MUC1-C represses the RASSF1A tumor suppressor in human carcinoma cells

Hasan Rajabi¹ · Tsuyoshi Hata¹ · Wei Li¹ · Mark D. Long^{1,2} · Qiang Hu^{1,2} · Song Liu² · Deepak Raina^{1,3} · Ling Kui¹ · Yota Yasumizu¹ · Deli Hong¹ · Mehmet Samur¹ · Donald Kufe¹

Received: 14 January 2019 / Revised: 16 May 2019 / Accepted: 21 May 2019 / Published online: 21 August 2019
© The Author(s) 2019. This article is published with open access

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.

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

Supplementary information The online version of this article (<https://doi.org/10.1038/s41388-019-0940-1>) contains supplementary material, which is available to authorized users.

✉ Donald Kufe
donald_kufe@dfci.harvard.edu

¹ Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA

² Department of Biostatistics & Bioinformatics, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA

³ Present address: Genus Oncology, Boston, MA, USA

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 $\rightarrow$ 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 (Fig. 1a, b; Supplemental Fig. S1A). Similar results were obtained in KRAS

Fig. 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 $\pm$ SD of three determinations) are expressed as relative mRNA levels compared with 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, h** HEK293 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 $\pm$ SD of three determinations) are expressed as relative mRNA levels compared with 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 .

mutant A549 and H460 NSCLC cells (Fig. 1c, d; 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 (Fig. 1e, f). In concert with these results, enforced expression of MUC1-C in MUC1-null HEK293 cells was associated with suppression of *RASSF1A* mRNA and protein (Fig. 1g, 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 (Fig. 2d, e). 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 Fig. S2A, B), 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 (Fig. 3a, 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 (Fig. 3d, e). 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 (Fig. 3f, g). 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 (p*RASSF1A*-Luc) containing the ZEB1 binding site (Fig. 4a). p*RASSF1A*-Luc activity was induced by silencing MUC1-C (Fig. 4b) or ZEB1

Fig. 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 $\pm$ SD of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (assigned a value of 1). **f, 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 $\pm$ SD of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (assigned a value of 1). The asterisk (*) denotes a *p*-value < 0.05

(Fig. 4c). Similar studies in A549 cells confirmed these effects of MUC1-C (Fig. 4d) and ZEB1 (Fig. 4e) on

Fig. 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 $\pm$ SD of three determinations) are expressed as relative mRNA levels as compared with that obtained with cells expressing the CshRNA (assigned a value of 1). **b**, **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 $\pm$ SD of three determinations) are expressed as relative mRNA levels as compared with 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

Fig. 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 $\pm$ SD of three determinations) are expressed as relative luciferase activity as compared with that obtained with pGL3 (assigned a value of 1). The asterisk (*) denotes a *p*-value < 0.05

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 Fig. S4A–C). These and the above findings that the MUC1-C $\rightarrow$ 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 (Fig. 5b, c). 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 with 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 $\rightarrow$ MEK $\rightarrow$ 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

Fig. 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, c** In re-ChIP analyses, anti-MUC1-C (**b**) or anti-ZEB1 (**c**) precipitates were released and re-immunoprecipitated with anti-DNMT3b or a control IgG. **d, 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 $\pm$ SD of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (assigned a value of 1). The asterisk (*) denotes a *p*-value < 0.05

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 Fig. 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

Fig. 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 $\pm$ SD of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (assigned a value of 1). **f, 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

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- κ B p65 pathway, binds to NF- κ B 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- κ B 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/

Fig. 7 Targeting MUC1-C and ZEB1 suppresses MEK→ERK signaling. **a, 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 RASSF1A siRNA 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- κ B 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

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 down-regulation 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 p*RASSF1A*-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 $\pm$ 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.

Acknowledgements This work was supported by Grants from the National Cancer Institute of the National Institutes of Health under award numbers CA97098, CA166480, CA216553, CA233084, and U24CA232979.

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., W.L., D.R., Y.Y., and D.H. performed the research and data analysis. M.L., Q.H., S.L., L.K., and M.S. performed bioinformatics analysis.

