Published in final edited form as:

Cancer Res. 2008 April 15; 68(8): 2726–2735. doi:10.1158/0008-5472.CAN-07-6654.

DNA Methyltransferase 1 and 3B Activate *BAG-1* Expression via Recruitment of CTCFL/BORIS and Modulation of Promoter Histone Methylation

Lunching Sun¹, Lei Huang¹, Phuongmai Nguyen¹, Kheem S. Bisht¹, Gil Bar-Sela¹, Allen S. Ho¹, C. Matthew Bradbury¹, Wenqiang Yu³, Hengmi Cui³, Sunmin Lee², Jane B. Trepel², Andrew P. Feinberg³, and David Gius¹

¹ Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland ² Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland ³ Department of Medicine and Center for Epigenetics, Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

In a previous genomic analysis, using somatic methyltransferase (*DNMT*) knockout cells, we showed that hypomethylation decreased the expression of as many genes as were observed to increase, suggesting a previously unknown mechanism for epigenetic regulation. To address this idea, the expression of the *BAG* family genes was used as a model. These genes were used because their expression was decreased in *DNMT1*^{-/-}, *DNMT3B*^{-/-}, and double knockout cells and increased in *DNMT1*-overexpressing and *DNMT3B*-overexpressing cells. Chromatin immunoprecipitation analysis of the *BAG-1* promoter in *DNMT1*-overexpressing or *DNMT3B*-overexpressing cells showed a permissive dimethyl-H3-K4/dimethyl-H3-K9 chromatin status associated with DNA-binding of CTCFL/BORIS, as well as increased *BAG-1* expression. In contrast, a nonpermissive dimethyl-H3-K4/dimethyl-H3-K9 chromatin status was associated with CTCF DNA-binding and decreased *BAG-1* expression in the single and double *DNMT* knockout cells. *BORIS* short hairpin RNA knockdown decreased both promoter DNA-binding, as well as *BAG-1* expression, and changed the dimethyl-H3-K4/dimethyl-H3-K9 ratio to that characteristic of a nonpermissive chromatin state. These results suggest that *DNMT1* and *DNMT3B* regulate *BAG-1* expression via insulator protein DNA-binding and chromatin dynamics by regulating histone dimethylation.

Introduction

One mechanism regulating the epigenome involves specific chromosomal methylation patterns controlled via a complex interplay of at least three independently encoded DNA methyltransferases (*DNMT*): *DNMT1*, *DNMT3A*, and *DNMT3B* (1,2). It has been shown previously that *DNMT1* and *DNMT3B* seem to cooperatively maintain DNA methylation and gene silencing, and genetic disruption of both significantly inhibited *DNMT* activity and reduced genomic DNA methylation by roughly 95% (3). Using these somatic knockout (*DNMT1*^{-/-}, *DNMT3B*^{-/-}) and double knockout (DKO) cell lines, our group has reported a

Requests for reprints: Andrew P. Feinberg, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, MD 21205. Phone: 410-614-3489; E-mail: afeinberg@jhu.edu or David Gius, Radiation Oncology Branch, National Cancer Institute, NIH, 9000 Rockville Pike, Bethesda, MD 20892. Phone: 301-496-5457; Fax: 301-480-5439; E-mail: giusd@mail.nih.gov.
L. Sun and L. Huang contributed equally to this manuscript.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

comprehensive study comparing the effects of altered DNMT activity on gene expression in the cancer epigenome (4).

One of the most interesting findings in this study involved the observation that a substantial fraction of genes was down-regulated, rather than up-regulated, after genetic *DNMT* knockout (4). These results suggest that a hypomethylated chromosomal status inhibits a subset of gene expression, whereas methylation may activate some of these same genes. Since this publication, data from another group (5) have also shown that overexpression of *DNMT1* increased the expression of a specific subset of genes. In this work, genomic analysis of Rat-1 cells overexpressing *DNMT1* showed an increased expression of slightly more genes than were observed to decrease, suggesting that regulation of the epigenome may be more complex than previously thought. However, neither of these studies suggests a mechanism to account for these surprising observations.

Chromatin insulators are DNA boundary elements that inhibit gene expression by partitioning chromosomal domains, and they function by blocking the effects of nearby enhancers in a position-dependent manner (6,7). Chromatin insulators binding proteins, such as CTCF and CTCFL/BORIS, bind to specific DNA boundary elements that isolate chromosomal domains from *cis*-acting transcriptional regions (6,7). CTCF is thought to be a ubiquitous, highly conserved, zinc-finger (ZF) DNA-binding protein that has multiple roles in gene regulation (6), as well as chromatin and X chromosome inactivation (8). A paralogue of *CTCF*, brother of the regulator of imprinted sites (*BORIS*), exhibits extensive homology to *CTCF* in the 11 ZF regions, suggesting a similar DNA-binding spectrum (9–11). However, there are significant differences in their NH₂ and COOH terminal domains (9), suggesting that these regions interact with different binding proteins to alter gene expression. *BORIS* is thought to be primarily a testis-specific protein (12); however, *BORIS* has also been shown to be increased in a variety of tumors (10). CTCF seems to repress gene expression of a specific subset of genes (13,14), whereas *BORIS* has been shown to activate gene expression (10).

