



E4BP4/NFIL3 modulates the epigenetically repressed RAS effector RASSF8 function through histone methyltransferases

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RAS proteins are major human oncogenes, and most of the studies are focused on enzymatic RAS effectors. Recently, non-enzymatic RAS effectors (RASSE, RAS association domain family) have garnered special attention because of their tumor-suppressive properties in contrast to the oncogenic potential of the classical enzymatic RAS effectors. Whereas most members of RASSF family are deregulated by promoter hypermethylation, RASSF8 promoter remains unmethylated in many cancers but the mechanism(s) of its down-regulation remains unknown. Here, we unveil E4BP4 as a critical transcriptional modulator repressing RASSF8 expression through histone methyltransferases, G9a and SUV39H1. In line with these observations, we noticed a negative correlation of RASSF8 and E4BP4 expression in primary breast tumor samples. In addition, our data provide evidence that E4BP4 attenuates RASSF8-mediated anti-proliferation and apoptosis, shedding mechanistic insights into RASSF8 down-regulation in breast cancers. Collectively, our study provides a better understanding on the epigenetic regulation of RASSF8 function and implicates the development of better treatment strategies.

RAS GTPase superfamily acts as a molecular switch to propagate signals in response to growth factors to mediate cellular processes like cell growth, migration, adhesion, and differentiation (1). Enzymatic effectors such as RAF, PI3K, and RIN1 associate with activated form of RAS through RAS association (RA)² domain and support oncogenic RAS functions (2). Recently identified nonenzymatic RAS effectors, RASSF proteins (RAS association domain family) are featured by the presence of a similar RA domain. Based on the position of RA domain, RASSF proteins are categorized into two groups:

Classical RASSFs (RASSF1–6) and N-terminal RASSFs (RASSF7–10). Interestingly, another important functional motif, SARAH (Salvador-RASSF-Hippo) domain, presents only in the classical RASSFs (3). In contrast, N-terminal RASSFs encode a coiled-coil domain in their C terminus to facilitate protein-protein interactions (4).

RASSF proteins play an important role in different cellular processes like cell cycle arrest, apoptosis, microtubule stability, and cell migration (5, 6). RASSF family members are frequently epigenetically silenced in cancers by promoter hypermethylation (7–10). Although functions and epigenetic regulation of C-terminal RASSF proteins are well-established, regulation of N-terminal RASSF expression and functions during tumorigenesis remains poorly understood. Recent report indicates that the N-terminal RASSF RASSF8 acts as a candidate tumor suppressor in lung cancer (11). This was further supported by the facts that RASSF8, involved in the maintenance of adherent junctions (13), induces cell cycle arrest and apoptosis (14). Interestingly, down-regulation of RASSF8 expression correlated with lymph node metastasis in esophageal squamous cell carcinoma, neuroblastoma, lung adenocarcinoma, male germ cell tumors, and leukemia despite insignificant promoter hypermethylation (7, 9, 11, 12, 15). These data suggest that an alternate epigenetic mechanism might regulate the RASSF8 expression and function. Data from the current investigation provide convincing evidence that transcriptional regulator E4BP4 represses RASSF8 expression through histone methylation. In addition, expression of E4BP4 and RASSF8 is inversely correlated in different tumor cell lines and primary tumor tissue samples. Furthermore, our data suggest that E4BP4 modulates RASSF8-mediated cellular apoptosis. Together, results from the present study provided in-depth insights on the transcriptional regulation and cellular function of RASSF8 in breast cancer.

Results

Identification of RASSF8 core promoter

To decipher the mechanism of transcriptional regulation of RASSF8, 2 kb of promoter sequence was retrieved from UCSC genome browser and was cloned into pGL3Basic luciferase reporter vector (Fig. S1A) as described under “Experimental procedures.” To identify the minimal promoter sequence, N-terminal deletion constructs were generated using RASSF8-

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This article contains Figs. S1–S7 and Table S1.

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² The abbreviations used are: RA, RAS association domain; 5-ADC, 5-aza deoxycytidine; t-Bid, truncated Bid; RT-qPCR, quantitative real-time PCR; RLU, relative light unit; TRAIL, TNF-related apoptosis-inducing ligand.

