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MUC1-C REPRESSES THE RASSF1A TUMOR SUPPRESSOR IN HUMAN CARCINOMA CELLS

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Abstract

RASSF1A encodes a tumor suppressor that inhibits the RAS→RAF→MEK→ERK pathway and is one of the most frequently inactivated genes in human cancers. MUC1-C is an oncogenic effector of the cancer cell epigenome that is overexpressed in diverse carcinomas. We show here that MUC1-C represses RASSF1A expression in KRAS wild-type and mutant cancer cells. Mechanistically, MUC1-C occupies the *RASSF1A* promoter in a complex with the ZEB1 transcriptional repressor. In turn, MUC1-C/ZEB1 complexes recruit DNA methyltransferase 3b (DNMT3b) to the CpG island in the *RASSF1A* promoter. Targeting MUC1-C, ZEB1 and DNMT3b thereby decreases methylation of the CpG island and derepresses *RASSF1A* transcription. We also show that targeting MUC1-C regulates KRAS signaling, as evidenced by RNA-seq analysis, and decreases MEK/ERK activation, which is of importance for RAS-mediated tumorigenicity. These findings define a previously unrecognized role for MUC1-C in suppression of RASSF1A and support targeting MUC1-C as an approach for inhibiting MEK→ERK signaling.

Keywords

MUC1; MUC1-C; RASSF1A; tumor suppressor; KRAS

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Author Contributions

H.R., T.H., D.R. and D.K. designed the research

D.R. generated the genetically silenced cell lines

H.R., T.H. and D.R. performed the research and data analysis

M.L., Q.H., S.L., L.K. and M.S. performed bioinformatics analysis

Competing Interests

Regarding potential conflicts of interest, D. Kufe has equity interests in, serves as a member of the board of directors of and is a paid consultant to Genus Oncology. The other authors disclosed no potential conflicts of interest.

Introduction

The *RAS Association Domain Family 1A* (*RASSF1A*) tumor suppressor gene (TSG) is localized to a region in chromosome 3 (3p21.3) that is deleted in human lung and certain other cancers (1, 2). *RASSF1A* expression is also repressed in diverse cancers by promoter hypermethylation (3, 4). Importantly, *RASSF1A* is one of the most frequently downregulated TSGs in human cancers (5–8). RASSF1A forms a complex with KRAS and regulates multiple downstream effectors, including suppression of the canonical RAF→MEK→ERK pathway (8–10). RASSF1A thereby relieves RAS→RAF-mediated suppression of the MST2 kinase (11, 12), linking RASSF1A to the HIPPO tumor suppressor pathway (13, 14). RASSF1A also promotes the formation of a complex between YAP and p73, resulting in the transcriptional activation of cell differentiation (15, 16). In addition, RASSF1A links KRAS to MOAP-1 and thereby activation of the proapoptotic BAX pathway (17, 18). Other studies have shown that RASSF1A depletion induces the epithelial-mesenchymal transition (EMT) and the metastatic potential of lung cancer cells (19). RASSF1A deficiency thus enhances the development of KRAS-driven lung tumors in association with induction of a proinflammatory response (20). These findings have supported the importance of RASSF1A in integrating (i) regulation of the KRAS pathway, (ii) activation of proapoptotic signaling, and (iii) suppression of inflammation, EMT and tumorigenesis.

MUC1-C is an oncoprotein that associates with receptor tyrosine kinases (RTKs) at the cell membrane and promotes activation of their downstream signaling pathways (21–24). MUC1-C also localizes to the nucleus (25), where it interacts with transcription factors, such as β-catenin/TCF4 (26–28) and p53 (29), and regulates expression of their target genes (24). The role of nuclear MUC1-C extends to the epigenetic repression of TSGs by activating (i) DNA methyltransferase 1 (DNMT1) and DNMT3b, and thereby DNA methylation (30) and (ii) function of Polycomb Repressive Complex 1 (PRC1) (31) and PRC2 (32) with downregulation of TSG transcription (33). MUC1-C thereby represses expression of the Crumbs CRB3 polarity factor (34), which functions as a tumor suppressor by activating the HIPPO cascade of MST1/2 and LATS1/2 signaling (35, 36). In this way, MUC1-C activates YAP and YAP/β-catenin-mediated induction of WNT target genes, such as *MYC* (34). In contrast to RASSF1A, MUC1-C binds directly to the BAX BH3 domain with inhibition of BAX function (37) and is of importance to induction of EMT and the cancer stem cell (CSC) state (33, 38). These findings have collectively supported the notion that MUC1-C plays an opposing role to that of RASSF1A in the regulation of pathways linked to cancer progression.

The *RASSF1A* promoter contains a CpG island that is frequently hypermethylated in lung (4), breast (39) and diverse other carcinomas (40). MUC1-C has been linked to TSG repression (30); however, there is no known association between MUC1-C and hypermethylation of the *RASSF1A* promoter. In addressing this issue, the present studies demonstrate that MUC1-C forms a complex with ZEB1 on the *RASSF1A* promoter, recruits DNMT3b and suppresses *RASSF1A* transcription. The results support a model in which MUC1-C is necessary for *RASSF1A* promoter methylation, downregulation of RASSF1A expression and activation of MEK→ERK signaling.

Results

MUC1-C suppresses RASSF1A expression

RASSF1A is repressed in diverse cancers (5–8). To investigate if MUC1-C is involved in *RASSF1A* regulation, we first studied the effects of silencing MUC1-C in BT-549 and MDA-MB-231 TNBC cells and, notably, found upregulation of RASSF1A mRNA and protein (Figs. 1A and 1B; Supplemental Fig. S1A). Similar results were obtained in KRAS mutant A549 and H460 NSCLC cells (Figs. 1C and 1D; Supplemental Fig. S1B), indicating that the effects of MUC1-C on *RASSF1A* are independent of KRAS status. These findings were not limited to TNBC and NSCLC cells in that downregulation of MUC1-C also induced RASSF1A expression in PC-3 prostate cancer cells (Figs. 1E and 1F). In concert with these results, enforced expression of MUC1-C in MUC1-null HEK293 cells was associated with suppression of RASSF1A mRNA and protein (Figs. 1G and H). These findings supported a role for MUC1-C in the repression of *RASSF1A* expression.

MUC1-C forms a complex with ZEB1 on the *RASSF1A* promoter

MUC1-C induces the ZEB1 transcriptional repressor in human cancer cells (41). In turn, MUC1-C binds to ZEB1 and promotes repression of ZEB1 target genes, such as *miR-200c* (41). The *RASSF1A* gene includes potential ZEB1 binding motifs upstream to the CpG island in the promoter region and in intron 1 (Fig. 2A). ChIP-qPCR studies of chromatin from BT-549 (Fig. 2B) and A549 (Fig. 2C) cells demonstrated that (i) MUC1-C and ZEB1 occupy the *RASSF1A* promoter region, and (ii) silencing MUC1-C decreases ZEB1 occupancy (Figs. 2D and 2E). We also found that MUC1-C and ZEB1 are detectable on *RASSF1A* intron 1 (Fig. 2F) and that MUC1-C silencing decreases the occupancy of ZEB1 in this region (Fig. 2G). Similar results were obtained in PC-3 cells (Supplemental Figs. S2A and S2B), consistent with a role for MUC1-C in enhancing ZEB1 binding to its target genes.

