after transfection. The results (mean±SD of 3 determinations) are expressed as the relative luciferase activity compared to that obtained with BAY11-7085-treated cells (assigned a value of 1). MCF-7/MUC1-C cells were treated with control vehicle (DMSO) or 10 μM BAY11-7085 for 24 h. Lysates were immunoblotted with the indicated antibodies (right).

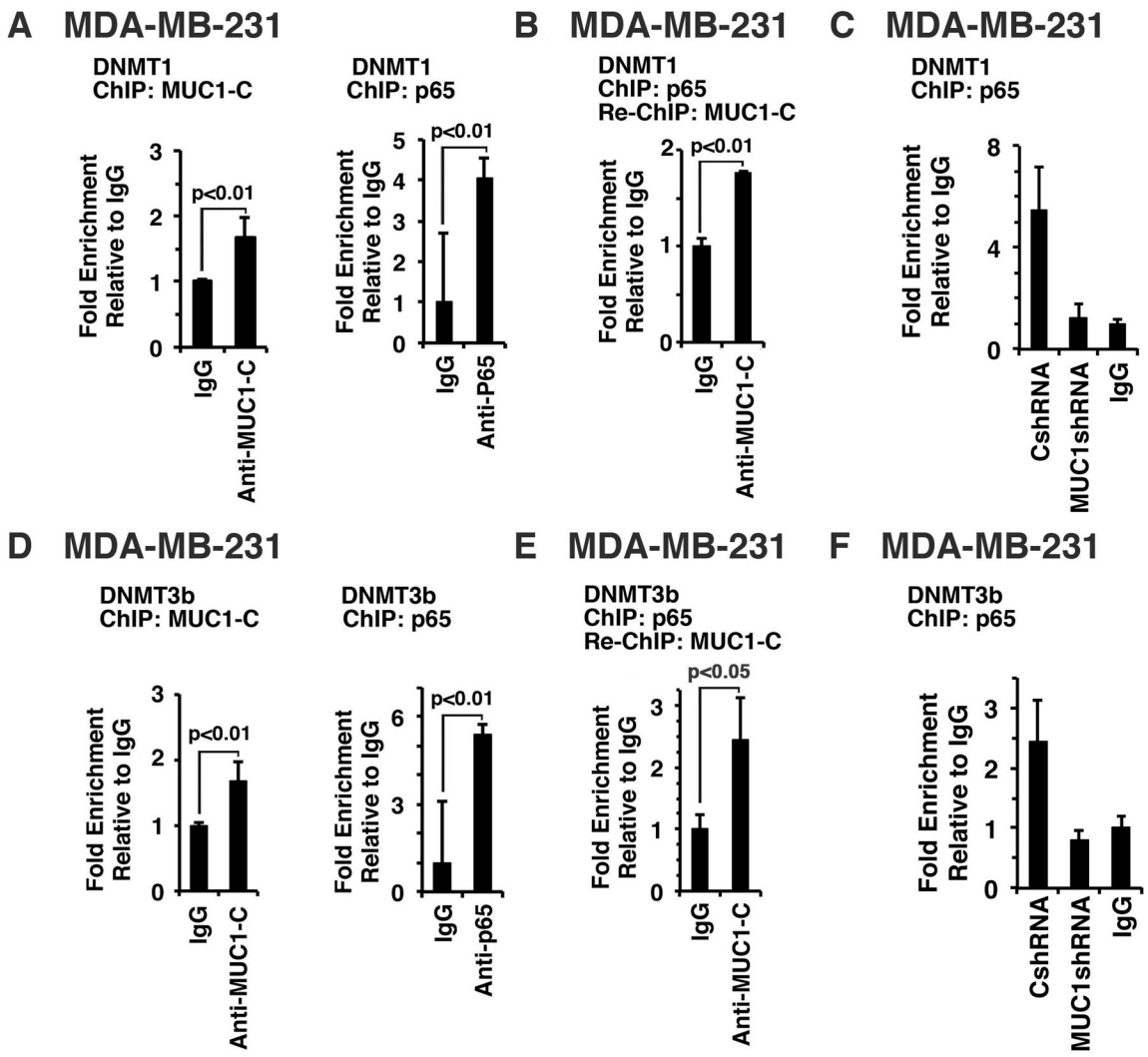

Figure 3. MUC1-C occupies the *DNMT1* and *DNMT3b* promoters

(A–F) (A and D) Soluble chromatin from MDA-MB-231 cells was precipitated with anti-MUC1-C (left), anti-p65 (right) or a control IgG. (B and E) In re-ChIP studies, the anti-p65 precipitates were released and reimmunoprecipitated with IgG or anti-MUC1-C. (C and F) Soluble chromatin from MDA-MB-231/CshRNA and MDA-MB-231/MUC1shRNA cells was precipitated with anti-p65 or a control IgG. (A–C) The final DNA samples were amplified by qPCR with pairs of primers (Table S2) for the NF-κB p65 binding site in the DNMT1 promoter. The results (mean±SD of 3 determinations) are expressed as the relative fold enrichment compared with that obtained for the IgG control (assigned a value of 1). (D–F) The final DNA samples were amplified by qPCR with pairs of primers (Table S2) for the NF-κB p65 binding sites in the *DNMT3b* promoter. The results (mean±SD of 3 determinations) are expressed as the relative fold enrichment compared with that obtained for the IgG control (assigned a value of 1).