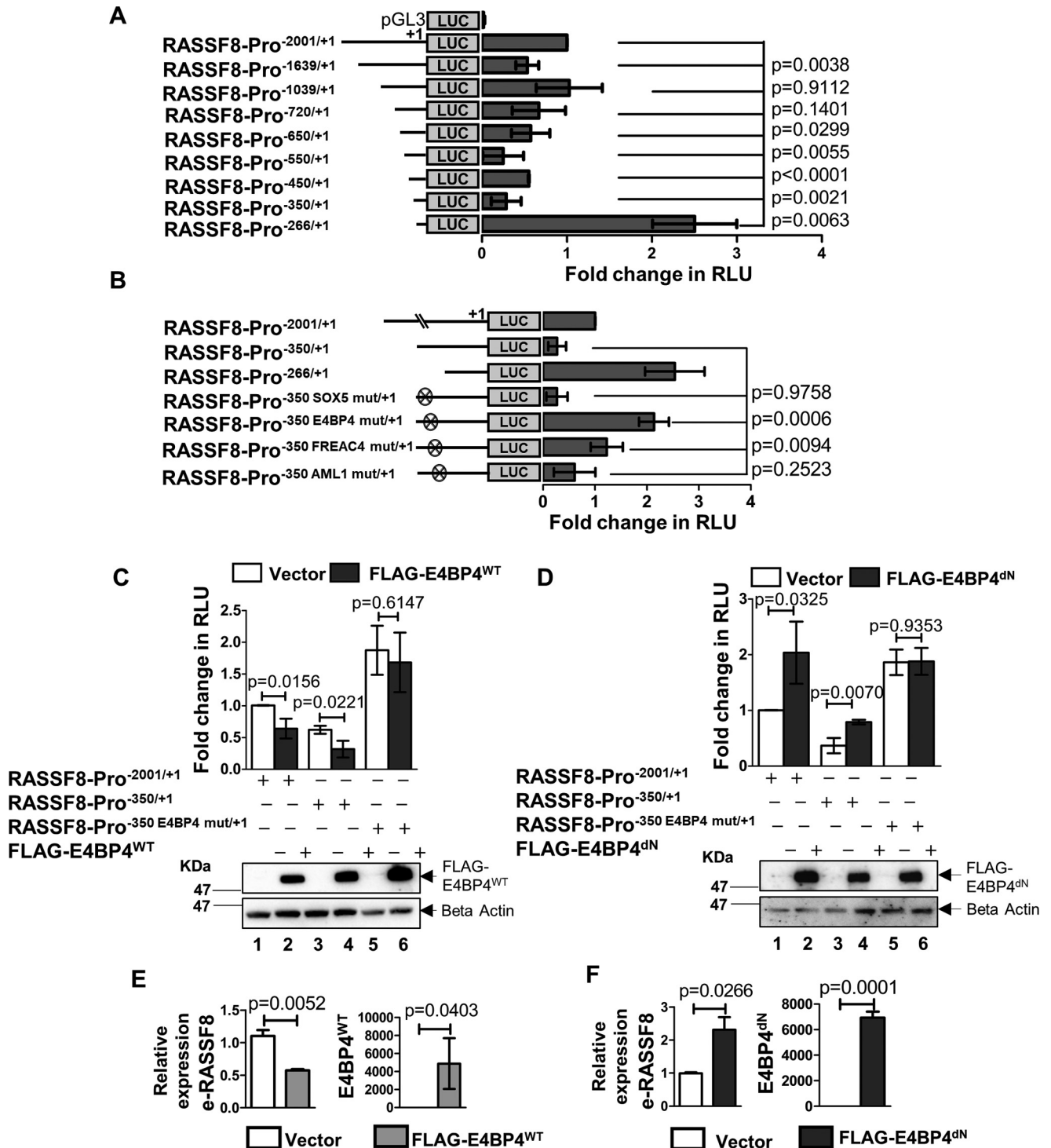


Figure 1. E4BP4 is a critical transcription factor modulating RASSF8 expression. *A*, activities of full-length and the indicated variants of RASSF8 promoter in HEK293 cells. Renilla luciferase was used as internal transfection control. Relative light unit (RLU) represents the ratio of firefly luminescence and Renilla luminescence. *B*, indicated transcription factor-binding motifs were exchanged in RASSF8-Pro^{-350/+1} and the promoter activity was determined in HEK293 cells. *C* and *D*, E4BP4 is a critical transcription factor modulating RASSF8 expression. RASSF8 promoter activity was determined in HEK293 cells cotransfected with FLAG-E4BP4^{WT} (*C*) and FLAG-E4BP4^{dN} (*D*). Western blot analysis was performed to check the expression of FLAG-E4BP4^{WT} and FLAG-E4BP4^{dN}. Beta actin was used as loading control. *E* and *F*, real-time PCR analysis indicates that ectopic expression of FLAG-E4BP4^{WT} down-regulates the expression of endogenous RASSF8 levels (*E*) and in contrast, FLAG-E4BP4^{dN} promotes RASSF8 mRNA levels (*F*) in HEK293 cells. The error bars are drawn from mean \pm SD from three independent experiments.

Pro^{-2001/+1} as template. All transfections for luciferase assay were performed in HEK293 cells. Our results indicate higher luciferase activity from cells transfected with RASSF8-Pro^{-266/+1} compared to RASSF8-Pro^{-2001/+1} and other pro-

motor deletion constructs (Fig. 1A and Fig. S1B). These data provide evidence that the core promoter elements are confined within -266/+1 sequence and are sufficient for RASSF8 expression.

E4BP4 modulates the function of RASSF8

E4BP4 is a transcriptional repressor for RASSF8

Our detailed analysis of the activity of RASSF8 promoter deletion constructs clearly indicates that a profound reduction in the promoter activity of RASSF8-Pro^{-350/+1} compared with RASSF8-Pro^{-2001/+1} and RASSF8-Pro^{-266/+1} (Fig. 1A). This convinced us to speculate that the sequences between -350 to -266 might harbor possible repressing elements. Analysis using various bioinformatics tools indicates that binding site for E4BP4, FREAC-4, AML-1, and SOX-5 transcription factors located between -350 and -266 (Fig. S1C). To define the role of these transcriptional modulators on RASSF8 expression, consensus sequences of each transcription factor in RASSF8-Pro^{-350/+1} were exchanged using site-directed mutagenesis (Fig. S1D). Results in Fig. 1B indicate that mutation of E4BP4-binding motif within RASSF8-Pro^{-350/+1} restored the promoter activity similar to the minimal promoter. In agreement with this finding, ectopic expression of FLAG-E4BP4^{WT} decreased the activity of RASSF8-Pro^{-2001/+1} and RASSF8-Pro^{-350/+1} in HEK293 cells (Fig. 1C). To define the specificity of E4BP4 activity on RASSF8 promoter, dominant negative form of E4BP4 (E4BP4^{dN}) was used, which forms nonfunctional dimers with endogenous E4BP4 and alters wildtype function. Previous reports have suggested that knockdown of E4BP4 and the expression of E4BP4^{dN} resulted in similar phenotype in neural cells (17). Interestingly, expression of E4BP4^{dN} significantly increased the activity of RASSF8-Pro^{-2001/+1} and RASSF8-Pro^{-350/+1} in HEK293 cells (Fig. 1D). Furthermore, these results are consistent with real-time quantitative PCR (RT-qPCR) data indicating that reduction of endogenous RASSF8 mRNA levels upon overexpression of E4BP4^{WT} (Fig. 1E). As expected, an opposite trend in the expression of RASSF8 mRNA level was observed upon expression of E4BP4^{dN} (Fig. 1F). Together, these results suggest that E4BP4 may be a critical factor to regulate RASSF8 expression at transcriptional level.

Toward understanding the relevance of E4BP4-mediated RASSF8 regulation in cancer, we first determined the expression status of RASSF8 and E4BP4 in different breast, neuronal, cervical, and colon cancer cell lines. RT-qPCR analysis indicates that an inverse correlation existed between RASSF8 and E4BP4 expression in all cell lines tested (Fig. S2A). These data suggest the possibility that E4BP4 may be a negative regulator of RASSF8 expression.

E4BP4 negatively regulates RASSF8 expression through histone methyltransferases, G9a and SUV39H1

Transcription factors modulate gene expression by potentially recruiting corepressors or coactivators. To date, RASSF8 stands unique from other members of the RASSF family by not being regulated by promoter hypermethylation in most cancers. Lack of data on the molecular function and epigenetic regulation of RASSF8 in breast cancer together with the reported overexpression of E4BP4 in breast cancer (18) prompted us to select breast cancer system in the current study to understand regulation of RASSF8 expression and function. To check the methylation status of RASSF8 promoter, methylation-specific PCR (MS-PCR) was performed in MCF-7, T47D,

BT-549, and SKBR-3 cell lines with the primers mentioned elsewhere (14). Interestingly, RASSF8 promoter was found to be unmethylated in all the cell lines tested (Fig. 2A). These data together with the previous reports suggest that an alternate epigenetic mechanism(s) is involved in RASSF8 regulation. A recent report suggests that E4BP4 interacts with a histone methyltransferase, G9a and occupies Fgf21 gene promoter to regulate its expression (19). Our results from the present study clearly reveal that E4BP4 is a potential modulator of RASSF8 expression. To define this further, different breast cancer cell lines (MCF-7, T47D, BT-549, and SKBR-3) were treated with DNA methylation inhibitor, 5-aza deoxycytidine (5-ADC) and G9a inhibitor, BIX-01294, and measured RASSF8 expression. RT-qPCR analysis showed that the treatment of 5-ADC did not alter the endogenous RASSF8 transcript levels whereas BIX-01294 significantly increased the endogenous RASSF8 levels in all the breast cancer cell lines tested (Fig. 2B). Treatment with different doses of BIX-01294 resulted in increased RASSF8 promoter activity in a dose-dependent manner (Fig. S3B). These data suggest that histone methylation but not DNA methylation modulates RASSF8 expression in breast cancer. Toward this, a systematic investigation was first carried out to define the possible role of histone methylation on RASSF8 expression. Methylation of histones at specific lysine residues leads to condensed chromatin which resulted in repression of gene expression. SUV39H1 is known to trimethylate the dimethylated lysine residue in histone H3 (20) (Fig. S3A). To investigate the role of E4BP4, G9a, and SUV39H1 on RASSF8 promoter activity, RASSF8-Pro^{-2001/+1} was cotransfected with E4BP4, G9a, and SUV39H1 expression plasmids in HEK293 cells. Results in Fig. 2C indicate that coexpression of E4BP4 with G9a and SUV39H1 decreased RASSF8 promoter activity, suggesting that E4BP4 alters RASSF8 promoter activity via histone methyltransferases.

Expression of E4BP4^{WT} decreased endogenous RASSF8 mRNA levels (Fig. S2B), whereas expression of E4BP4^{dN} increased the RASSF8 levels in MCF-7 cells (Fig. S2C). Furthermore, MCF-7 cells were transfected with E4BP4^{WT}, G9a, and SUV39H1 alone or in combination. As expected, RT-qPCR analysis indicated that expression of E4BP4^{WT}, G9a, and SUV39H1 efficiently repressed the RASSF8 mRNA levels (Fig. 2D). Together, these results provided evidence that E4BP4 possibly recruits G9a and SUV39H1 to RASSF8 promoter and regulates RASSF8 expression.