MUC1-C suppresses *RASSF1A* activation by a ZEB1-mediated mechanism

As shown for MUC1-C, stable silencing of ZEB1 in BT-549 cells was associated with upregulation of RASSF1A mRNA and protein (Figs. 3A and B). We also found that silencing MUC1-C or ZEB1 was associated with comparable increases in RASSF1A expression (Fig. 3C). ZEB1 silencing in A549 cells similarly resulted in RASSF1A induction (Figs. 3D and 3E). In the HEK293 cell model, MUC1-C-induced repression of RASSF1A was attenuated by silencing ZEB1, confirming involvement of the MUC1-C→ZEB1 pathway in suppressing RASSF1A expression (Figs. 3F and 3G). In concert with these findings, overexpression of MUC1-C in MCF-10A breast epithelial cells was associated with induction of ZEB1 and repression of RASSF1A (Supplemental Fig. S3).

To further assess these effects of MUC1-C and ZEB1, BT-549 cells were transfected to express a *RASSF1A* promoter-luciferase reporter (pRASSF1A-Luc) containing the ZEB1 binding site (Fig. 4A). pRASSF1A-Luc activity was induced by silencing MUC1-C (Fig. 4B) or ZEB1 (Fig. 4C). Similar studies in A549 cells confirmed these effects of MUC1-C (Fig. 4D) and ZEB1 (Fig. 4E) on pRASSF1A-Luc activation, supporting the premise that MUC1-C represses the *RASSF1A* promoter by a ZEB1-mediated mechanism.

MUC1-C/ZEB1 recruit DNMT3b to the *RASSF1A* promoter

Methylation of *RASSF1A* promoter has been identified as one mechanism responsible for suppression of RASSF1A expression (3, 4). Carcinoma cells under study here were therefore treated with decitabine (DEC; 5-aza-2'-deoxycytidine) to assess whether DNA methylation contributes to *RASSF1A* repression. As anticipated, we found upregulation of RASSF1A in response to DEC treatment (Supplemental Figs. S4A–S4C). These and the above findings that the MUC1-C→ZEB1 pathway represses *RASSF1A* activation thus invoked the possibility that MUC1-C/ZEB1 complexes contribute to *RASSF1A* promoter methylation. MUC1-C drives DNMT3b expression and changes in DNA methylation patterns in cancer cells (30). In addition, ZEB1 has been associated with recruitment of DNMT3b (42, 43), supporting a potential model in which MUC1-C/ZEB1 complexes associate with DNMT3b on the *RASSF1A* promoter. Indeed, ChIP studies demonstrated that, like MUC1-C and ZEB1, DNMT3b occupies the *RASSF1A* promoter (Fig. 5A). In re-ChIP experiments, we also found that MUC1-C and ZEB1 form complexes with DNMT3b on the *RASSF1A* promoter (Figs. 5B and 5C). Moreover, silencing MUC1-C (Fig. 5D) or ZEB1 (Fig. 5E) was associated with decreases in DNMT3b occupancy, indicating that MUC1-C/ZEB1 complexes recruit DNMT3b to the *RASSF1A* promoter. In support of these findings, DNMT3b occupancy was significantly increased in HEK293/MUC1-C, as compared to HEK293/vector, cells (Fig. 5F).

MUC1-C drives DNMT3b-mediated methylation of the *RASSF1A* promoter

To assess function of the MUC1-C/ZEB1/DNMT3b complexes, we studied the effects of silencing MUC1-C on methylation of the CpG island in the *RASSF1A* promoter (Fig. 6A). Immunoprecipitation of methylated DNA (MeDIP) followed by qPCR demonstrated that silencing MUC1-C (Fig. 6B), ZEB1 (Fig. 6C) or DNMT3B (Fig. 6D) decreases CpG island methylation. In addition, *RASSF1A* promoter methylation was increased in HEK293 cells expressing MUC1-C (Fig. 6E), confirming involvement of the MUC1-C/ZEB1/DNMT3b pathway. In concert with these findings, silencing DNMT3b was associated with increases in RASSF1A expression in BT-549 (Fig. 6F), A549 (Supplemental Fig. S5) and HEK293/MUC1-C (Fig. 6G) cells. Other work has demonstrated that *RASSF1* CpG island methylation is linked to activation of RASSF1C expression (44). In concert with those findings, silencing MUC1-C with decreases in *RASSF1* promoter methylation was associated with suppression of RASSF1C mRNA levels (Supplemental Fig. S6).

MUC1-C regulates the RAS→MEK→ERK pathway

RNA-seq analysis further demonstrated that targeting MUC1-C in BT-549 cells is highly associated with regulation of KRAS signaling as determined by gene set enrichment analysis from the Hallmarks Molecular Signature Database (Fig. 7A; Supplemental Fig. S7)(45). Targeting MUC1-C expression in A549 cells was also significantly associated with the Hallmark RAS Signaling gene set (Fig. 7B; Supplemental Fig. S7). In concert with this involvement of MUC1-C in KRAS signaling and MUC1-C-mediated repression of RASSF1A, we found that downregulation of MUC1-C in BT-549 cells has no apparent effect on KRAS activity (Supplemental Fig. S8), but is associated with decreases in MEK and ERK phosphorylation (Fig. 7C), consistent with the role of RASSF1A in suppression of

the MEK→ERK pathway (8–10). Moreover, silencing RASSF1A in BT-549/MUC1shRNA cells attenuated the suppression of pMEK and pERK levels (Fig. 7D), confirming dependence on RASSF1A for this response. Similar effects of targeting MUC1-C signaling on downregulation of pMEK and pERK were observed in A549 (Fig. 7E; Supplemental Figure S9) and PC-3 (Fig. 7F) cells. As further support for MUC1-C→ZEB1→RASSF1A signaling in driving the MEK→ERK pathway, expression of MUC1-C in HEK293 cells increased pMEK and pERK levels (Supplemental Fig. S10A) and this response was attenuated by ZEB1 silencing (Supplemental Fig. S10B).

Discussion

Epigenetic silencing of TSGs is considered an early event in oncogenesis and is universally found in human cancers (46). MUC1-C, a widely overexpressed oncogenic protein in human carcinomas (23, 24), has been linked to the epigenetic downregulation of TSGs, such as *CDH1*, *CDKN2A*, *PTEN* and *BRCA1*, by mechanisms involving in part PRC1/2-mediated suppression (30–33, 47). The present findings have identified a role for MUC1-C in downregulation of the *RASSF1A* TSG, which is reportedly one of the most frequently inactivated genes in over 30 types of cancers (5, 7). Studies in normal human mammary epithelial cells identified a role for the Sp1 transcription factor in activation of the *RASSF1A* promoter, such that decreases in Sp1 occupancy were associated with downregulation of RASSF1A expression (48). Our results demonstrate that silencing MUC1-C in breast, NSCLC and prostate cancer cells is associated with induction of RASSF1A mRNA and protein. In support of these observations, enforced expression of MUC1-C in MUC1-low MCF-10A mammary epithelial cells or in MUC1-null HEK293 cells resulted in suppression of RASSF1A expression. We also found that MUC1-C occupies the *RASSF1A* promoter and intron 1, suggesting that MUC1-C plays a direct role in repressing *RASSF1A* transcription. In concert with this notion, we found that MUC1-C suppresses activation of the *RASSF1A* promoter. These findings provided support for the premise that overexpression of MUC1-C, as found in human carcinomas, contributes to repression of the *RASSF1A* gene.