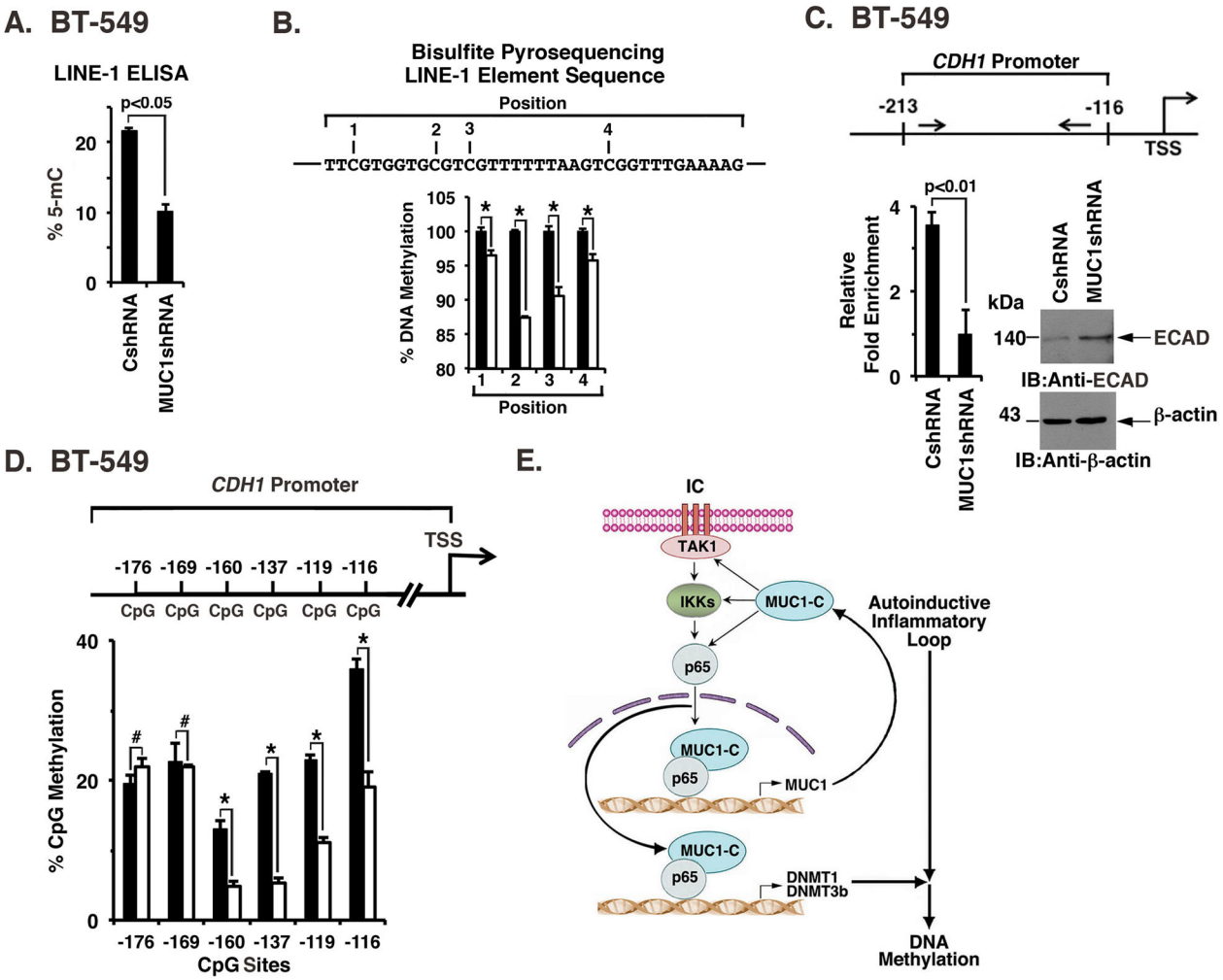

Figure 4. MUC1-C induces LINE-1 and *CDH1* promoter DNA methylation

(A) Genomic DNA from the indicated BT-549 cells was analyzed using the LINE-1 global DNA methylation ELISA kit. The results are expressed as percentage 5-mC (mean±SD of 3 determinations) based on extrapolation from the methylated and unmethylated genomic standards provided in the kit. (B) Genomic DNA from BT-549/CshRNA (closed bars) and BT-549/MUC1shRNA (open bars) was analyzed by bisulfite conversion, PCR amplification and pyrosequencing of 4 CpG positions in the LINE-1 element. The results are expressed as the percentage DNA methylation (mean±SD of 3 determinations) at each of the 4 positions as compared to that obtained in BT-549/CshRNA cells (assigned a value of 100%). The asterisk (*) denotes a p value of <0.05. (C) Genomic DNA from BT-549 cells was subjected to immunoprecipitation of methylated DNA (MeDIP) and the precipitates were analyzed by qPCR of the *CDH1* promoter (positions −213 to −116). The results (mean±SD of 3 determinations) are expressed as relative fold enrichment compared to that obtained from MUC1shRNA expressing cells (assigned a value of 1)(left panel). Lysates from the indicated cells were analyzed by immunoblotting (right panel). (D) Genomic DNA from BT-549/CshRNA (closed bars) and BT-549/MUC1shRNA (open bars) was analyzed by bisulfite

conversion, PCR amplification and pyrosequencing of the indicated CpG sites in the *CDH1* promoter. The results are expressed as the percentage DNA methylation (mean±SD of 3 determinations) at each of the 6 CpG sites. The hashtag (#) and asterisk (*) denote p values of >0.05 and <0.05, respectively. (E) Schema depicting the proposed MUC1-C-induced expression of DNMT1 and DNMT3b, and thereby DNA methylation in human cancer cells. MUC1-C induces an autoinductive inflammatory circuit involving activation of the TAK1→IKK→NF-κB p65 pathway (21–23). MUC1-C promotes NF-κB p65-mediated induction of *DNMT1* and *DNMT3b* transcription by interacting with NF-κB p65 on their respective promoters and increasing NF-κB p65 occupancy. In turn, MUC1-C links the inflammatory response with induction of DNA methylation and regulation of gene expression. Thus, targeting MUC1-C in cancer cells with silencing or inhibitors suppresses (i) DNMT1 and DNMT3b expression, and (ii) global and promoter-specific DNA methylation.