E4BP4 recruits methyltransferases G9a and SUV39H1 to RASSF8 promoter and promotes histone methylation to modulate RASSF8 expression

To understand whether E4BP4 directly binds to RASSF8 promoter, we first carried out electrophoretic mobility shift assay (EMSA) with nuclear protein extract from MCF-7 cells. Retarded DNA-protein complex was observed only in the presence of wildtype not mutant oligos of E4BP4-binding motif derived from RASSF8 promoter (Fig. 3A, lanes 2 and 5). Competition with cold mutant not wildtype oligo failed to reduce the DNA-protein complex formation (Fig. 3A, lanes 3 and 4). To emphasize the specificity of E4BP4 binding with RASSF8 promoter, super-shift assay was performed. Results in Fig. 3B

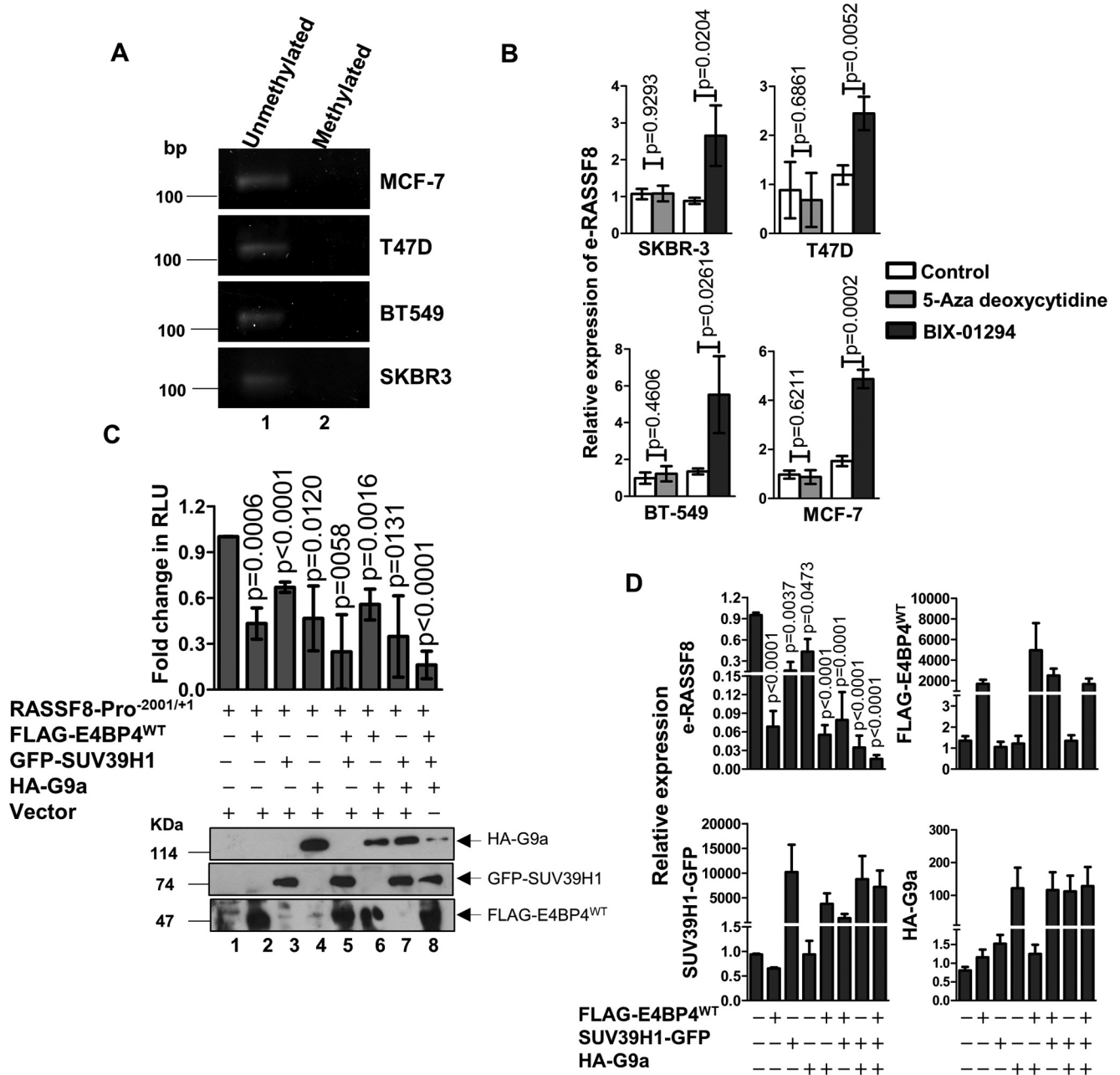


Figure 2. Histone methylation but not DNA methylation down-modulates RASSF8 expression. *A*, methylation-specific PCR was performed in bisulfite-converted genomic DNA from MCF-7, T47D, SKBR3, and BT549 cells. PCR was performed using primers mentioned elsewhere (14). *B*, RASSF8 expression was determined by RT-qPCR in indicated breast cancer cell lines treated with 5-aza deoxycytidine (5 μ M, 72 h) or BIX-01294 (6 μ M, 6 h). *C*, expression of E4BP4, SUV39H1, and G9a represent the RASSF8-Pro^{-2001/+1} activity. Western blot analysis was performed to test the expression of E4BP4, SUV39H1, and G9a. *D*, FLAG-E4BP4^{WT}, G9a, and SUV39H1 were expressed alone or in combinations in MCF-7 cells and endogenous RASSF8 mRNA levels were determined by RT-qPCR. Beta actin was used as internal control. Values represent mean \pm S.D. from three independent experiments.

clearly indicated the formation of high molecular weight complexes upon addition of anti-E4BP4 antibody (Fig. 3B, lane 3). Collectively, these data provide evidence that E4BP4 directly binds to RASSF8 promoter sequence and support the notion that E4BP4 binding may be critical to regulate RASSF8 transcription.

Further, to determine the *in vivo* occupancy of E4BP4, SUV39H1, and G9a on RASSF8 promoter, ChIP-PCR was performed in MCF-7 cells overexpressed with E4BP4^{WT} and E4BP4^{dN}, using primers designed in the region flanking E4BP4-binding site within RASSF8 promoter (Fig. S3, C and D). Results

from ChIP-PCR clearly indicate the occupancy of endogenous E4BP4, SUV39H1, G9a, and histone methyl signatures on RASSF8 promoter. Ectopic expression of E4BP4^{WT} increased the occupancy of E4BP4, G9a, SUV39H1, and respective histone methyl marks (H3K9Me2 and H3K9Me3) on RASSF8 promoter compared with vector control. Interestingly, this occupancy was significantly attenuated upon expression of E4BP4^{dN} in MCF-7 cells (Fig. 3C). Together, these results provide strong evidence that the recruitment of G9a and SUV39H1 by E4BP4 is critical to promote histone methylation to down-regulate RASSF8 expression.