In the current study, we show that regulation of *BAG-1* expression by CTCF and *BORIS* through CTCF-binding sites located in its upstream promoter region. Chromatin immunoprecipitation (ChIP) analysis of the *BAG-1* upstream regulatory region in *DNMT* somatic knockout cells showed a nonpermissive chromatin status, indicated by a lower ratio of dimethyl-H3-K4/dimethyl-H3-K9. In addition, we showed preferential CTCF binding to the promoter region, as well as decreased *BAG-1* expression in *DNMT* somatic knockout cells. In contrast, ChIP analysis of the *BAG-1* promoter in cell lines overexpressing either *DNMT1* or *DNMT3B* showed a permissive chromatin status, indicated by a higher ratio of dimethyl-H3-K4/dimethyl-H3-K9. Furthermore, preferential *BORIS* binding to the promoter and increased *BAG-1* expression were observed in these cells. *BORIS* short hairpin RNA (shRNA) knockdown decreased *BORIS* binding to the *BAG-1* promoter, decreased *BAG-1* expression, and changed the ratio of dimethyl-H3-K4/dimethyl-H3-K9 to a nonpermissive chromatin state. Finally, transient cotransfection experiments also showed that *BORIS* activated and *CTCF* inhibited *BAG-1* promoter activity. The results of these experiments suggest that (a) cellular *DNMT* expression seems to influence histone methylation, as reflected by changes in the dimethyl-H3-K4/dimethyl-H3-K9 ratio, (b) there is a reciprocity of *BORIS* and *CTCF* function to coordinately dictate gene expression via histone methylation, and (c) DNA methylation may activate specific genes or gene families via a mechanism involving *BORIS* binding to the promoter.

Materials and Methods

Cell culture and permanent cell lines

DNMT1 knockout (DNMT1^{-/-}) and DNMT3B knockout (DNMT3B^{-/-}) cell lines and DNMT1 and DNMT3B DKO cell lines, as well as a parental colon cancer cell line (HCT116), were described (3) and grown in McCoy's 5A medium (Invitrogen). HCT116 cells overexpressing DNMT1 (DNMT1⁺), DNMT3B (DNMT3B⁺), DNMT1, and DNMT3B double knock-in (DKI) were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 750 µg/mL G418. Rat-1 and Rat-1 CMV-DNMT1⁺ cell lines (a kind gift from Dr. Tom Curran) were cultured in MEM (α modification), containing 5% FBS, penicillin (100 units/mL), streptomycin (100 µg/mL), and G418 (300 µg/mL) in a humidified 37°C incubator with 5% CO₂.

RNA extraction and quantitative real-time PCR

Total cellular RNA was isolated as described previously (4). Reverse transcription-PCR (RT-PCR) and quantitative real-time RT-PCR were carried out as previously described (15). RT-PCR reactions were done thrice for each sample, as previously described (16). Gene-specific primers were purchased from Applied Biosystems as premade TaqMan gene expression assays: *BAG-1*-Hs00185390_m1, *BAG-3*-Hs00188713_m1, *BAG-4*-Hs00362193_m1, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-HS99999905_m1. The relative amounts of transcript of the tested genes were normalized by *GAPDH*. The average ratios of three replicate runs were calculated.

Preparation of whole-cell extracts, SDS-PAGE, and immunoblot analysis

Nuclear extracts were prepared using a modification of the previously described method (17). No reducing agents were added to the buffers used to make extracts. Protein concentrations were determined using the Bradford assay (Bio-Rad Labs.) and a Beckman DU-640 spectrophotometer (18). After preparation and protein analysis, samples were stored at -80°C and thawed on ice immediately before use.

After preparation of nuclear extracts, equal protein amounts (10–20 µg/sample) were mixed with Laemmli lysis buffer, boiled for 5 min, and loaded into denaturing SDS-polyacrylamide gels for electrophoresis, as previously described (19). Membranes were probed with antibodies against BAG-1 (Santa Cruz Biotechnology) and BAG3 [a kind gift from Elise Kohn, National Cancer Institute (NCI)] and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Bands were analyzed using an enhanced chemiluminescence protocol (Amersham Biosciences) and visualized on radiographic film (Eastman-Kodak). Figures are representative of the outcome in at least two independent experiments.

Plasmids, cotransfections, and luciferase assays

The 827-bp fragment encompassing -854 to -28 of the *hBAG-1* promoter was PCR amplified from an Invitrogen BAC clone (RP11-366F19). The fragment was subcloned into pGL3-Basic (Promega) to construct the pBAG-1-luc plasmid. All internal deletions and truncations of the *hBAG-1* promoter were done by subcloning various PCR fragments into either pBAG-1-FL-luc or pGL3-Basic. Two complementary oligos (ggtaccAGGCTGGGCGGCGGgagct-cacgcgtCCGCGAAGAAACgctagcctcgagCCTCCTGGCGTTTtagatct) carrying all three putative CTCF-binding sites that reside in the *hBAG-1* promoter were synthesized, annealed, and subcloned into pTAL-Luc (Clontech) to construct p3x-CTCF-tk-LUC.

BORIS shRNA was obtained from OriGene and transfected into HCT116 cells using FuGENE (Roche) according to the manufacturer's recommendation. At 48 h after transfection, cells were

put in selective media containing 1 µg/mL puromycin. Colonies were isolated after 3 wk of antibiotic selection, and Western blotting was performed to screen for positive colonies with decreased BORIS expression. HCT116-based and Rat-1-based cells were plated at 2×10^5 cells per 60-mm plate and transfected using FuGENE according to manufacturer's protocol. For each transfection, 1 µg of either pBAG-1-Luc or p3x-CTCF-tk-LUC was used. As an internal control, 1 µg of the β-galactosidase expression plasmid (pCMV-β-gal) was used, and carrier DNA (pUC) was added to each sample to maintain a total of 6 µg per transfection. For all transfections, the total amount of CMV promoter-containing plasmids was adjusted so that the same amount was present in each reaction. Thirty-six hours after transfection, cells were harvested and prepared for luciferase and β-galactosidase assay (Promega). The total amount of luciferase and β-galactosidase activities were measured independently using a Wallac plate reader (Perkin-Elmer) equipped with the appropriate filters. The relative fold induction of luciferase activity was normalized to β-galactosidase activity. The *BORIS* expression vector, a kind gift from Dr. David Schrump (NCI), and the *CTCF* expression vector, a kind gift from Dr. Carl Barrett (NCI), were subcloned into pCIneo.