RASSF1A is epigenetically silenced by promoter hypermethylation (8). In this respect, studies in human carcinoma cells have shown that MYC/EZH2/DNMT3b complexes occupy the *RASSF1A* promoter and are necessary for its methylation and inactivation (49). Of potential relevance to those findings, MUC1-C drives MYC (34, 50, 51), EZH2 (32) and DNMT3b (30) expression in cancer cells and could thereby contribute to the formation of MYC/EZH2/DNMT3b complexes. MUC1-C also activates the inflammatory NF-κB p65 pathway, binds to NF-κB p65 and induces transcription of *ZEB1* (38, 41, 52)(Fig. 7G). In turn, MUC1-C forms a complex with ZEB1 and promotes ZEB1-mediated transcriptional repression (41). MUC1-C and ZEB1 thus cooperate in suppression of the *miR-200c* gene and thereby the induction of EMT in human cancer cells (30, 38). The present studies extend the importance of the MUC1-C→NF-κB p65→ZEB1 pathway by demonstrating that MUC1-C and ZEB1 also occupy the *RASSF1A* promoter and suppress its activation. In addition, we found that MUC1-C/ZEB1 complexes recruit DNMT3b to the *RASSF1A* promoter and that MUC1-C, ZEB1 and DNMT3b are necessary for its methylation (Fig. 7G). In this way, MUC1-C/ZEB1-mediated recruitment of DNMT3b could integrate with

that conferred by MYC/EZH2 (49) and thereby further enhance methylation of the *RASSF1A* promoter. Moreover, MUC1-C binds to EZH2 and increases H3K27 trimethylation (32). Therefore, MUC1-C could directly contribute to the function of MYC/EZH2/DNMT3b complexes by interacting with EZH2 (49). Our findings thus (i) support a model in which MUC1-C→ZEB1→DNMT3b signaling contributes to repression of *RASSF1A,* (ii) invoke the possibility for functional integration of MUC1-C/ZEB1/DNMT3b and MYC/EZH2/DNMT3b complexes on the *RASSF1A* promoter, and (iii) provide evidence for a potential link between ZEB1-mediated induction of EMT and downregulation of RASSF1A expression (Fig. 7G). Our findings may also provide the basis for studies of other TSGs, such as *HIC1* (53), that are hypermethylated in cancer cells.

RASSF1A plays an important role in the regulation of RAS signaling and downstream effectors, such as the MEK→ERK pathway (8, 12, 15, 54–56). In this capacity and as determined using the Hallmarks Molecular Signature Database, we found that targeting MUC1-C in TNBC and NSCLC cells is highly associated with the RAS signaling gene set. To our knowledge, MUC1-C has not been previously linked to regulation of the RAS pathway. Notably, these findings do not preclude a role for MUC1-C in other pathways, such as GRB2/SOS (21), that like RASSF1A contribute to the control of RAS signaling. Further studies will thus be needed to more precisely address other potential relationships between MUC1-C and RAS. Along these lines, we found that (i) targeting MUC1-C in carcinoma cells is associated with suppression of the MEK→ERK pathway, and (ii) overexpression of MUC1-C in HEK293 cells with suppression of RASSF1A results in activation of MEK and ERK. Importantly, RAF→MEK→ERK signaling is necessary for RAS-induced oncogenesis (57) and inhibiting this pathway has represented a major focus of drug development (58, 59). However, additional weapons are needed for the treatment of RAS-driven carcinomas. Therefore, targeting the MUC1-C→ZEB1→DNMT3b pathway with derepression of RASSF1A could represent an alternative strategy for inhibiting MEK→ERK signaling in cancer cells (Fig. 7H). In this context, previous work demonstrated that targeting MUC1-C is associated with marked synergy in combination with MEK inhibitors (60). These findings were attributed to the effects of targeting MUC1-C on downregulation of BCL-XL (60). The present results demonstrating that targeting MUC1-C induces RASSF1A and suppresses pMEK and pERK therefore provide new insights regarding the potential basis for synergy with MEK inhibitors. Of additional importance, RAS signaling in cancer is MYC dependent (57, 61). In this respect, MUC1-C drives MYC expression in carcinoma cells (34, 50, 51) and, accordingly, targeting MUC1-C could suppress integration of the RAS and MYC pathways in promoting cancer progression.

Materials and Methods

Cell culture

Human BT-549 TNBC, A549 (mutant KRAS) NSCLC, H460 (mutant KRAS) NSCLC and embryonic kidney HEK293 cells were cultured in RPMI1640 medium (ATCC, Manassas, VA, USA). MDA-MB-231 (mutant KRAS) TNBC cells were grown in Dulbecco's modified Eagle's medium (Corning, Manassas, VA, USA). PC-3 prostate cancer cells were grown in F-12K medium (ATCC). MCF-10A cells were cultured in MEGM medium (Lonza,

Walkersville, MD, USA). Media were supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell authentication was performed by short tandem repeat analysis. Cells were monitored for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, MA, USA). Cells stably expressing a control scrambled shRNA (CshRNA), MUC1shRNA, ZEB1shRNA, DNMT3bshRNA, empty vector or MUC1-C were generated as described (30–32). Cells were transfected to express a control siRNA (AM4611; ThermoFisher Scientific, Waltham, MA, USA) or RASSF1A siRNA (AM16708; ThermoFisher Scientific) in the presence of Lipofectamine RNAimax reagent (Invitrogen, Carlsbad, CA, USA).

Real-time quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen). cDNAs were synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY, USA) (32). Samples were amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7300 Realtime PCR System (Applied Biosystems). Primers used for qRT-PCR analysis are listed in the Supplemental Table S1.

Immunoblot analysis

Whole cell lysates were prepared in NP-40 buffer containing protease inhibitor cocktail (ThermoFisher Scientific). Immunoblotting was performed with anti-MUC1-C (62), anti-RASSF1A (Abcam, Cambridge, MA, USA), anti-β-actin (Sigma), anti-ZEB1, anti-DNMT3b, anti-pMEK(S217/S221), anti-MEK, anti-pERK(T202/Y204) and anti-ERK (Cell Signaling Technologies, Danvers, MA, USA).

RASSF1A promoter luciferase reporter assays

Cells were transfected with (i) an empty pGL3 vector, (ii) a pRASSF1A-Luc vector containing *RASSF1A* promoter sequences –600 to +19 relative to the TSS, and (iii) SV-40-*Renilla*-Luc in the presence of Lipofectamine 3000 Reagent (Invitrogen). At 48 h after transfection, cell extracts were prepared using the Luciferase Assay System (Promega, Madison, WI, USA). Luminescence was detected with the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation (ChIP) assay

Soluble chromatin was precipitated with anti-MUC1-C (NeoMarkers, Fremont, CA, USA), anti-ZEB1, anti-DNMT3b or a control non-immune IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In re-ChIP studies, complexes from the primary anti-MUC1-C or anti-ZEB1 ChIPs were eluted and re-immunoprecipitated with anti-DNMT3b. The precipitates were analyzed by ChIP-PCR using the Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7300 Realtime PCR System (Applied Biosystems). Primers used for ChIP-PCR are listed in the Supplemental Table S2. Data are reported as the fold-enrichment relative to IgG (32).