E4BP4 modulates the function of RASSF8

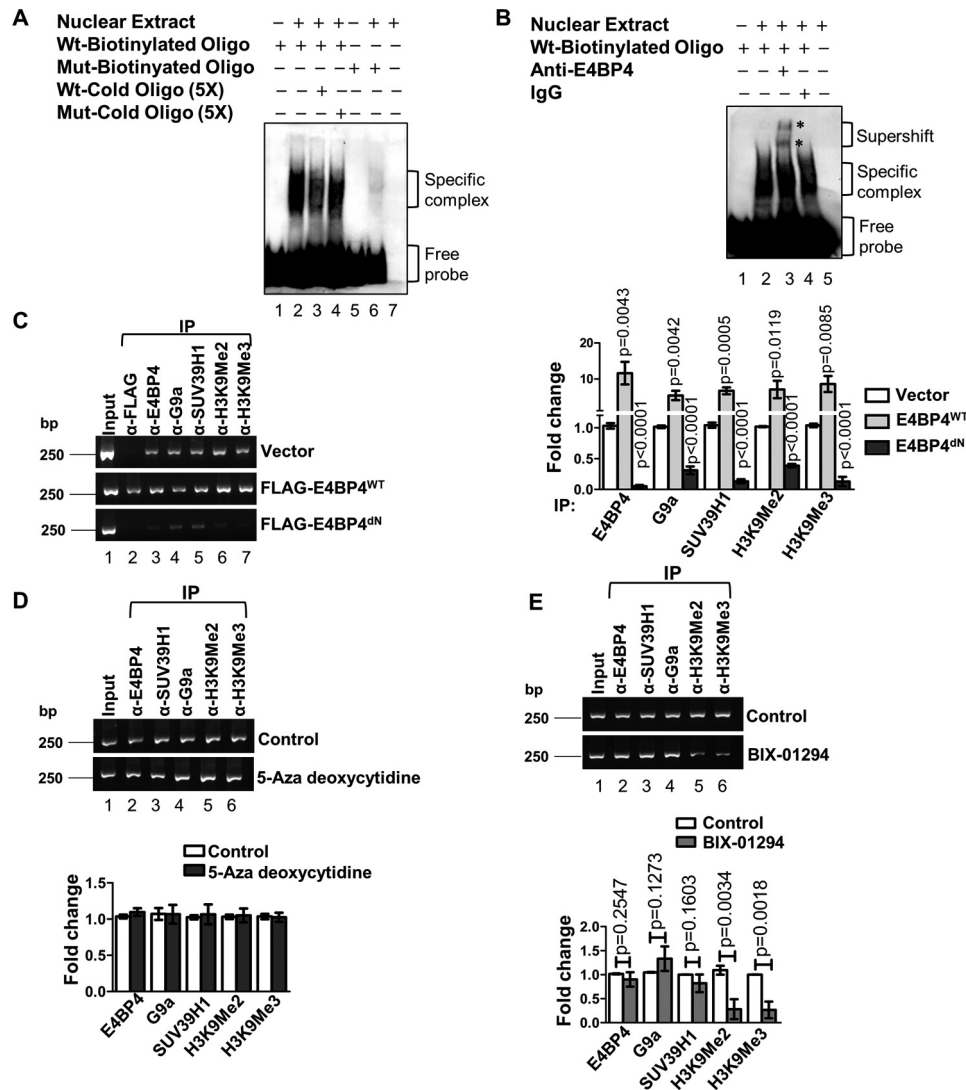


Figure 3. Occupancy of E4BP4 in RASSF8 promoter. *A*, EMSA was performed as described under “Experimental procedures.” Unlabeled wildtype or mutant oligonucleotides were used for competition in EMSA. Oligonucleotides with no nuclear extract were used as negative control. *B*, super-shift assay was performed by incubating the annealed oligonucleotides and nuclear extract of MCF-7 cells with anti-E4BP4 antibodies. The super-shifted complexes are indicated by an asterisk. *C*, MCF-7 cells ectopically expressed with E4BP4^{WT} and E4BP4^{dN} were fixed 48 h post transfection and subjected to ChIP-PCR. *D* and *E*, MCF-7 cells were treated with 5-aza deoxycytidine (5 μ M, 72 h) and BIX-01294 (6 μ M, 6 h), respectively, and performed ChIP-PCR. RT-qPCR was performed with DNA immunoprecipitated from ChIP assay and normalized with input. The graph represents -fold change with respect to control from three independent experiments, and error bars represent S.D.

Having demonstrated E4BP4, G9a, and SUV39H1 occupancy in RASSF8 promoter, we next investigated the presence of H3K9Me2 and H3K9Me3 signatures upon inhibition of histone methyltransferase activity by BIX-01294 in MCF-7 and T47D cells. The addition of BIX-01294 not 5-ADC altered the presence of these methyl signatures in RASSF8 promoter (Fig. 3, *D* and *E* and Fig. S4). Because BIX-01294 inhibits the enzymatic activity of G9a, it reduces methyl signatures without altering the occupancy of E4BP4, SUV39H1, or G9a in RASSF8 promoter (Fig. 3, *D* and *E* and Fig. S4). Together, these data provided evidence that methylation of H3K9 is critical to down-regulate RASSF8 expression in breast cancer cells.

E4BP4 attenuates anti-proliferative activity of RASSF8

Results from the current investigation provided evidence that E4BP4 down-regulates RASSF8 expression via histone meth-

ylation. To define the role of E4BP4 on RASSF8 function, we first determined the anti-proliferative function of RASSF8. Toward this end, RASSF8 expression frequency in different cancer samples was first analyzed using BioXpress database and showed a significant down-regulation of RASSF8 in many cancers (Fig. 4A). In addition, results from GENT database analysis indicate that RASSF8 mRNA expression level was down-regulated in breast cancer tissues compared with normal (Fig. 4B). These data suggest a possible growth regulatory function for RASSF8. MCF7 and T47D cells expressing RASSF8 exhibited lesser formation of formazan crystals compared with vector control in MTT assay (Fig. 4C and Fig. S5A). In contrast, knock-down of RASSF8 with specific shRNA exhibited increased formation of formazan crystals in both MCF-7 and T47D cells (Fig. 4D and Fig. S5B). Further to confirm, colony formation assay was performed in MCF-7 cells and the results showed reduced