Chromatin immunoprecipitation

Cells were cultured for 24 h and prepared using a ChIP assay kit from Upstate Biotechnology, Inc., according to the manufacturer's recommendations. Briefly, cells were fixed in 1% formaldehyde at 37°C for 10 min before being washed, scraped, and pelleted in ice-cold PBS. Cells were lysed in SDS lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1)] for 10 min on ice. Cell lysates were sonicated using a Misonix 3000 sonicator equipped with a cup horn for 2×2 min, 20 s pulse, and 20 s rest interval at setting 4. Sonicated cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C. An aliquot of the supernatant was subjected to reverse cross-linking with 5 mol/L NaCl to check for sonication efficiency. Samples with DNA sheared between 1000 and 200 bp were used for subsequent ChIP reactions.

Immunoprecipitations were performed using CTCF (C-20) antibody from Santa Cruz Biotechnology, Inc., or BORIS antibody (Supplementary Figs. S2 and S3). ChIP-enriched DNA along with input DNA was purified by phenol/chloroform extraction and ethanol precipitation. Purified DNA samples were analyzed by quantitative real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) with the ABI Prism 7500 Detection System (Applied Biosystems) with the following primer pair: 5'-GTCTTCCGGG-GATGGAGAGCA-3' and 5'-TTGACCGCCAGCGATGG-3'. The conditions for ChIP-PCR were 40 two-step cycles of 95°C for 15 sec and 60°C for 60 sec. Data were collected at 60°C and analyzed by comparative C_t methods (20). All results for ChIP experiments (including those described below) are presented as fold enrichment relative units that is calculated as the mean $\pm$ SD fold enrichment of immunoprecipitated DNA relative to no antibody (BORIS or CTCF) IgG controls, which represents ChIP specificity, done in duplicate for each experiment.

ChIP assay for histone 3 K4 and K9 methylation in BAG-1 CTCF-binding sites

Immunoprecipitations were performed using either an antibody to histone H3 dimethyl K4 or histone H3 dimethyl K9 (Abcam). ChIP was performed as described previously (21). Each experiment was repeated at least twice independently. Pulled down DNA was analyzed using real-time PCR normalized by input DNA. The sequences of primers at *BAG-1/CTCF*-binding sites 1 and 2 are forward, 5'-GTGCCAATGCAGTCAGTCA and reverse, 5'-TGGTTGCTCTAGTGT-TTGGG; the sequences of primers at *BAG-1/CTCF*-binding site 3 are forward, 5'-ACCAGAAACGGA-AGCAGAGT and reverse, 5'-CCTCCCACTCCTTCTACCAA.

Electrophoretic mobility shift assay

Protein samples were prepared, stored, and quantified by methods described earlier. No reducing agents were added to the electrophoretic mobility shift assays (EMSA) or the buffers used to make nuclear extracts. EMSAs were performed as previously described (22) using a ^{32}P -radiolabeled oligonucleotide corresponding to the second CTCF DNA-binding site in the *BAG-1* promoter. Nuclear extracts (10 μg) were incubated with poly dI-dC for 10 min on ice, followed by the addition of radiolabeled oligonucleotide (100,000 cpm of radiolabeled probe per reaction) and incubation at 25°C for 20 min. Samples were electrophoresed on a 6% nondenaturing PAGE gel, dried, exposed to a phosphorimager screen, and analyzed using a Typhoon 8600 Phosphor-imager (Molecular Dynamics).

Results

BAG family gene is decreased in *DNMT1*^{-/-}, *DNMT3B*^{-/-}, and DKO cell lines

We have previously shown that roughly equal numbers of genes were observed to increase as those shown to decrease after genetic knockout of the *DNMT* genes (4). Interestingly, an additional genomic study of *DNMT1* overexpression in Rat-1 cells showed an increase in expression of roughly 380 genes while ~300 genes were decreased (5). Taken together, these results implied that DNA methylation may increase, as well as, silence specific genes in a coordinate manner. To address this issue, a more complete analysis of our microarray data was done to examine genes that exhibit decreased gene expression in *DNMT* somatic knockout cells. This analysis identified a family of prosurvival genes (*BAG* gene family) that was decreased in *DNMT1*^{-/-} and *DNMT3B*^{-/-} cell lines; this decrease was even greater in the DKO cells (Table 1). In contrast, an increase in *BAG-1*, *BAG-3*, and *BAG-4* gene expression was observed in HCT116 cells overexpressing either *DNMT1* (*DNMT1*⁺) or *DNMT3B* (*DNMT3B*⁺). Because the expression of all three of the *BAG* genes was altered as a function of *DNMT* status, it seemed logical to use the *BAG* family genes as a model.

Decreased *BAG* expression in *DNMT1*^{-/-}, *DNMT3B*^{-/-}, and DKO cells is transcriptional

To confirm the microarray results, RNA was isolated from the HCT116, *DNMT1*^{-/-}, *DNMT3B*^{-/-}, and DKO cell lines and was used for quantitative real-time RT-PCR analysis for *BAG-1*, *BAG-3*, and *BAG-4* expression. These experiments authenticated the microarray data and showed that gene expression for the *BAG* genes is intermediately down-regulated in the *DNMT1*^{-/-} and *DNMT3B*^{-/-} cells and significantly decreased in the DKO cells (Fig. 1A). Western blot analysis with antibodies against either BAG-1 (Santa Cruz Biotechnology) or BAG-3 (a kind gift from Dr. Elise Kohn) showed that the BAG-1 and BAG-3 protein levels were decreased in the somatic knockout cell lines and further decreased in the DKO cells (Fig. 1B).