MeDIP analysis

Promoter methylation analysis was performed using the Methylation DNA IP (MeDIP) kit (Active Motif) as described (30). Primers used for MeDIP are listed in Supplemental Table S3. Data are reported as the fold-enrichment relative to IgG (32).

RNA-seq analysis

Total RNA from cells cultured in triplicates was isolated using Trizol reagent (Invitrogen). TruSeq Stranded mRNA (Illumina, San Diego, CA, USA) was used for library preparation.

RNA-seq data analysis

Raw sequencing reads were aligned to the human genome (GRCh38.74) using STAR (20.1×10^6 uniquely mapped reads per sample). Raw feature counts were normalized and differential expression analysis using DESeq2. Differential expression rank order was utilized for subsequent GSEA, performed using the fgsea (v1.8.0) package in R. Gene sets queried included the Hallmark Gene Sets available through the Molecular Signatures Database (MSigDB) (45).

KRAS activation assays

Lysates were assayed for KRAS activation according to the manufacturer's instructions (Cat. #STA-400-K; Cell Biolabs, San Diego, CA).

Statistical analysis

Each experiment was repeated at least three times. Data are expressed as the mean±SD. The unpaired Student's t-test was used to examine differences between means of two groups. A p-value of <0.05 was considered a statistically significant difference.

Data and software availability

The accession number for the RNA-seq data reported in this paper is GEO ACCESSION GSE123860.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MUC1	mucin 1
MUC1-C	MUC1 C-terminal transmembrane subunit
TNBC	triple negative breast cancer

NSCLC	non-small cell lung cancer
PC	prostate cancer
RASSF1A	RAS Association Domain Family 1A
MST	mammalian sterile twenty kinase
TSG	tumor suppressor gene
DNMT	DNA methyltransferase
PRC	polycomb repressive complex
CRB3	Crumbs Polarity Factor 3
YAP	Yes-associated protein
TAZ	transcriptional activator with PDX-binding motif
DEC	decitabine
MeDIP	immunoprecipitation of methylated DNA

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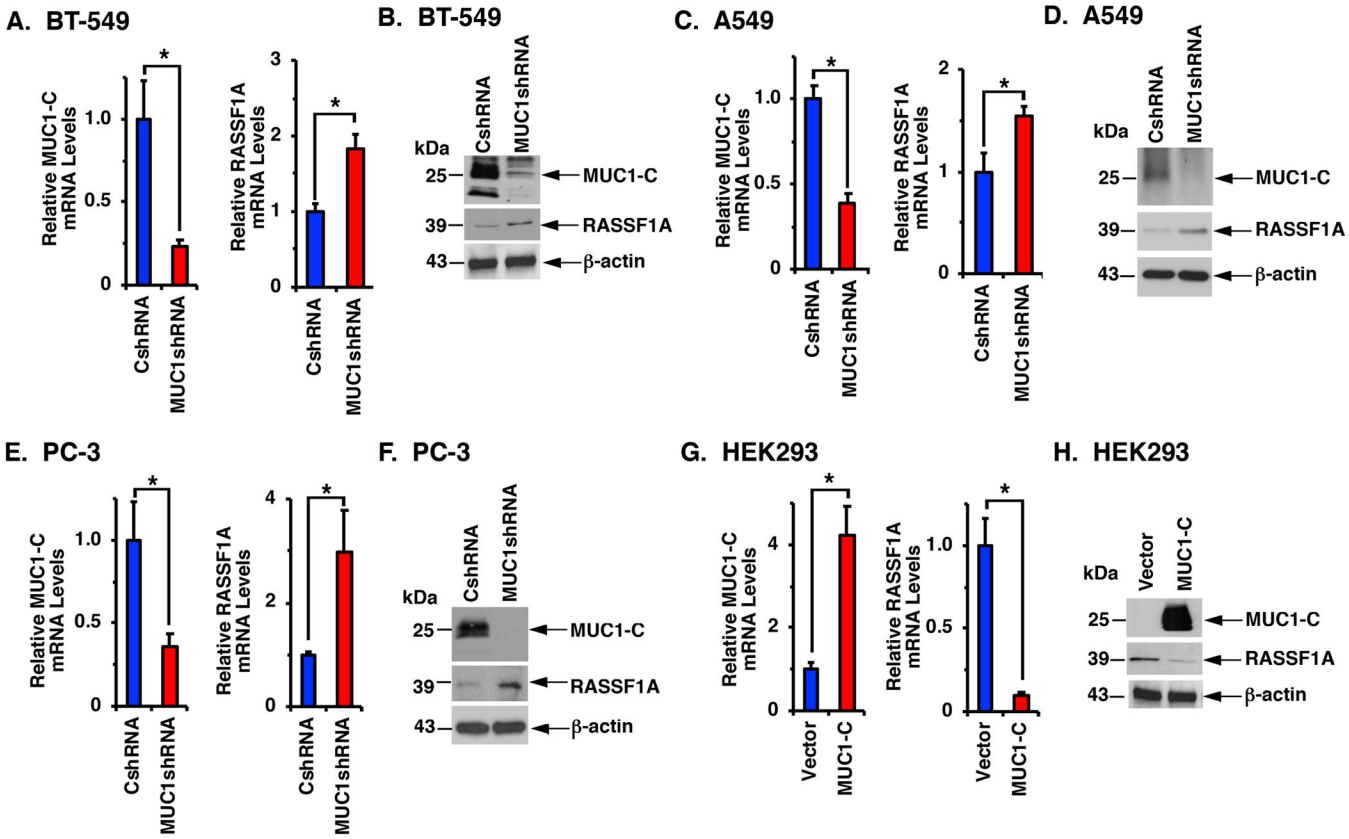

Figure 1. Targeting MUC1-C represses RASSF1A expression.

A-F. BT-549 TNBC (A,B), A549 NSCLC (C,D) and PC-3 PC (E,F) cells were transduced to stably express a CshRNA or MUC1shRNA. The cells were analyzed for MUC1-C and RASSF1A mRNA levels by qRT-PCR using primers listed in Supplemental Table S1. The results (mean±SD of three determinations) are expressed as relative mRNA levels compared to that obtained with cells expressing the CshRNA (assigned a value of 1)(A,C,E). Lysates were immunoblotted with antibodies against the indicated proteins (B,D,F). G and H. HEK293/MCF-10A cells were transduced to stably express an empty vector or one encoding MUC1-C. Cells were analyzed for MUC1-C and RASSF1A mRNA levels by qRT-PCR. The results (mean±SD of three determinations) are expressed as relative mRNA levels compared to that obtained with cells expressing the empty vector (assigned a value of 1)(G). Lysates were immunoblotted with antibodies against the indicated proteins (H). The asterisk (*) denotes a p-value <0.05.