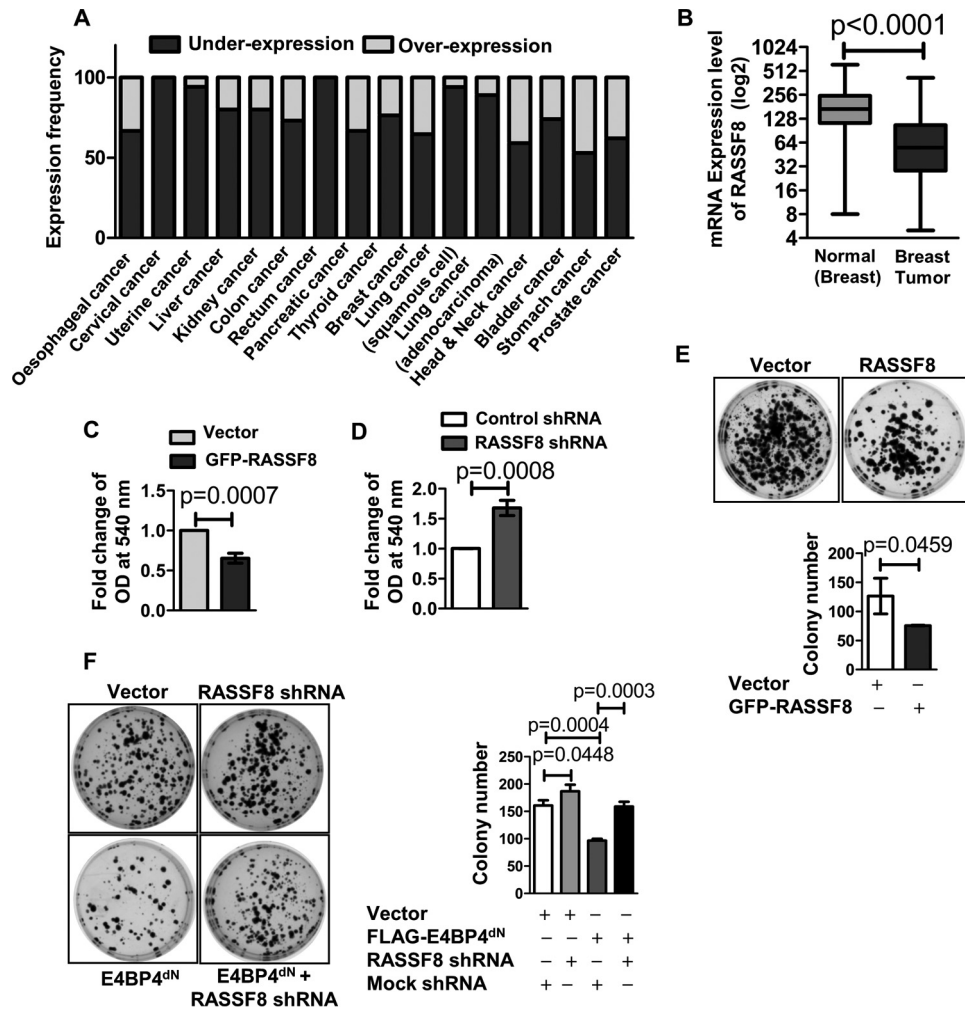


Figure 4. RASSF8 inhibits cell proliferation. A, the expression frequency of RASSF8 in different cancers was retrieved from BioXpress database. The expression frequency was normalized with respective normal samples. B, RASSF8 expression in normal and breast cancer in different datasets was retrieved from GENT database and represented in *bar graph*. C and D, MCF-7 cells were transfected with GFP-RASSF8 (C) and RASSF8 shRNA (D) and the cell viability was determined by MTT assay as per manufacturer's instructions. E, GFP-RASSF8 was ectopically expressed in MCF-7 cells and the GFP-positive cells were sorted using MoFlo Astrios EQ cell sorter (Beckman). The assay was performed as described under "Experimental procedures." F, MCF-7 cells were overexpressed with FLAG-E4BP4^{dN} and RASSF8 was knocked down using shRNA. GFP (1:10 ratio) was cotransfected in all indicated transfection experiments for sorting cells for colony formation assay. Sorted cells (2500/well) were plated onto six-well plates and selected with G418 (40 μ g/ml) for 14 days. The colonies were stained with crystal violet and counted using ImageJ software. Values in the bar graph are from biological triplicates (error bars \pm S.D.).

number of colonies in RASSF8-expressing cells compared with vector control (Fig. 4E). Collectively, these results provide a direct evidence for RASSF8 function on cell proliferation.

To further understand RASSF8 function, endogenous RASSF8 level was first altered by masking E4BP4 function with E4BP4^{dN}. Results from RT-qPCR indicate that expression of E4BP4^{dN} promoted endogenous RASSF8 expression which resulted in significant decrease in the number of colonies (Fig. 4F and Fig. S6A). In contrast, knockdown of endogenous RASSF8 resulted in increased colony numbers (Fig. 4F). Comparatively, knockdown of RASSF8 in MCF-7 cells expressing E4BP4^{dN} resulted in significantly reduced colony numbers (Fig. 4F). To support these results, a significant increase in the rate of cellular apoptosis was observed upon ectopic expression of E4BP4^{dN} (Fig. 5A). Increase in RASSF8 mRNA level was observed upon overexpression of E4BP4^{dN} (Fig. S6B). Conversely, expression of E4BP4^{dN} under RASSF8 knockdown condition resulted in significant reduction of apoptosis (Fig. 5A). Together, these results suggest that E4BP4 modulates the anti-

proliferative function of RASSF8 by repressing its expression in breast cancer cells.

RASSF8 induces apoptosis in caspase-dependent manner

To define the mechanism by which RASSF8 reduces cell proliferation, we performed annexin V staining and measured apoptotic population from RASSF8-expressing cells by flow cytometry. Results in Fig. S5, C and D reveal that RASSF8 expression induces apoptosis in both MCF-7 and T47D cell lines. Interestingly, we observed RASSF8-induced apoptosis in MCF-7 cells which are caspase-3 negative. To understand whether RASSF8 induces apoptosis in caspase-3-negative cells through other caspases or by caspase-independent pathway, RASSF8-expressing MCF-7 cells were treated with two pan-caspase inhibitors (100 μ M Z-VAD-FMK and 20 μ M Q-VD-OPh) and measured apoptosis. Interestingly, RASSF8-induced apoptosis was significantly reduced upon inhibition of caspases (Fig. 5B and Fig. S7C) suggesting that RASSF8 induces apoptosis through