The upstream regulatory regions of *BAG-1*, *BAG-3*, and *BAG-4* were analyzed, and interestingly, the 5'upstream regulatory regions of all three *BAG* genes revealed several common sequences, including CpG islands (Fig. 1C). To investigate a mechanism for *BAG* gene regulation, the *BAG-1* promoter region was cloned into a luciferase reporter vector (pBAG-1-Luc) and transient transfection assays were done in the *DNMT1*^{-/-}, *DNMT3B*^{-/-}, DKO, and *DNMT1*⁺ cells. Consistent with the RNA and protein data, cells lacking *DNMT* genes transfected with pBAG-1-Luc showed decreased *BAG-1* promoter activity (Fig. 1D, vertical columns 1–4). In contrast, HCT116 cells overexpressing *DNMT1* (*DNMT1*⁺ cells) displayed a roughly 2-fold increase in luciferase activity (column 5). In addition, a pair of matched Rat-1 fibroblast cell lines overexpressing *DNMT1* (23) showed a similar increase in the *BAG-1* promoter activity (Fig. 1D, vertical columns 6 versus 7). An increase in *BAG-1* promoter activity was also seen in HCT116 cell lines overexpressing *DNMT3B* or both

DNMT1 and *DNMT3B* (DKI; Supplementary Fig. S1), suggesting that overexpression of *DNMT* genes also alters *BAG-1* gene expression.

CTCF *cis*-acting elements regulate *BAG-1* gene expression

To define the critical DNA *cis*-acting sequence(s) in the *BAG-1* promoter responsible for regulating gene expression, a series of progressive *BAG-1* promoter 5' deletion mutants were constructed. HCT116 cells transiently transfected with these plasmids (Fig. 2A) clearly showed that the transcriptional regulatory region of interest is located within the *BAG-1* CpG island. Similar results were observed in HeLa cells (data not shown). A series of internal deletion mutants were constructed that systematically remove various parts of the CpG island, including the three CTCF DNA-binding sites. Transient transfection experiments showed that deletion of the two upstream CTCF-binding sites (Fig. 2B) increased expression by roughly 2-fold (third column from the top), and the removal of all three CTCF-binding sites (fifth column from the top) resulted in an additional, but very modest, increase in the luciferase activity. These data suggest two points: (a) the CTCF DNA-binding sites in the CpG island are potentially the *cis*-acting DNA sequence inhibiting *BAG-1* gene expression and (b) the first and second CTCF-binding sites have a more significant effect on gene expression than the third.

Effect of *DNMT* status on *BAG-1* promoter heterochromatin features

To determine a potential mechanism, we examined the methylation pattern of the *BAG-1* promoter in both the somatic *DNMT* knockout and the *DNMT*-overexpressing cells. Multiple regions of the *BAG-1* promoter, including the CpG island and CTCF-binding sites, were analyzed by bisulfite sequencing and failed to show any significant differences in methylation (Fig. 3A). We also looked at histone methylation to determine if changes in chromatin compaction might account for the changes in *BAG-1* gene expression. Histone H3 dimethylation at residue K4 (H3-K4) or histone H3 dimethylation at residue K9 (H3-K9) have been shown to define distinct chromatin regions either permissive or nonpermissive for gene expression, respectively. ChIP analysis of the *BAG-1* first, second (Fig. 3B), and third (Fig. 3C) CTCF-binding sites in *DNMT* somatic knockout cells showed a nonpermissive chromatin status indicated by a low dimethyl-H3-K4/dimethyl-H3-K9 ratio compared with that of control HCT116 cells (Fig. 3B and C). In contrast, a permissive chromatin status with a high dimethyl-H3-K4/dimethyl-H3-K9 ratio was observed in *DNMT1*⁺ and *DNMT3B*⁺ cells (Fig. 3B and C). Interestingly, no change in the dimethyl-H3-K4/dimethyl-H3-K9 ratio was observed for the third CTCF DNA-binding site in the *DNMT3B*^{-/-} cells (Fig. 3B), whereas the first and second region did change (Fig. 3C). This result matches with the RT-PCR data where only a very modest decrease in *BAG-1* expression was observed in the *DNMT3B*^{-/-} cells (Fig. 1A). The results of these experiments suggest that *DNMT1* and *DNMT3B* may selectively influence H3-K4 and H3-K9 dimethylation, and this may be one mechanism for the regulation of *BAG-1* gene expression.

Increased CTCF DNA-binding corresponds with a reciprocal decrease in *BAG-1* expression

It has been reported that CTCF can block the transcriptional activity of enhancer elements (13,24). Thus, it seemed logical to determine if CTCF associates with the *BAG-1* promoter and if this might play a role in the regulation of gene expression. CTCF ChIP analysis of the *BAG-1* promoter in HCT116, *DNMT1*^{-/-}, *DNMT3B*^{-/-}, and DKI cells showed an increase in CTCF binding to the *BAG-1* promoter in the *DNMT1*^{-/-} and *DNMT3B*^{-/-} cells and a further increase in the DKI cells (Fig. 4A). In contrast, CTCF binding to the *BAG-1* promoter was markedly decreased in *DNMT1*⁺, *DNMT3B*⁺, and DKI cells (Fig. 4B). Overall, these results showed an inverse relationship between CTCF binding and the expression of *BAG-1*, suggesting a potential mechanism whereby CTCF inhibits transcription and lack of binding prevents repression and increases the expression of *BAG-1* promoter.

The above experiments used primers that flank the *BAG-1* promoter CTCF-binding sites, but these results do not *a priori* show that the CTCF-binding site is the target sequence. To address this, electrophoretic mobility shift assays (EMSAs) were done using the CTCF-binding site oligos. These experiments showed a significant increase in CTCF-binding activity with protein extracts harvested from HCT116, DNMT1^{-/-}, and DNMT3B^{-/-} cells (Fig. 4C, right, lane 1 versus lanes 2 and 3). Surprisingly, instead of an expected decrease in DNA binding, a significant increase in binding was observed in the DNMT1⁺, DNMT3B⁺, and DKI cells (Fig. 4C, lanes 4–7) compared with that observed in the HCT116 cells (lane 1) or the somatic cell knockouts (lanes 2 and 3). In addition, not only was there an increase in DNA-binding in the *DNMT*-overexpressing cells, but the shifted band ran slightly lower than that seen in the somatic cell knockout cells. These experiments suggest that another protein and/or protein complex is binding to the CTCF sites in the *BAG-1* promoter in cells overexpressing *DNMT*.