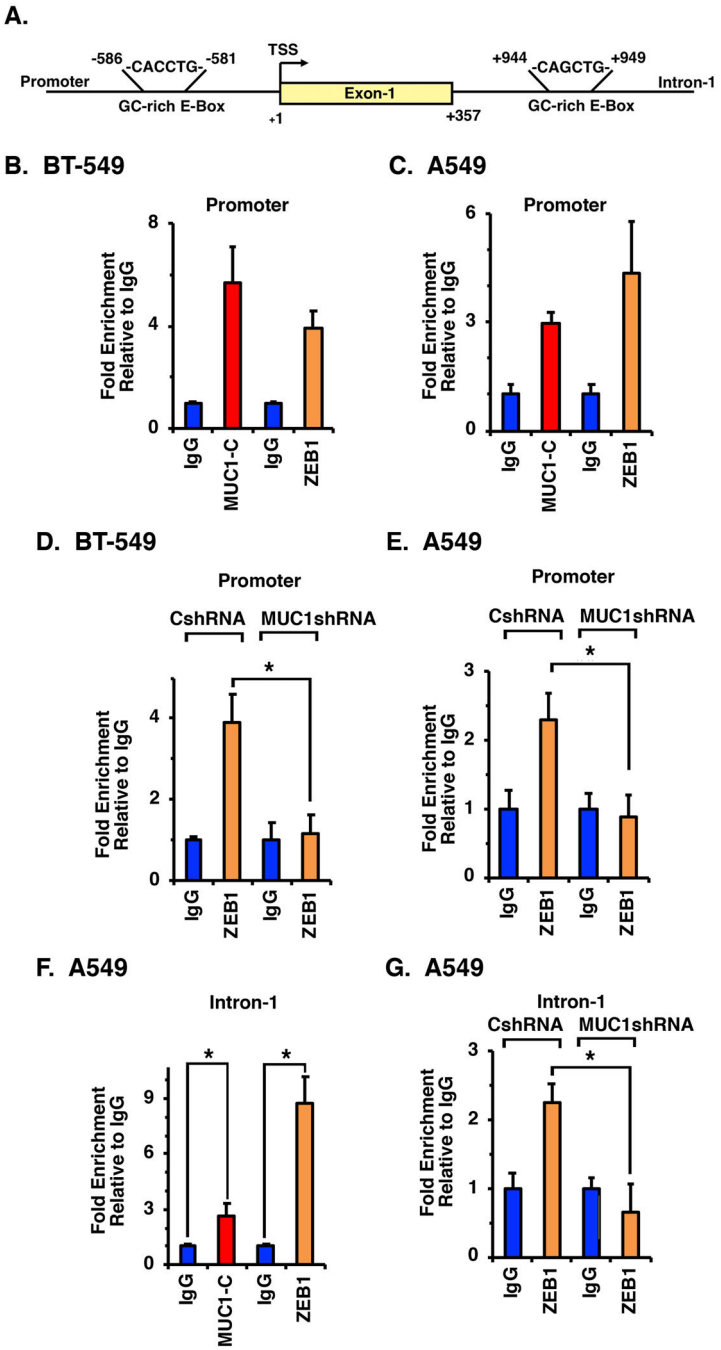

Figure 2. MUC1-C occupies the *RASSF1A* promoter and intron 1 in a complex with ZEB1.

A. Schema of the *RASSF1A* promoter and intron 1 with highlighting localization of GC-rich E-boxes. B-C. Soluble chromatin from BT-549 (B) and A549 (C) cells was precipitated with anti-MUC1-C, anti-ZEB1 or a control IgG. D-E. Soluble chromatin from BT-549 (D) and A549 (E) cells expressing a CshRNA or MUC1shRNA was precipitated with anti-ZEB1 or a control IgG. The final DNA samples were amplified by qPCR with primers for the *RASSF1A* promoter (listed in Supplemental Table S2). The results (mean±SD of three determinations) are expressed as the relative fold enrichment compared to that obtained with

the IgG control (assigned a value of 1). F and G. Soluble chromatin from A549 (F) and A549/CshRNA or A549/MUC1shRNA (G) cells was precipitated with anti-MUC1-C, anti-ZEB1 or a control IgG. The final DNA samples were amplified by qPCR with primers for the *RASSF1A* intron 1 region (listed in Supplemental Table S2). The results (mean±SD of three determinations) are expressed as the relative fold enrichment compared to that obtained with the IgG control (assigned a value of 1). The asterisk (*) denotes a p-value <0.05.

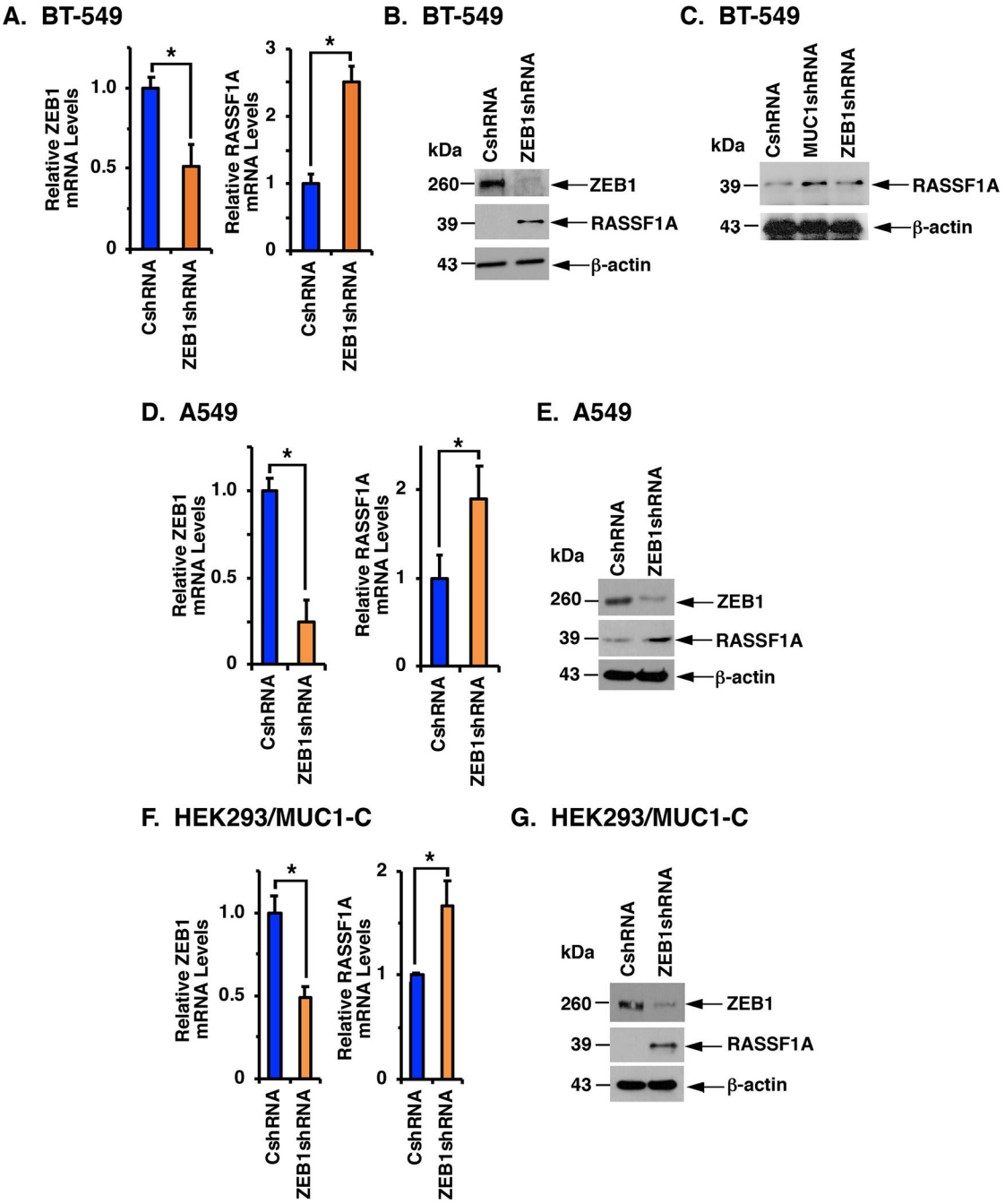

Figure 3. ZEB1 represses RASSF1A expression.