Compliance with ethical standards

Conflict of interest 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 declare that they have no conflict of interest.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

References

1. Sekido Y, Ahmadian M, Wistuba II, Latif F, Bader S, Wei MH. et al. Cloning of a breast cancer homozygous deletion junction narrows the region of search for a 3p21.3 tumor suppressor gene. *Oncogene*. 1998;16:3151–7.
2. Kok K, Naylor SL, Buys CH. Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. *Adv Cancer Res*. 1997;71:27–92.
3. Richter AM, Pfeifer GP, Dammann RH. The RASSF proteins in cancer; from epigenetic silencing to functional characterization. *Biochim Biophys Acta*. 2009;1796:114–28.
4. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet*. 2000;25:315–9.
5. Donninger H, Vos MD, Clark GJ. The RASSF1A tumor suppressor. *J Cell Sci*. 2007;120(Pt 18):3163–72.

6. Agathangelou A, Cooper WN, Latif F. Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer Res.* 2005;65:3497–508.
7. van der Weyden L, Adams DJ. The Ras-association domain family (RASSF) members and their role in human tumorigenesis. *Biochim Biophys Acta.* 2007;1776:58–85.
8. Donniger H, Schmidt ML, Mezzanotte J, Barnoud T, Clark GJ. Ras signaling through RASSF proteins. *Semin Cell Dev Biol.* 2016;58:86–95.
9. Vos MD, Ellis CA, Bell A, Birrer MJ, Clark GJ. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. *J Biol Chem.* 2000;275:35669–72.
10. Matallanas D, Romano D, Al-Mulla F, O'Neill E, Al-Ali W, Crespo P, et al. Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wild-type K-Ras. *Mol Cell.* 2011;44:893–906.
11. O'Neill E, Rushworth L, Baccarini M, Kolch W. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. *Science.* 2004;306:2267–70.
12. Romano D, Nguyen LK, Matallanas D, Halasz M, Doherty C, Kholodenko BN, et al. Protein interaction switches coordinate Raf-1 and MST2/Hippo signalling. *Nat Cell Biol.* 2014;16:673–84.
13. Oh HJ, Lee KK, Song SJ, Jin MS, Song MS, Lee JH, et al. Role of the tumor suppressor RASSF1A in Mst1-mediated apoptosis. *Cancer Res.* 2006;66:2562–9.
14. Guo C, Zhang X, Pfeifer GP. The tumor suppressor RASSF1A prevents dephosphorylation of the mammalian STE20-like kinases MST1 and MST2. *J Biol Chem.* 2011;286:6253–61.
15. Matallanas D, Romano D, Yee K, Meissl K, Kucerova L, Piazzolla D, et al. RASSF1A elicits apoptosis through an MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein. *Mol Cell.* 2007;27:962–75.
16. Papaspyropoulos A, Bradley L, Thapa A, Leung CY, Toskas K, Koennig D, et al. RASSF1A uncouples Wnt from Hippo signalling and promotes YAP mediated differentiation via p73. *Nat Comm.* 2018;9:424.
17. Vos MD, Dallol A, Eckfeld K, Allen NP, Donniger H, Hesson LB, et al. The RASSF1A tumor suppressor activates Bax via MOAP-1. *J Biol Chem.* 2006;281:4557–63.
18. Baksh S, Tommasi S, Fenton S, Yu VC, Martins LM, Pfeifer GP, et al. The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to Bax conformational change and cell death. *Mol Cell.* 2005;18:637–50.
19. Dubois F, Keller M, Calvayrac O, Soncin F, Hoa L, Hergovich A, et al. RASSF1A suppresses the invasion and metastatic potential of human non-small cell lung cancer cells by inhibiting YAP activation through the GEF-H1/RhoB pathway. *Cancer Res.* 2016;76:1627–40.
20. Schmidt ML, Hobbing KR, Donniger H, Clark GJ. RASSF1A deficiency enhances RAS-driven lung tumorigenesis. *Cancer Res.* 2018;78:2614–23.
21. Pandey P, Kharbanda S, Kufe D. Association of the DF3/MUC1 breast cancer antigen with Grb2 and the Sos/Ras exchange protein. *Cancer Res.* 1995;55:4000–3.
22. Raina D, Kharbanda S, Kufe D. The MUC1 oncoprotein activates the anti-apoptotic PI3K/Akt and Bcl-xL pathways in rat 3Y1 fibroblasts. *J Biol Chem.* 2004;279:20607–12.