Increased *BAG-1* gene expression corresponds with BORIS DNA-binding

CTCF/BORIS, a testis-specific protein that is structurally similar to CTCF, shares most of its homology in the ZF DNA-binding domain (9–11). This similarity suggested that the change in the shifted protein-DNA band in Fig. 4C (lanes 4–7 versus lanes 1–3) might be due to BORIS binding to the CTCF-binding sequence. To address this idea, ChIP analysis of the *BAG-1* promoter with anti-BORIS antibody (Supplementary Figs. S2 and S3) was performed and showed an increase in BORIS binding to the *BAG-1* promoter region in the DNMT1⁺, DNMT3B⁺, and DKI cells (Fig. 4D) compared with HCT116 cells. This observation matches the increase in *BAG-1* gene expression (Fig. 1D and Supplementary Fig. S1). Very little, if any, BORIS binding to the *BAG-1* promoter was observed in the analysis using the HCT116 somatic *DNMT* knockout cells (data not shown).

shRNA knockdown of *BORIS* decreases *BAG-1* and alters chromatin status

The above experiments suggest a role for BORIS in the regulation of *BAG-1* expression; however, no cause and effect was established. To address this idea more critically, tissue culture cells were treated with shRNA to knockdown *BORIS* expression. Treatment of HCT116 cells with *BORIS* shRNA resulted in a decrease in BORIS protein levels (Fig. 5A), and ChIP analysis with an anti-BORIS antibody showed a decrease in BORIS binding to the *BAG-1* promoter (Fig. 5B). Finally, *BORIS* shRNA knockdown changed the dimethyl-H3-K4/dimethyl-H3-K9 ratio at the *BAG-1* promoter (Fig. 5C) to a nonpermissive chromatin status and decreased *BAG-1* expression (Fig. 5D). The result of these experiments suggests a connection between BORIS binding to the *BAG-1* promoter, histone methylation status, and *BAG-1* expression.

BORIS activates and CTCF inhibits *BAG-1* expression in transient assays

To determine if *BORIS* and *CTCF* can alter *BAG-1* expression, a series of transient cotransfection experiments were done, using either pBAG-1-Luc or a reporter plasmid containing three CTCF DNA-binding sites in tandem (p3x-CTCF-tk-Luc). Transient cotransfection using a *CTCF* expression vector (pCMV-CTCF) with pBAG-1-Luc (Fig. 6A) or p3x-CTCF-tk-LUC (Fig. 6B) showed decreased luciferase activity. In contrast, cotransfection using a *BORIS* expression vector (pCMV-BORIS) with pBAG-1-Luc (Fig. 6C) or p3x-CTCF-tk-Luc (Fig. 6D) showed increased luciferase activity. The results of these experiments extend the results above, suggesting that BORIS and CTCF use the same *cis*-acting DNA site to either repress (through binding by CTCF) or induce (through binding by BORIS) *BAG-1* gene expression.

Discussion

Epigenetic inheritance involves three primary mechanisms, all of which seem to play a role in carcinogenesis: DNA methylation; posttranslational modification of histones, including

methylation, phosphorylation, or acetylation; and modifications by specific DNA-binding proteins, such as insulators binding to boundary elements (6,7). One of the mechanisms that regulate chromatin compaction involves chromatin insulators, which demarcate specific semiautonomous genomic regions into units (25). The boundaries created by the insulators seem to block or alter the interaction between *cis*-acting enhancers or silencers in a position-dependent manner (26,27). We have previously shown that genetic inhibition of *DNMT* down-regulates, as well as up-regulates, an equal number of genes (4). *DNMT1* overexpression also increases the expression of as many genes as are silenced (data not shown), which others have also shown (5). However, the explanation for this observation is likely complex, and given the intricate nature of the epigenome, this may be due to indirect, as well as direct, effects. To address this, we used the *BAG* gene family, more specifically *BAG-1*, as a model to determine a potential mechanism to explain this gene expression pattern.

The results presented here suggest that BORIS binding to the *BAG-1* promoter coincides with changes in the dimethyl-H3-K4/dimethyl-H3-K9 ratio, which indicates a permissive chromatin status for the expression of *BAG-1*. This was further suggested by the *BORIS* shRNA knockdown experiments that showed decreased BORIS binding to the *BAG-1* promoter and decreased *BAG-1* expression, as well as a nonpermissive chromatin status indicated by the change in the dimethyl-H3-K4/dimethyl-H3-K9 ratio. Furthermore, CTCF binding to the *BAG-1* promoter corresponds with a decrease in gene expression, as well as a nonpermissive chromatin status reflected by a decrease in the dimethyl-H3-K4/dimethyl-H3-K9 ratio. A 5' and internal deletion analysis of the *BAG-1* promoter clearly showed that the CTCF-binding sites in the *BAG-1* CpG island are the target sequences for this regulatory process. As such, these results suggest that insulator proteins, such as BORIS and CTCF, may be critical to the regulation of *BAG-1* and very likely to the other *BAG* genes as well.

These data showed that CTCF binding to the *BAG-1* promoter increases as the *DNMT* gene is deleted. In addition, BORIS binding increases as a function of *DNMT* overexpression. However, the mechanism underlying these changes as a function of *DNMT* status is unclear. One possibility is that BORIS and CTCF compete for binding to CTCF-binding sites, and binding is a function of overall intracellular protein levels. However, the protein levels of BORIS and CTCF are roughly equal in both the *DNMT* somatic knockout and the *DNMT*-overexpressing cells (Supplementary Fig. S4). While this question, in the context of this manuscript, remains unanswered, it does seem that histone methylation, and likely chromatin remodeling, is associated with the specific insulator protein bound to the *BAG-1* promoter.