A. BT-549 cells expressing a CshRNA or ZEB1shRNA were analyzed for ZEB1 and RASSF1A mRNA levels. The results (mean±SD of three determinations) are expressed as relative mRNA levels as compared to that obtained with cells expressing the CshRNA (assigned a value of 1). B and C. Lysates from BT-549 cells expressing a CshRNA, ZEB1shRNA or MUC1shRNA were immunoblotted with antibodies against the indicated proteins. D-G. A549 (D, E) and HEK293/MUC1-C (F,G) cells stably expressing a CshRNA or ZEB1shRNA were analyzed for ZEB1 and RASSF1A mRNA levels (D,F). The results

(mean±SD of three determinations) are expressed as relative mRNA levels as compared to that obtained with cells expressing the CshRNA (assigned a value of 1). Lysates were immunoblotted with antibodies against the indicated proteins (E,G). The asterisk (*) denotes a p-value <0.05.

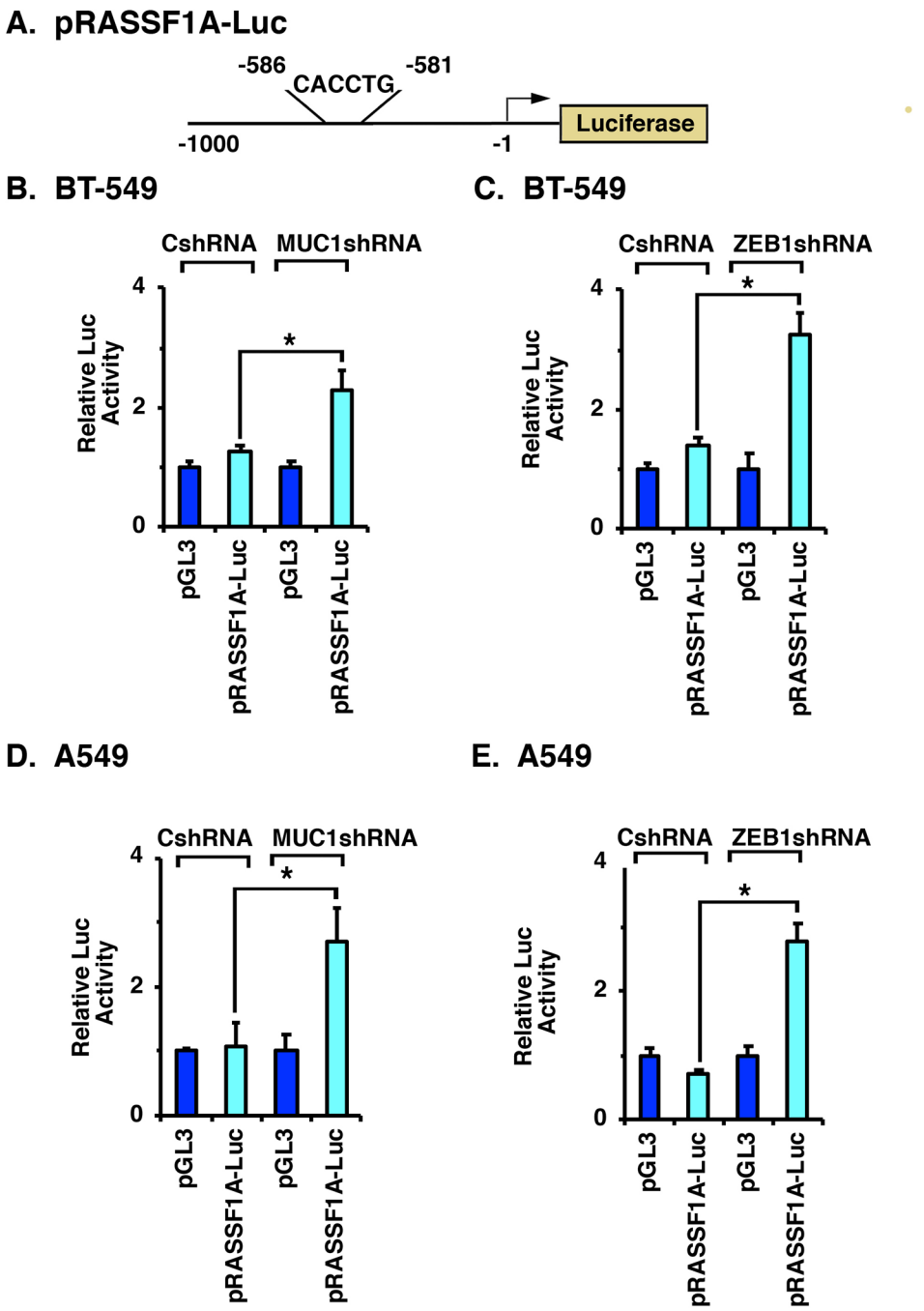

Figure 4. MUC1-C/ZEB1 complexes repress activation of the *RASSF1A* promoter.

A. Schema of the *RASSF1A* promoter-luciferase reporter (pRASSF1A-Luc). B-E. The indicated BT-549 (B,C) and A549 (D,E) cells expressing a CshRNA, MUC1shRNA or ZEB1shRNA were transfected with pGL3-Basic Luc or pRASSF1A-Luc vectors for 48 h and then analyzed for luciferase activity. The results (mean±SD of three determinations) are expressed as relative luciferase activity as compared to that obtained with pGL3 (assigned a value of 1). The asterisk (*) denotes a p-value <0.05.