23. Kufe D. Mucins in cancer: function, prognosis and therapy. *Nat Rev Cancer.* 2009;9:874–85.
24. Kufe D. MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. *Oncogene.* 2013;32:1073–81.
25. Li Y, Liu D, Chen D, Kharbanda S, Kufe D. Human DF3/MUC1 carcinoma-associated protein functions as an oncogene. *Oncogene.* 2003;22:6107–10.
26. Yamamoto M, Bharti A, Li Y, Kufe D. Interaction of the DF3/MUC1 breast carcinoma-associated antigen and β -catenin in cell adhesion. *J Biol Chem.* 1997;272:12492–4.
27. Huang L, Chen D, Liu D, Yin L, Kharbanda S, Kufe D. MUC1 oncoprotein blocks GSK3 β -mediated phosphorylation and degradation of β -catenin. *Cancer Res.* 2005;65:10413–22.
28. Rajabi H, Ahmad R, Jin C, Kosugi M, Alam M, Joshi M, et al. MUC1-C oncoprotein induces TCF7L2 transcription factor activation and promotes cyclin D1 expression in human breast cancer cells. *J Biol Chem.* 2012;287:10703–13.
29. Wei X, Xu H, Kufe D. Human MUC1 oncoprotein regulates p53-responsive gene transcription in the genotoxic stress response. *Cancer Cell.* 2005;7:167–78.
30. Rajabi H, Tagde A, Alam M, Bouillez A, Pitroda S, Suzuki Y, et al. DNA methylation by DNMT1 and DNMT3b methyltransferases is driven by the MUC1-C oncoprotein in human carcinoma cells. *Oncogene.* 2016;35:6439–45.
31. Hiraki M, Maeda T, Bouillez A, Alam M, Tagde A, Hinohara K, et al. MUC1-C activates BMI1 in human cancer cells. *Oncogene.* 2016;36:2791–801.
32. Rajabi H, Hiraki M, Tagde A, Alam M, Bouillez A, Christensen CL, et al. MUC1-C activates EZH2 expression and function in human cancer cells. *Sci Rep.* 2017;7:7481.
33. Rajabi H, Hiraki M, Kufe D. MUC1-C activates polycomb repressive complexes and downregulates tumor suppressor genes in human cancer cells. *Oncogene.* 2018;37:2079–88.
34. Alam M, Bouillez A, Tagde A, Ahmad R, Rajabi H, Maeda T, et al. MUC1-C represses the Crumbs complex polarity factor CRB3 and downregulates the Hippo pathway. *Mol Cancer Res.* 2016;14:1266–76.
35. Martin-Belmonte F, Perez-Moreno M. Epithelial cell polarity, stem cells and cancer. *Nat Rev Cancer.* 2012;12:23–38.
36. Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. *Nat Rev Cancer.* 2013;13:246–57.
37. Ahmad R, Alam M, Rajabi H, Kufe D. The MUC1-C oncoprotein binds to the BH3 domain of the proapoptotic BAX protein and blocks BAX function. *J Biol Chem.* 2012;287:20866–75.
38. Rajabi H, Kufe D. MUC1-C oncoprotein integrates a program of EMT, epigenetic reprogramming and immune evasion in human carcinomas. *BBA Rev Cancer.* 2017;1868:117–22.
39. Dammann R, Yang G, Pfeifer GP. Hypermethylation of the CpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. *Cancer Res.* 2001;61:3105–9.
40. Dammann RH, Richter AM, Jimenez AP, Woods M, Kuster M, Witharana C. Impact of natural compounds on DNA methylation levels of the tumor suppressor gene RASSF1A in cancer. *Int J Mol Sci.* 2017;18:E2160.
41. Rajabi H, Alam M, Takahashi H, Kharbanda A, Guha M, Ahmad R, et al. MUC1-C oncoprotein activates the ZEB1/miR-200c regulatory loop and epithelial-mesenchymal transition. *Oncogene.* 2014;33:1680–9.
42. Zhou C, Cui F, Li J, Wang D, Wei Y, Wu Y, et al. MiR-650 represses high-risk non-metastatic colorectal cancer progression via inhibition of AKT2/GSK3 β /E-cadherin pathway. *Oncotarget.* 2017;8:49534–47.
43. Zhou C, Jiang H, Zhang Z, Zhang G, Wang H, Zhang Q, et al. ZEB1 confers stem cell-like properties in breast cancer by targeting neurogenin-3. *Oncotarget.* 2017;8:54388–401.
44. Vlahov N, Scrase S, Soto MS, Grawenda AM, Bradley L, Panikova D, et al. Alternate RASSF1 transcripts control SRC activity, E-cadherin contacts, and YAP-mediated invasion. *Curr Biol.* 2015;25:3019–34.

45. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst*. 2015;1:417–25.
46. Kazanets A, Shorstova T, Hilmi K, Marques M, Witcher M. Epigenetic silencing of tumor suppressor genes: paradigms, puzzles, and potential. *Biochim Biophys Acta*. 2016;1865:275–88.
47. Tagde A, Rajabi H, Stroopinsky D, Gali R, Alam M, Bouillez A, et al. MUC1-C induces DNA methyltransferase 1 and represses tumor suppressor genes in acute myeloid leukemia. *Oncotarget*. 2016;7:38974–87.
48. Strunnikova M, Schagdarsurengin U, Kehlen A, Garbe JC, Stampfer MR, Dammann R. Chromatin inactivation precedes de novo DNA methylation during the progressive epigenetic silencing of the RASSF1A promoter. *Mol Cell Biol*. 2005;25:3923–33.
49. Palakurthy RK, Wajapeyee N, Santra MK, Gazin C, Lin L, Gobeil S, et al. Epigenetic silencing of the RASSF1A tumor suppressor gene through HOXB3-mediated induction of DNMT3B expression. *Mol Cell*. 2009;36:219–30.
50. Bouillez A, Rajabi H, Pitroda S, Jin C, Alam M, Kharbanda A, et al. Inhibition of MUC1-C suppresses MYC expression and attenuates malignant growth in KRAS mutant lung adenocarcinomas. *Cancer Res*. 2016;76:1538–48.
51. Maeda T, Hiraki M, Jin C, Rajabi H, Tagde A, Alam M, et al. MUC1-C induces PD-L1 and immune evasion in triple-negative breast cancer. *Cancer Res*. 2017;78:205–15.
52. Ahmad R, Raina D, Joshi MD, Kawano T, Kharbanda S, Kufe D. MUC1-C oncoprotein functions as a direct activator of the NF- κ B p65 transcription factor. *Cancer Res*. 2009;69:7013–21.
53. Teng IW, Hou PC, Lee KD, Chu PY, Yeh KT, Jin VX, et al. Targeted methylation of two tumor suppressor genes is sufficient to transform mesenchymal stem cells into cancer stem/initiating cells. *Cancer Res*. 2011;71:4653–63.
54. Ram RR, Mendiratta S, Bodemann BO, Torres MJ, Eskioçak U, White MA. RASSF1A inactivation unleashes a tumor suppressor/oncogene cascade with context-dependent consequences on cell cycle progression. *Mol Cell Biol*. 2014;34:2350–8.
55. Thaler S, Hahnel PS, Schad A, Dammann R, Schuler M. RASSF1A mediates p21Cip1/Waf1-dependent cell cycle arrest and senescence through modulation of the Raf-MEK-ERK pathway and inhibition of Akt. *Cancer Res*. 2009;69:1748–57.
56. Cox AD, Der CJ. Ras history: the saga continues. *Small GTPases*. 2010;1:2–27.
57. Cox AD, Fesik SW, Kimmelman AC, Luo J, Der CJ. Drugging the undruggable RAS: Mission possible? *Nat Rev Drug Disco*. 2014;13:828–51.
58. Tolcher AW, Peng W, Calvo E. Rational approaches for combination therapy strategies targeting the MAP kinase pathway in solid tumors. *Mol Cancer Ther*. 2018;17:3–16.
59. Kidger AM, Siphthorp J, Cook SJ. ERK1/2 inhibitors: New weapons to inhibit the RAS-regulated RAF-MEK1/2-ERK1/2 pathway. *Pharm Ther*. 2018;187:45–60.
60. Takahashi H, Jin C, Rajabi H, Pitroda S, Alam M, Ahmad R, et al. MUC1-C activates the TAK1 inflammatory pathway in colon cancer. *Oncogene*. 2015;34:5187–97.
61. Vaseva AV, Blake DR, Gilbert TSK, Ng S, Hostetter G, Azam SH, et al. KRAS suppression-induced degradation of MYC is antagonized by a MEK5-ERK5 compensatory mechanism. *Cancer Cell*. 2018;34:807–22 e7.
62. Panchamoorthy G, Rehan H, Kharbanda A, Ahmad R, Kufe D. A monoclonal antibody against the oncogenic mucin 1 cytoplasmic domain. *Hybridoma*. 2011;30:531–5.