It has been suggested that hypermethylation, presumably via the activation of *DNMT* genes, may play a role in carcinogenesis via the silencing of genes, such as *p16* and *RB* (28,29). In this model, the loss of gene function results in a genomic environment that is permissive for cellular damage, such as genomic instability, and this is an early step in carcinogenesis. However, cellular transformation also requires the activation of pro-survival and proliferative genes. It is well established that *in vitro* *DNMT1* overexpression can induce immortalized cells to transform; however, the mechanisms are complex (23). The results presented above suggest that two contrasting DNA methylation phenomena may coexist, causing epigenetic silencing of tumor suppressor genes, such as *p16* and *RB*, and the activation of oncogenes, such as *BAG* family genes; both of which may be required for transformation. This idea is being pursued.

This observation is interesting because *BAG* genes seem to be evolutionarily conserved gene family with homologues found in yeast, amphibians, plants, and mammals. The human genome contains four *BAG* genes: *BAG-1*, *BAG-3* (*CAIR-1*), *BAG-4* (*SOD*), and *BAG-6* (*Scythe*, *BAT3*; refs. 30–32). *BAG* proteins contain at least one copy of a conserved domain, allowing for an interaction with HSP70 (33,34), and operate as cochaperones that ultimately affect

cellular processes, including division, migration, differentiation, and pro-survival pathways (35–37). The BAG family proteins, thus, might be viewed as molecular bridges, with the NH₂ terminal domains serving as scaffolds for interactions with specific proteins and the COOH terminal BAG domain serving as a recruit for HSP70 (38).

Our data raise an intriguing question: can changes in the regulation of the epigenome silence tumor suppressor genes and simultaneously activate proliferative/survival pathways, creating a permissive environment for genomic instability and conferring a cell growth advantage? Although BORIS is initially thought to be primarily a testis-specific protein (11), it is also expressed in a wide variety of tumors (9), suggesting a potential role in carcinogenesis. Our results suggest that at least one potential mechanism for cellular transformation may involve BORIS-induced activation of a series of preprogrammed prosurvival/proliferative genes or gene families that favor cell division, creating a permissive environment for division. These results also suggest that *DNMT1* and *DNMT3B* expression seems to influence the reciprocity of BORIS and CTCF function to coordinately dictate gene expression via histone methylation as reflected by changes in the dimethyl-H3-K4/dimethyl-H3-K9 ratio.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: NIH grants CA65145 (A.P. Feinberg), CA72602 (D. Gius), and CA75556 (D. Gius). This research was supported in part by Intramural Research Program of the NIH National Cancer Institute Center for Cancer Research.

We thank Ina Rhee and Bert Vogelstein for the HCT116 knockout cell lines.

References

1. Chen T, Li E. Establishment and maintenance of DNA methylation patterns in mammals. *Curr Top Microbiol Immunol* 2006;301:179–201. [PubMed: 16570848]
2. James SR, Link PA, Karpf AR. Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b. *Oncogene* 2006;25:6975–85. [PubMed: 16715135]
3. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 2002;416:552–6. [PubMed: 11932749]
4. Gius D, Cui H, Bradbury CM, et al. Distinct effects on gene expression of chemical and genetic manipulation of the cancer epigenome revealed by a multimodality approach. *Cancer Cell* 2004;6:361–71. [PubMed: 15488759]
5. Ordway JM, Williams K, Curran T. Transcription repression in oncogenic transformation: common targets of epigenetic repression in cells transformed by Fos, Ras or Dnmt1. *Oncogene* 2004;23:3737–48. [PubMed: 14990994]
6. Bell AC, West AG, Felsenfeld G. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell* 1999;98:387–96. [PubMed: 10458613]
7. Ohlsson R, Renkawitz R, Lobanikov V. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *Trends Genet* 2001;17:520–7. [PubMed: 11525835]
8. Ishihara K, Oshimura M, Nakao M. CTCF-dependent chromatin insulator is linked to epigenetic remodeling. *Mol Cell* 2006;23:733–42. [PubMed: 16949368]
9. Klenova EM, Morse HC III, Ohlsson R, Lobanikov VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. *Semin Cancer Biol* 2002;12:399–414. [PubMed: 12191639]
10. Hong JA, Kang Y, Abdullaev Z, et al. Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. *Cancer Res* 2005;65:7763–74. [PubMed: 16140944]

11. Vatolin S, Abdullaev Z, Pack SD, et al. Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. *Cancer Res* 2005;65:7751–62. [PubMed: 16140943]
12. Loukinov DI, Pugacheva E, Vatolin S, et al. BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. *Proc Natl Acad Sci U S A* 2002;99:6806–11. [PubMed: 12011441]
13. Bell AC, Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* 2000;405:482–5. [PubMed: 10839546]
14. Schoenherr CJ, Levorsen JM, Tilghman SM. CTCF maintains differential methylation at the Igf2/H19 locus. *Nat Genet* 2003;33:66–9. [PubMed: 12461525]
15. Cui H, Horon IL, Ohlsson R, Hamilton SR, Feinberg AP. Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability. *Nat Med* 1998;4:1276–80. [PubMed: 9809551]
16. Cui H, Cruz-Correa M, Giardiello FM, et al. Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. *Science* 2003;299:1753–5. [PubMed: 12637750]
17. Diamond DA, Parsian A, Hunt CR, et al. Redox factor-1 (Ref-1) mediates the activation of AP-1 in HeLa and NIH 3T3 cells in response to heat shock. *J Biol Chem* 1999;274:16959–64. [PubMed: 10358044]
18. Curry HA, Clemens RA, Shah S, et al. Heat shock inhibits radiation-induced activation of NF- κ B via inhibition of I- κ B kinase. *J Biol Chem* 1999;274:23061–7. [PubMed: 10438474]
19. Smart DK, Ortiz KL, Mattson D, et al. Thioredoxin reductase as a potential molecular target for anticancer agents that induce oxidative stress. *Cancer Res* 2004;64:6716–24. [PubMed: 15374989]
20. Litt MD, Simpson M, Recillas-Targa F, Prioleau MN, Felsenfeld G. Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *EMBO J* 2001;20:2224–35. [PubMed: 11331588]
21. Li J, Lin Q, Yoon HG, et al. Involvement of histone methylation and phosphorylation in regulation of transcription by thyroid hormone receptor. *Mol Cell Biol* 2002;22:5688–97. [PubMed: 12138181]
22. Wei SJ, Botero A, Hirota K, et al. Thioredoxin nuclear translocation and interaction with redox factor-1 activates the activator protein-1 transcription factor in response to ionizing radiation. *Cancer Res* 2000;60:6688–95. [PubMed: 11118054]
23. Bakin AV, Curran T. Role of DNA 5-methylcytosine transferase in cell transformation by fos. *Science* 1999;283:387–90. [PubMed: 9888853]
24. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorsen JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* 2000;405:486–9. [PubMed: 10839547]
25. Moon H, Filippova G, Loukinov D, et al. CTCF is conserved from Drosophila to humans and confers enhancer blocking of the Fab-8 insulator. *EMBO Rep* 2005;6:165–70. [PubMed: 15678159]
26. Murrell A, Heeson S, Reik W. Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. *Nat Genet* 2004;36:889–93. [PubMed: 15273689]
27. Kurukuti S, Tiwari VK, Tavoosidana G, et al. CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. *Proc Natl Acad Sci U S A* 2006;103:10684–9. [PubMed: 16815976]
28. Greger V, Passarge E, Hopping W, Messmer E, Horsthemke B. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum Genet* 1989;83:155–8. [PubMed: 2550354]
29. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev* 2002;3:415–28.
30. Brive L, Takayama S, Briknarova K, et al. The carboxyl-terminal lobe of Hsc70 ATPase domain is sufficient for binding to BAG1. *Biochem Biophys Res Commun* 2001;289:1099–105. [PubMed: 11741305]
31. Briknarova K, Takayama S, Homma S, et al. BAG4/SODD protein contains a short BAG domain. *J Biol Chem* 2002;277:31172–8. [PubMed: 12058034]