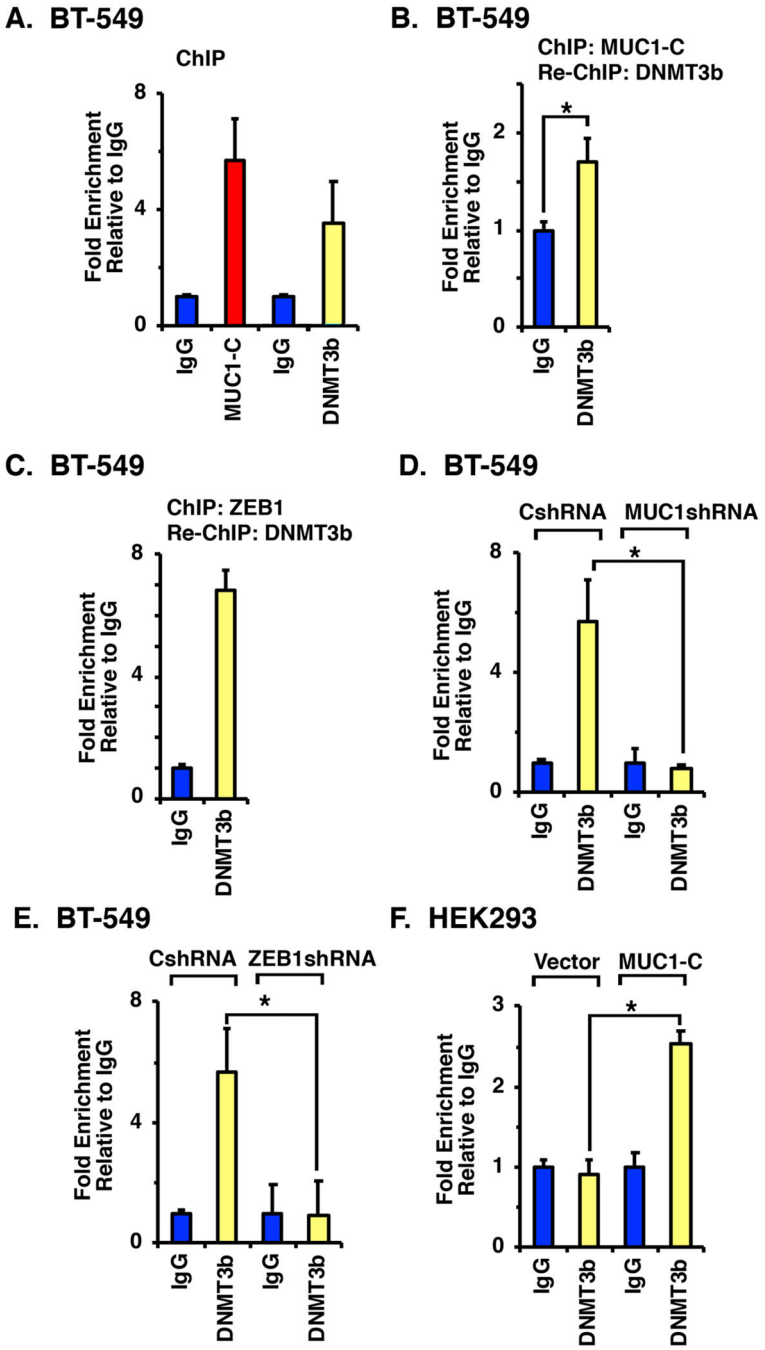

Figure 5. MUC1-C/ZEB1 form complexes with DNMT3b on the *RASSF1A* promoter.

A. Soluble chromatin from BT-549 cells was precipitated with anti-MUC1-C, anti-DNMT3b or a control IgG. B and C. In re-ChIP analyses, anti-MUC1-C (B) or anti-ZEB1 (C) precipitates were released and reimmunoprecipitated with anti-DNMT3b or a control IgG. D and E. Soluble chromatin from BT-549 cells expressing a CshRNA, MUC1shRNA (D) or ZEB1shRNA (E) was precipitated with anti-DNMT3b or a control IgG. F. Soluble chromatin from HEK293/vector and HEK293/MUC1-C cells was precipitated with anti-DNMT3b or a control IgG. The final DNA samples were amplified by qPCR with primers

for the *RASSF1A* promoter (listed in Supplemental Table S2). The results (mean±SD of three determinations) are expressed as the relative fold enrichment compared to that obtained with the IgG control (assigned a value of 1). The asterisk (*) denotes a p-value <0.05.

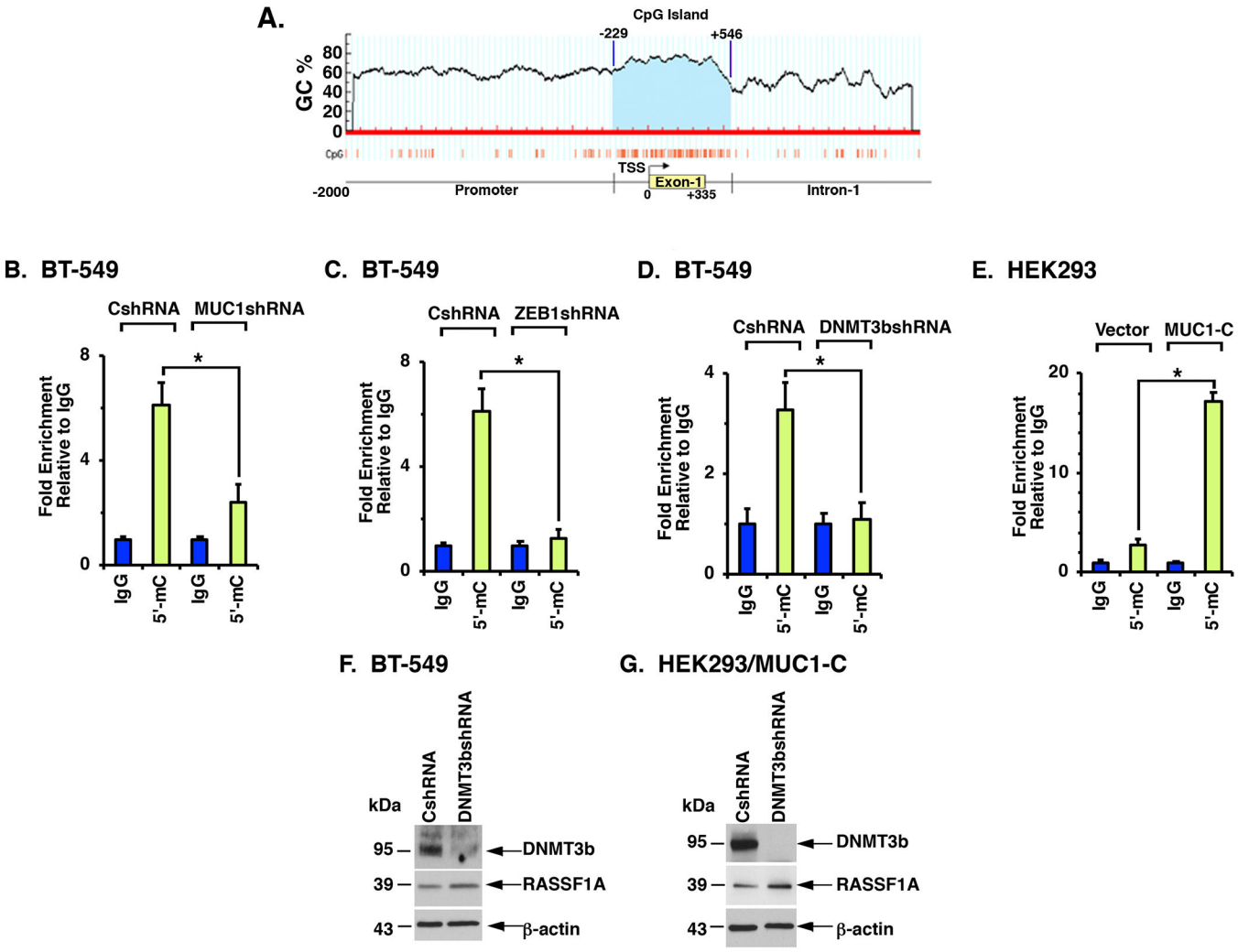

Figure 6. MUC1-C→ZEB1→DNMT3b pathway drives methylation of the *RASSF1A* promoter.