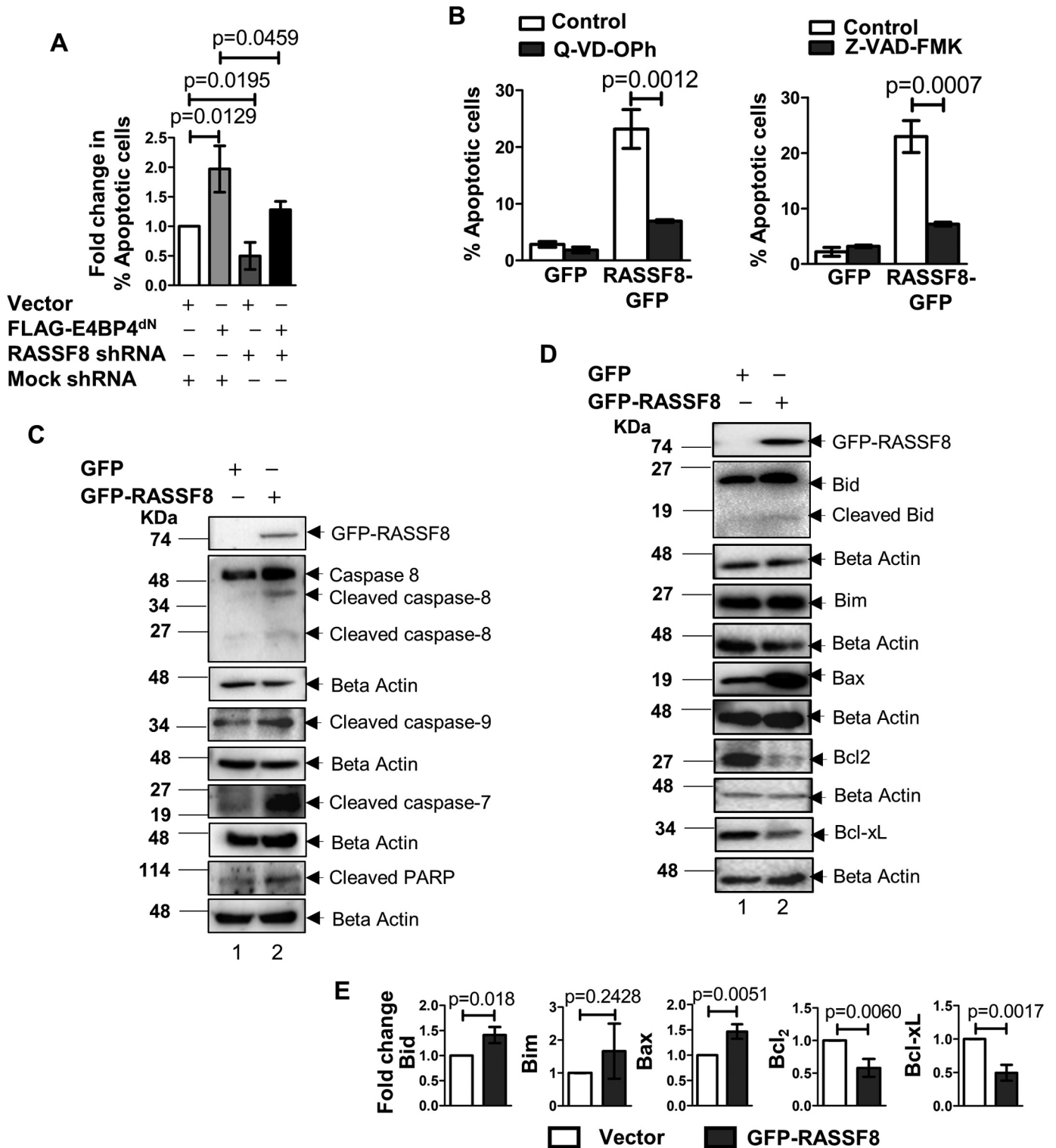


Figure 5. RASSF8-induced apoptosis is modulated by E4BP4. *A*, RASSF8 knockdown was performed in MCF-7 cells with or without ectopic expression of FLAG-E4BP4^{dN}. Transfected cells were subjected to annexin V staining and analyzed using flow cytometry to determine the status of apoptosis. *B*, RASSF8 expressed in MCF-7 cells was treated with pan-caspase inhibitors Z-VAD-FMK and Q-VD-OPh. Cells were stained with annexin V and status of apoptosis was measured using flow cytometry analysis. *Bar graphs* indicate the percentage apoptotic cells. Values are from biological triplicates; error bars represent \pm S.D. *C*, MCF-7 cells were ectopically expressed with RASSF8 and the cleavage status of caspases and poly (ADP-ribose) polymerase (PARP) was determined by Western blotting using indicated antibodies. *D*, RASSF8 was ectopically expressed in MCF-7 cells and the indicated Bcl2 family of pro-apoptotic and anti-apoptotic proteins levels were determined by Western blot analysis using respective antibodies. *E*, the graphs indicate the -fold change of the band intensity of indicated proteins in *D* normalized to beta actin using ImageJ software. The error bars indicate S.D. of three independent experiments.

caspase-dependent mechanism. Furthermore, results in Fig. 5C clearly indicate that RASSF8 promotes cleavage of caspases 7, 8, and 9. Increased levels of cleaved poly (ADP-ribose) polymerase (PARP) was observed upon RASSF8 expression compared with vector-transfected cells (Fig. 5C, lane 2). Together, these data suggest that RASSF8 induces apoptosis in caspase-dependent manner.

RASSF8 modulates the expression of Bcl2 family proteins to promote apoptosis

Commitment of cells to undergo apoptosis is determined by the Bcl2 family proteins. The BH domain-containing family comprises pro-apoptotic (Bax, Bid, Bim, Bak, and Bik) and anti-apoptotic (Bcl2, BclxL, and Mcl) proteins (21). Any alteration in the physiological levels of these proteins contributes to a significant change in mitochondrial membrane permeabilization which alters the rate of apoptosis in cells. In the present investigation, expression of RASSF8 significantly reduced the levels of anti-apoptotic Bcl2 and BclxL proteins and increased pro-apoptotic Bax and Bid protein levels in both MCF-7 and T47D cells (Fig. 5, D and E and Fig. S7A). Furthermore, we observed a significant reduction of Bcl2 and BclxL mRNA levels and, in contrast, Bax and Bid mRNA levels were increased in MCF-7 cells upon RASSF8 expression (Fig. S7B). Caspase 8 cleaves Bid into truncated Bid (t-Bid) which localizes to the mitochondria from cytosol and induces mitochondrial damage to promote apoptotic cell death (22). The observed increased level of t-Bid upon RASSF8 overexpression (Fig. 5, D and E and Fig. S7A) further supports the involvement of caspase 8 on RASSF8-induced apoptosis. These data suggest that Bcl2 family proteins are critical for RASSF8-induced apoptosis.

Clinical relevance of RASSF8 and E4BP4 expression in breast cancer

Having elucidated the E4BP4-mediated regulation of RASSF8 function, we next investigated the clinical relevance of this regulation in cancers. To this end, the expression profile of RASSF8, E4BP4, G9a, and SUV39H1 was analyzed from BioXpress database which utilizes TCGA RNA-seq datasets. The observed negative Pearson coefficient signifies an inverse correlation of RASSF8 with E4BP4, G9a, and SUV39H1 expression in breast cancer patient samples (Fig. 6A). These data support the results from the current investigation that E4BP4, G9a, and SUV39H1 are negative regulators of RASSF8 expression. To further demonstrate its clinical implication, Kaplan-Meier survival plot analysis was performed using RASSF8 and E4BP4 expression levels from TCGA, GEO DataSets by online KM plotter tool. Results from this analysis clearly indicate better patient survival with higher RASSF8 expression (Fig. 6B). On the other hand, higher E4BP4 expression resulted in poor survival of breast cancer patients (Fig. 6B). Collectively, these observations suggest the clinical significance of E4BP4 and its transcriptional target, RASSF8 in breast cancer prognosis.

Discussion

RASSF8 belongs to N-terminal Ras-association domain family of proteins and recently emerged as a potential tumor suppressor. Down-regulation of RASSF family members in cancers

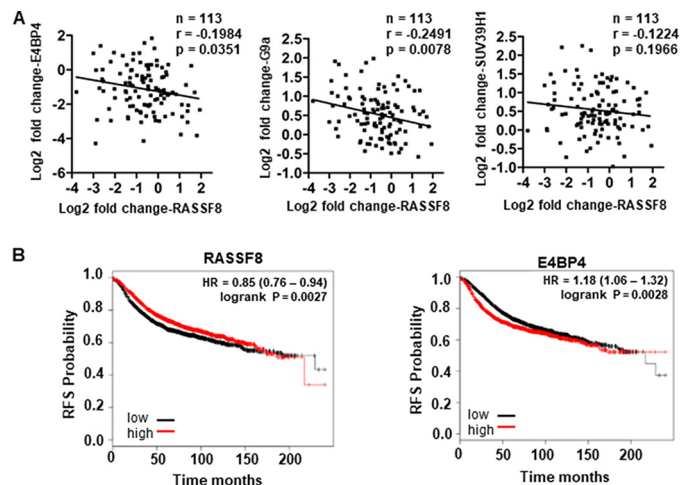


Figure 6. Expression of RASSF8 negatively correlates with the expression status of E4BP4 and histone methyltransferases in primary breast cancers. A, expression levels of RASSF8, E4BP4, G9a, and SUV39H1 were retrieved from BioXpress database. The -fold change of expression were plotted in a correlation plot. Pearson co-efficient was calculated using GraphPad Prism to show the inverse correlation of RASSF8 against E4BP4, G9a, and SUV39H1 expression in breast cancer datasets. B, differential expression levels of RASSF8 and E4BP4 determine the survival status of breast cancer patients. Kaplan-Meier survival plot indicates the better survival of patients with high levels of RASSF8, which negatively correlates with E4BP4 expression.

has been observed because of promoter hypermethylation (5–10). Surprisingly, RASSF8 promoter was found to be unmethylated in most cancers (9, 11, 13) except a small subset of leukemia and melanoma (7, 14) but the mechanism remains unexplored. Data from the present investigation provides evidence for the first time that transcriptional modulator E4BP4 represses RASSF8 expression in breast cancer by promoting histone methylation through G9a and SUV39H1. In addition, loss of G9a-dependent H3K9 methylation facilitates active transcription of RASSF8. Furthermore, our results suggest that E4BP4 inhibits RASSF8-induced apoptosis by modulating its expression. Additionally, negative correlation of E4BP4 and RASSF8 expression in primary breast tumors was observed which supports the results from the present study that E4BP4 negatively regulates RASSF8 expression in breast cancer.