32. Doong H, Rizzo K, Fang S, Kulpa V, Weissman AM, Kohn EC. CAIR-1/BAG-3 abrogates heat shock protein-70 chaperone complex-mediated protein degradation: accumulation of polyubiquitinated Hsp90 client proteins. *J Biol Chem* 2003;278:28490–500. [PubMed: 12750378]
33. Takayama S, Xie Z, Reed JC. An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. *J Biol Chem* 1999;274:781–6. [PubMed: 9873016]
34. Takahashi N, Yanagihara M, Ogawa Y, Yamanoha B, Andoh T. Down-regulation of Bcl-2-interacting protein BAG-1 confers resistance to anti-cancer drugs. *Biochem Biophys Res Commun* 2003;301:798–803. [PubMed: 12565851]
35. Liao Q, Ozawa F, Friess H, et al. The anti-apoptotic protein BAG-3 is overexpressed in pancreatic cancer and induced by heat stress in pancreatic cancer cell lines. *FEBS Lett* 2001;503:151–7. [PubMed: 11513873]
36. Takayama S, Reed JC. Molecular chaperone targeting and regulation by BAG family proteins. *Nat Cell Biol* 2001;3:E237–41. [PubMed: 11584289]
37. Doong H, Vrillas A, Kohn EC. What's in the 'BAG'? -A functional domain analysis of the BAG-family proteins. *Cancer Lett* 2002;188:25–32. [PubMed: 12406544]
38. Kanelakis KC, Murphy PJ, Galigniana MD, et al. hsp70 interacting protein Hip does not affect glucocorticoid receptor folding by the hsp90-based chaperone machinery except to oppose the effect of BAG-1. *Biochemistry* 2000;39:14314–21. [PubMed: 11087380]

Figure 1.

Confirmation of *BAG* gene family expression. **A**, validation of microarray data using real-time RT-PCR assays in HCT116, DNMT1^{-/-}, DNMT3B^{-/-}, and DKO cells. Total RNA was isolated and purity checked, and levels of *BAG-1*, *BAG-3*, and *BAG-4* RNA were determined using Taqman probes. Results are presented as *BAG* relative RNA levels compared with control HCT116 cells. All experiments were normalized to *GAPDH*. All results are the mean of at least three separate experiments. Error bars represent 1 SD about the arithmetic mean. Statistical significance was established by Student's *t* test compared with wild-type HCT116 cells. *, *P* < 0.05; **, *P* < 0.01. **B**, *BAG-1* and *BAG-3* protein levels in the HCT116 DNMT somatic knockout cells. Nuclear extracts were isolated from HCT116, DNMT1^{-/-}, DNMT3B^{-/-}, and DKO cells, and 20 μg of protein were separated by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using either an anti-*BAG-1* or anti-*BAG-3* antibody. Blots were reprobed with actin antibody to ensure equal protein loading. **C**, promoter region analyses of the *BAG* gene family. The 5' upstream regulatory regions of *BAG-1*, *BAG-3*, and *BAG-4* were aligned, and potential *cis*-acting DNA-binding/enhancer sites and potential CpG islands were identified using software available at Entrez Genome and Genetics Computer Group. **D**, analysis of the *BAG-1* promoter. The 5' promoter region of *BAG-1* was cloned upstream of the luciferase gene (pBAG-1-Luc) and transfected into HCT116, DNMT1^{-/-}, DNMT3B^{-/-}, DKO, DNMT1⁺,

Rat-1, or Rat-1-DNMT1⁺ cells. Cells were also transfected with CMV- β -gal, and results are presented as luciferase/ β -galactosidase relative units and represent the relative fold change compared with sham-treated HCT116 control. All results are the mean of at least three separate experiments all done in duplicate. The results of individual transfections varied by <25%. Error bars around data points represent 1 SD about the arithmetic mean. Statistical significance was established by Student's *t* test compared with transfected wild-type HCT116 cells. *, $P < 0.05$; **, $P < 0.01$.

Figure 2.