A. Schema of the *RASSF1A* promoter and exon 1 highlighting the CpG islands (blue) with the region (−130 to −31) analyzed for CpG methylation. B-D. Soluble chromatin from BT-549 cells expressing a CshRNA or MUC1shRNA (B), ZEB1shRNA (C) and DNMT3bshRNA (D) was precipitated with anti-5'-mC or a control IgG. E. Soluble chromatin from HEK293/vector and HEK293/MUC1-C cells was precipitated with anti-5'-mC or a control IgG. The final DNA samples were amplified by qPCR with primers for the *RASSF1A* promoter (listed in Supplemental Table S2). The results (mean±SD of three determinations) are expressed as the relative fold enrichment compared to that obtained with the IgG control (assigned a value of 1). F and G. Lysates from BT-549 (F) and HEK293/MUC1-C (G) cells expressing a CshRNA or DNMT3bshRNA were immunoblotted with antibodies against the indicated proteins. The asterisk (*) denotes a p-value <0.05.

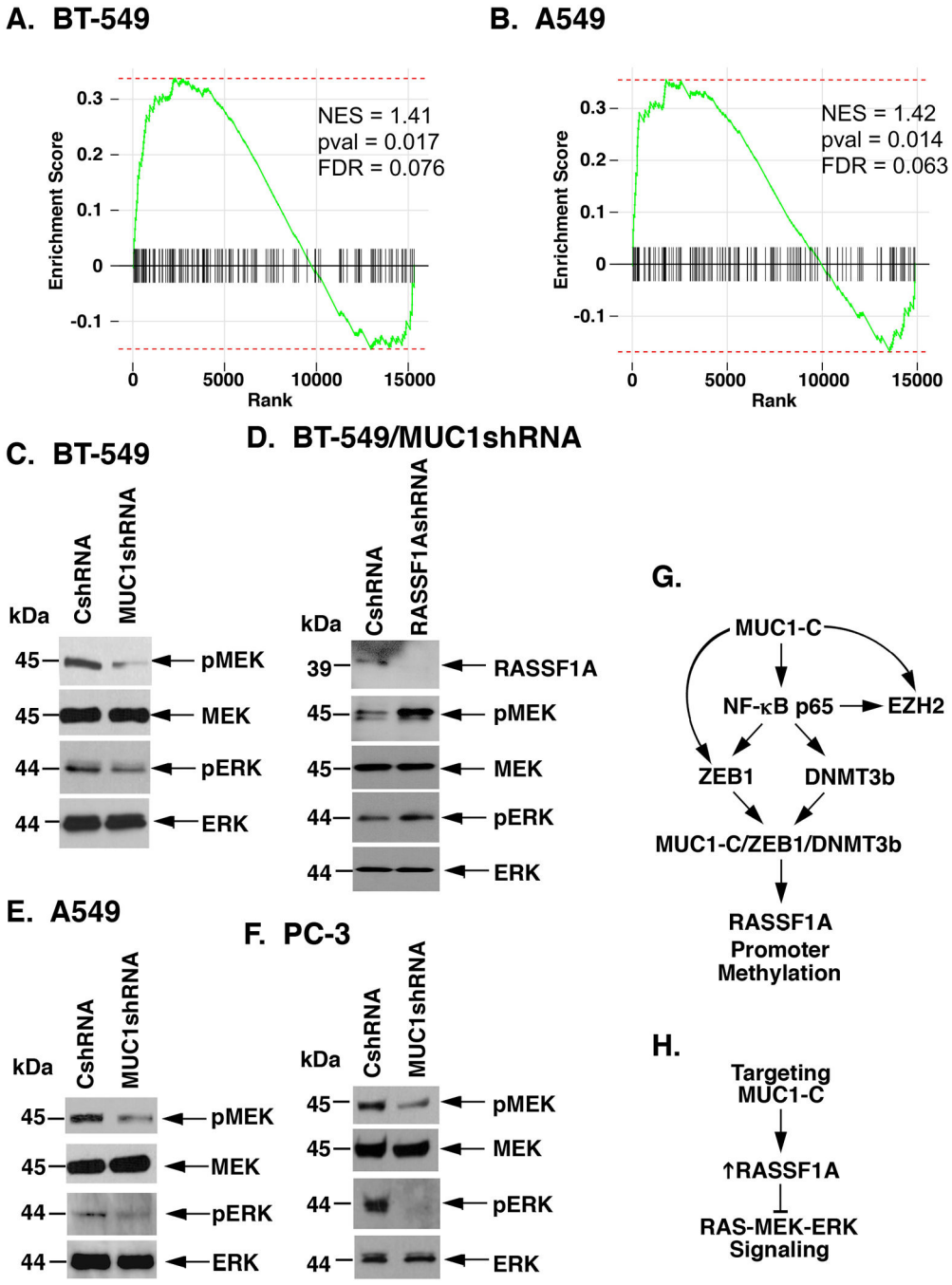

Figure 7. Targeting MUC1-C and ZEB1 suppresses MEK→ERK signaling.

A and B. RNA-seq was performed in triplicate on (A) BT-549/CshRNA and BT-549/MUC1shRNA cells, and (B) A549/CshRNA and A549/MUC1shRNA cells. The BT-549 and A549 datasets were analyzed using the Hallmark gene signature collection. MUC1-C expression was significantly associated with regulation of the KRAS pathway. C. Lysates from BT-549 cells expressing a CshRNA or MUC1shRNA were immunoblotted with antibodies against the indicated proteins. D. Lysates from BT-549/MUC1shRNA cells transfected with a CsiRNA or RASSF1AsiRNA were immunoblotted with antibodies against

the indicated proteins. E. Lysates from A549 cells expressing a CshRNA or MUC1shRNA were immunoblotted with antibodies against the indicated proteins. F. PC-3 cells expressing a CshRNA or MUC1shRNA were immunoblotted with antibodies against the indicated proteins. G-H. Proposed model depicting the roles of MUC1-C in driving repression of the *RASSF1A* gene and activation of the RAS→RAF→MEK→ERK pathway. G. MUC1-C activates the NF-κB p65 TF and thereby induction of the *ZEB1, DNMT3b* and *EZH2* genes (30, 41, 52). In turn, MUC1-C binds to ZEB1 and MUC1-C/ZEB1 complexes contribute to repression of *miR-200c* with induction of EMT. MUC1-C/ZEB1 complexes also occupy the *RASSF1A* promoter, recruit DNMT3b and drive *RASSF1A* methylation and inactivation. H. Targeting MUC1-C derepresses RASSF1A, which results in downregulation of RAF→MEK→ERK signaling. These findings support the potential involvement MUC1-C in linking the induction of ZEB1 and EMT with downregulation of RASSF1A expression and activation of the RAS→MEK→ERK pathway.