E4BP4 is a key signaling component in a myriad of cellular processes including metabolism, nerve regeneration, immune development, and cancer. E4BP4 has emerged as a novel factor that contributes to cancer by repressing the expression of pro-apoptotic TRAIL (18). Additionally, knockdown of E4BP4 up-regulated TRAIL expression in breast cancer (18). E4BP4 represses the transcription of several viral and cellular genes (23). However, there is no direct evidence of cross-talk between E4BP4 and RAS effectors on cell growth regulation. Current study is the first to demonstrate that E4BP4 negatively regulates RASSF8 transcription. Abrogation of E4BP4 binding to the RASSF8 promoter resulted in increased RASSF8 transcription. DNA and the associated histone proteins in the chromatin face a large number of encounters with chromatin-modifying enzymes to acquire posttranslational modifications such as acetylation, methylation, and phosphorylation (24–27). Histone and DNA modifiers coordinate chromatin condensation through direct interactions (28, 29). In prostate cancer, ChIP on

E4BP4 modulates the function of RASSF8

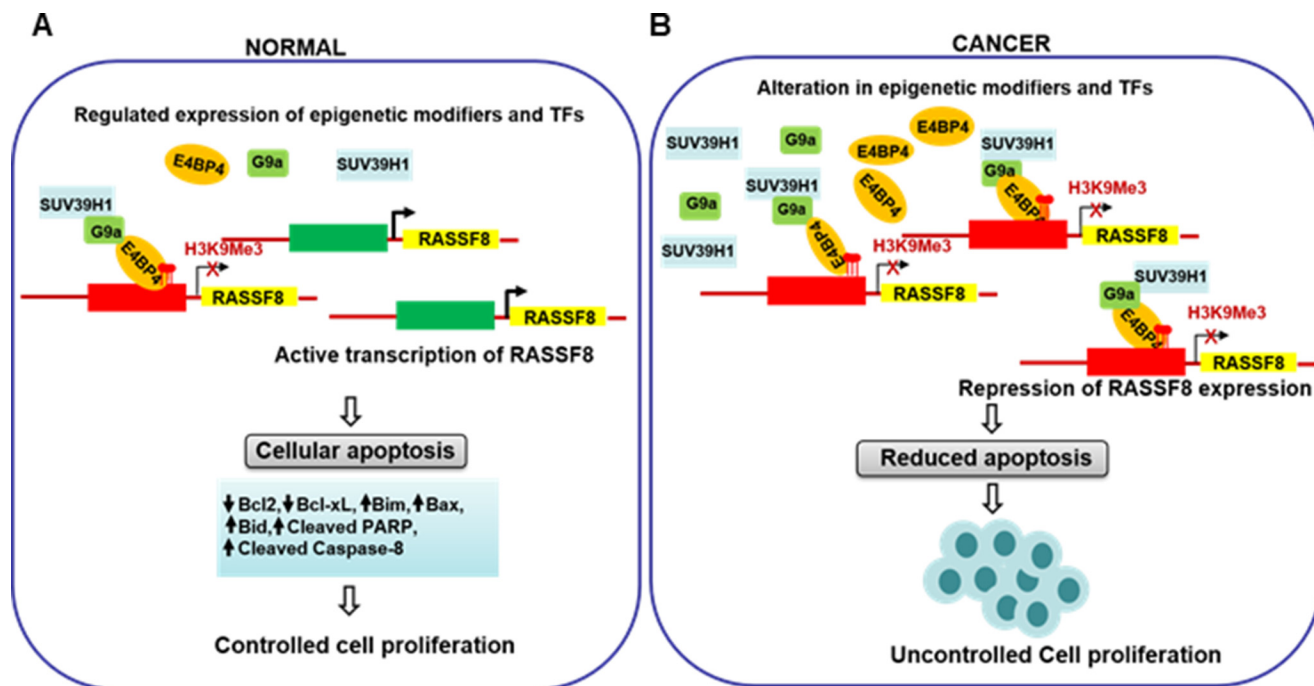


Figure 7. Proposed schematic model summarizing the role of E4BP4 and epigenetic modifiers on RASSF8 expression and apoptotic function. A, in normal conditions, RASSF8 expression was not altered by because of the basal level expression of E4BP4, G9a, and SUV39H1. RASSF8 regulates cellular apoptosis by modulating the expression of Bcl2 proteins and caspases. B, in cancer, there is an aberrant expression of E4BP4, G9a, and SUV39H1 which promotes their active participation in epigenetic repression of RASSF8 and resulted in uncontrolled cell proliferation.

ChIP experiments indicated that 5% of promoters (16% CpG islands and 84% non-CpG islands) was enriched with H3K27Me3 signature. The non-CpG regions possessed histone methylation, and gene silencing was observed in those regions (30). Recent reports showed that microRNA-139 was silenced by H3K27Me3 independent of DNA methylation in lung cancer (31). Data available from the existing literature on E4BP4 interaction with histone methyltransferase G9a (19), together with observed down-regulation of RASSF8 expression by E4BP4 in the present investigation suggest the possibility that histone methylation may regulate RASSF8 expression and function. Results from this study reveal that inhibition of the histone methyltransferase activity of G9a increased RASSF8 expression in breast cancer cells and suggest that G9a is a novel epigenetic modifier of RASSF8 expression. G9a catalyzes monomethylation and dimethylation of H3K9 (32). However, the trimethylated H3K9 is a more potent inhibitory signature leading to chromatin compaction. G9a in cooperation with SUV39H1 trimethylates histones (20). The current study demonstrates that the recruitment of G9a and SUV39H1 by E4BP4 induced H3K9me2 and H3K9me3 signatures in RASSF8 promoter. These data unravel the novel role of histone methylation in transcriptional regulation of RASSF8. Dynamic epigenetic modification in RASSF8 promoter might be critical to achieve a robust proliferation of breast cancer cells. Collectively, our data suggest that coordinated activities of E4BP4, G9a, and SUV39H1 is critical to down-regulate RASSF8 expression in cancer. Future work is warranted to understand how E4BP4 expression is induced to modulate RASSF8 transcription during tumorigenesis.

Results from the current study demonstrate that RASSF8 induces apoptosis by modulating the levels of pro-apoptotic

(Bax and Bid) and anti-apoptotic proteins (Bcl2 and BclxL). Previous reports suggest that Bid is cleaved into t-Bid by caspase-8 and induces changes in mitochondrial membrane potential to promote apoptosis (22, 33). We observed ectopic expression of RASSF8 resulted in the increased accumulation of t-Bid and cleaved caspase 8 in breast cancer cells. It is known that cleaved caspase-8 and t-Bid activate caspase-9 and caspase-7 to promote apoptosis (34, 35). Results from our study clearly suggest that the activation of caspases-7, -8, and -9 and Bid is essential for RASSF8-induced apoptosis. Collectively, these data provide evidence that RASSF8 promotes caspase-dependent apoptosis in breast cancer cells.

E4BP4 has emerged as a survival factor that blocks the induction of apoptosis in numerous systems from B cells to motor neurons (36, 37). Reports indicate that E4BP4 induces the expression of anti-apoptotic Bcl2 in rat embryonic fibroblasts (36) and altered the Fas ligand (FasL)-induced motor neuron cell death upon trophic factor deprivation (37). These data suggest the possibility that E4BP4 might alter the apoptotic function of RASSF8. Interestingly, results from the clonogenic assays indicate that E4BP4^{dN} alters RASSF8 mediated cell proliferation. In addition, ectopic expression of E4BP4^{dN} promoted apoptosis and was reversed upon RASSF8 knockdown. Collectively, based on these data, we propose a model suggesting that E4BP4 modulates anti-proliferative function of RASSF8 and further provides evidence that RASSF8 is a potential tumor suppressor in breast cancer (Fig. 7). Under normal condition, RASSF8 mRNA is actively expressed and controls cell proliferation because of the basal level expression of E4BP4 and epigenetic modifiers (Fig. 7A). It is known that E4BP4, G9a, and SUV39H1 levels are up-regulated in many cancers (18, 38–43). The observed down-regulation of RASSF8 may be

because of the aberrant expression of E4BP4, G9a, and SUV39H1 during tumorigenesis (Fig. 7B). Recent reports suggest that microRNA-224 is involved in regulating RASSF8 expression in cervical, gastric, and lung cancers (44–46) and DNA methylation in melanoma (14). The present investigation is the first report to demonstrate that histone methylation is one of the epigenetic regulations contributing to the differential expression of RASSF8 in breast cancer. Deregulation in RASSF8 expression contributes to uncontrolled cell proliferation during tumorigenesis by reducing the rate of cellular apoptosis (Fig. 7B). In conclusion, E4BP4 represses RASSF8 expression via histone modification by recruiting histone methyltransferases, G9a, and SUV39H1 to RASSF8 promoter. Expression of RASSF8 and E4BP4 was inversely correlated in breast cancers and may be used as a potential target to identify anti-cancer therapeutics.

Experimental procedures

Plasmid construction

RASSF8 (NM_001164746) and E4BP4 (NM_001289999.1) were cloned in pcDNA3 as GFP and FLAG fusions, respectively. RASSF8 promoter was cloned in promoterless pGL3Basic vector. Site-specific substitution mutations in RASSF8-Pro^{-350/+1} were introduced using QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Dominant negative E4BP4 expression construct (nine amino acid substitutions in DNA-binding domain: YWEKRRKNNEAAKRSRE → YWEQSQQYSEPPQRSRE) was a generous gift from Dr. Toshiya Inaba, Hiroshima University, Japan (47). SUV39H1-EGFP plasmid was a kind gift from Dr. Sanjeev Khosla, Centre for DNA Fingerprinting and Diagnostics, India. pCMV-G9a-HA was procured from Addgene (plasmid no. 33024). The primers used for cloning are listed in Table S1. For the knockdown studies, control shRNA (SHC016) and RASSF8 shRNA (TRCN0000140473, TRCN0000122298) were procured from Sigma-Aldrich.

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 and fetal bovine serum (FBS) were from Invitrogen Life Technologies. Annexin V-APC was procured from BD Biosciences. Caspase inhibitors, 5-aza deoxycytidine, BIX-01294 were from Sigma. The antibodies used for ChIP-PCR were from Abcam, UK, and for caspases and Bcl2 proteins were from Cell Signaling Technology (Danvers, MA). All other reagents used in this study are of molecular biology grade and were procured from standard suppliers.

Cell lines, culture conditions, and transfections

HEK293, MCF-7, T47D, and BT-549 cell lines were maintained in DMEM; SKBR-3 cell line was maintained in RPMI 1640 media supplemented with 10% FBS at 37 °C under 5% CO₂. Cells were seeded at about 60% confluency and all the transfections were performed with linear polyethylene imine (PEI) (Polysciences Inc., Warrington, PA).

Luciferase assay

Reporter constructs were cotransfected with pRL-TK (Renilla Luciferase under thymidine kinase constitutive pro-

motor) plasmid in HEK293 in 1:10 ratio as an internal transfection control. Luciferase assay was performed as described elsewhere (48).

ChIP-PCR

ChIP-PCR was performed in MCF-7/T47D cells as described previously (49). Antibodies against G9a (ab133482), SUV39H1 (ab12405), E4BP4 (ab93785), H3K9Me2 (ab1220), and H3K9Me3 (ab8898) were used in this assay. The eluted DNA was then purified using phenol-chloroform and used for PCR analysis. The primers used for PCR are listed in Table S1. RT-qPCR was carried out using SYBR Green PCR mix (Takara Bio, Kusatsu, Japan) and the calculations were performed using the percent input method.

EMSA

Nuclear extract from MCF-7 cells was prepared using NEPER Nuclear and Cytoplasmic Extraction Kit (Thermo Fischer Scientific) according to manufacturer's protocol. The single-stranded oligos were biotinylated using biotin-11-dUTP (Thermo Fischer Scientific) and treated with terminal transferase (New England Biolabs). Anti-E4BP4 (ab93785, Abcam) was used in super-shift assay. Assay was carried out per manufacturer's instructions (LightShift Chemiluminescent EMSA Kit, Thermo Fischer Scientific).

Methylation-specific PCR

Genomic DNA was isolated from MCF-7, T47D, BT549, and SKBR3 cells using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Genomic DNA (1 µg) was bisulfite-modified using EpiTect Bisulfite kit (Qiagen) according to manufacturer's instructions. PCR amplifications were performed using primers used elsewhere (14).

Flow cytometry

Apoptosis assay was performed using APC annexin V (BD Biosciences) as per manufacturer's instructions. Stained population was measured using FACSCanto II and analyzed in FACSDIVA™ software (BD Biosciences).

MTT assay, RT-qPCR, and Western blotting

MTT assay, RT-qPCR, and Western blotting were performed as described previously (50). Primers used for RT-qPCR are listed in Table S1.

Colony formation assay

After 48 h of transfection, GFP-positive MCF-7 cells were sorted using MoFlo Astrios EQ Cell Sorter (Beckman Coulter, Indianapolis, IN) in six-well plates as triplicates and cultured with G418 (40 µg/ml). For FLAG and shRNA constructs, GFP-pcDNA3 was cotransfected in 1:10 ratio to facilitate sorting of GFP-positive cells. After 14 days of selection, the cells were fixed with 10% neutral buffered formalin solution and stained with 0.01% crystal violet and photographed using ChemiDoc MP Imaging System (Bio-Rad). The number of colonies were counted using ImageJ software.

Public data sources: Survival and gene expression analysis

RASSF8 expression levels in normal and cancer tissues were obtained from GENT (Gene Expression database of Normal and Tumor tissues) database which utilizes data from GEO DataSets. The expression dataset is created by utilizing Affymetrix U133A and U133 Plus 2 platforms from public resources in the GENT database (51). RASSF8 expression data in normal *versus* tumor in GSE 10810, GSE 3744, GSE 5764, GSE 8977, GSE 20086, and GSE 5364 were collected from the GENT database. Normal (87) and tumor (277) sample data from these datasets were plotted as box plots using GraphPad software. For correlation analysis, expression -fold change of RASSF8, E4BP4, SUV39H1, and G9a in breast cancer samples was retrieved from BioXpress database (<http://hive.biochemistry.gwu.edu/tools/bioexpress>)³ (52). The expression frequency plotted was calculated from the -fold change in tumor samples relative to respective normal samples. Kaplan-Meier analysis for survival of the breast cancer patients was performed using the online Kaplan-Meier plotter database (<http://www.Kmplot.com>)³ (16).

Statistical analysis

All statistical analysis was performed using GraphPad Prism 5.0 software. Error bars represent mean \pm S.D. from three independent experiments. Statistical significance was obtained by Student's unpaired *t* test using GraphPad Prism 5.0. For colony formation assays and apoptosis assay with caspase inhibitors, data are representative of three independent experiments and error bars represent mean \pm S.D. from biological triplicates. For KM plots, statistical significance was projected from the log rank *p* value.

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