The *BAG-1* CTCF DNA-binding sites are critical to the regulation of *BAG-1* gene expression. **A**, genetic analysis of the 5'*BAG-1* upstream regulatory region. Eight separate 5'*BAG-1* promoter deletion mutants were constructed and sequenced to determine the correct bp deletions. These 5' deletion mutants were subsequently cotransfected with CMV- β -gal into HCT116 cells, harvested after 36 h, and processed for luciferase and β -gal assay. The CpG island is shown in gray. **B**, internal deletion analysis of the *BAG-1* promoter CpG island. The three CTCF DNA-binding sites are shown as light spotted boxes. We constructed a series of internal deletion mutants in the *BAG-1* promoter that systematically removed various parts of the CpG island, including the two 5' and one 3' CTCF DNA-binding sites. The CpG island deletion mutant plasmids and CMV- β -gal were cotransfected into HCT116 cells. Results are presented as luciferase/ β -galactosidase relative units and as relative fold induction over the sham-treated control. All results are the mean of at least three separate experiments all done in duplicate. The results of individual transfections varied by <25%. Error bars around data points represent 1 SD about the mean. Statistical significance was established by Student's *t* test compared with transfected wild-type HCT116 cells. *, $P < 0.05$.

Figure 3. Effect of cellular DNA methyltransferase status on *BAG-1* promoter chromatin remodeling. Analysis of DNA methylation in HCT116 somatic cell knockouts and *DNMT*-overexpressing cell lines. A, the methylation status of the CpG island in the *BAG-1* upstream promoter region in HCT116, *DNMT1*^{-/-}, *DNMT3B*^{-/-}, DKO, *DNMT1*^{-/-}, and *DNMT3B*^{-/-}, and DKI cell lines were analyzed by bisulfite genomic sequencing. *BAG-1* gene expression is linked to histone methylation. HCT116, *DNMT1*⁺, *DNMT3B*⁺, *DNMT1*^{-/-}, *DNMT3B*^{-/-}, and DKO cells were harvested, followed by ChIP analysis using either a histone H3 dimethyl K4 or a dimethyl K9 antibody (Abcam), as described (20). Primers to the first and second CTCF-binding sites (B) and third CTCF-binding (C) were subsequently used in quantitative RT-PCR. *Diagonal-lined column*, alterations of H3-K4 methylation; *dark-spotted columns*, changes of H3-K9 methylation. All results for ChIP experiments are presented as fold enrichment relative units calculated as the mean $\pm$ SD fold enrichment of immunoprecipitated DNA relative to IgG control, which represents ChIP specificity, done in duplicate for each experiment. All results are the mean of at least three separate experiments. Error bars around data points represent 1 SD about the mean. Statistical significance was established by Student's *t* test compared with transfected wild-type HCT116 cells. *, *P* < 0.05.

Figure 4.

Chromatin immunoprecipitation assay to determine CTCF and BORIS binding to the *BAG-1* promoter. **A**, ChIP analysis to determine CTCF binding to the *BAG-1* promoter in HCT116-based *DNMT* somatic knockout cell lines. HCT116, DNMT1^{-/-}, DNMT3B^{-/-}, and DKO cells were exposed to formaldehyde to cross-link protein-DNA interactions. Chromatins were immunoprecipitated with anti-CTCF antibody. CTCF binding was quantified using real-time PCR with primers that covered the *BAG-1* CpG island. Experimental results are the mean of at least three separate experiments. **B**, ChIP analysis to determine CTCF binding to the *BAG-1* promoter in HCT116-based cells overexpressing *DNMT1*, *DNMT3B*, or both. HCT116, DNMT1⁺, DNMT3B⁺, and DKI cells as described above. **C**, EMSA analysis of protein complex binding to the second *BAG-1* CTCF DNA (oligo)-binding site. Nuclear lysates from HCT116, DNMT1^{-/-}, DNMT3B^{-/-}, DNMT1⁺, DNMT3B⁺, and DKI cells were incubated with a ³²P-labeled CTCF-binding site oligo on ice for 30 min, followed by separation on a nondenaturing polyacrylamide gel. Sections of fluorograms from native gels, obtained using a TYPHOON Phosphorimager, as shown. Arrows, position of the CTCF shifted band and the free probe. **D**, ChIP analysis to determine BORIS binding to the *BAG-1* promoter in HCT116-based cells overexpressing *DNMT1*, *DNMT3B*, or both. Chromatins were immunoprecipitated with anti-BORIS antibody, and BORIS binding was quantified by real-time PCR as described above. ChIP experiments are presented as fold enrichment relative units calculated as the mean $\pm$ SD fold enrichment of immunoprecipitated DNA relative to IgG control, which represents ChIP specificity, done in duplicate for each experiment. Error bars around data points represent 1 SD about the arithmetic mean and statistical significance was established by Student's *t* test compared with transfected wild-type HCT116 cells. *, *P* < 0.05; **, *P* < 0.01.

Figure 5.

shRNA knockdown of *BORIS* decreases *BAG-1* expression and alters chromatin status. **A**, shRNA knockdown of *BORIS* decreases intracellular protein levels. HCT116 cells were transfected with either vector only (*Cont*) or a *BORIS* hairpin loop shRNA, and cells were harvested, separated by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using an anti-BORIS antibody. **B**, shRNA knockdown of *BORIS* decreases *BORIS* binding to the *BAG-1* CpG island. HCT116 cells were transfected with either vector only or *BORIS* shRNA and harvested for ChIP analysis with an anti-BORIS antibody, as described above. **C**, shRNA knockdown of *BORIS* alters *BAG-1* promoter chromatin status. Control vector or *BORIS* shRNA-transfected HCT116 cells were harvested to determine histone H3-K4 (dark columns) or H3-K9 (check light gray columns) methylation status via ChIP analysis, using either a histone H3 dimethyl K4 or H3 dimethyl K9 antibody (Abcam), as described above. **D**, shRNA knockdown of *BORIS* decreases *BAG-1* expression. HCT116 cells were transfected with either vector only or *BORIS* shRNA. Total RNA was isolated and purity checked, and levels of *BAG-1* RNA were determined using real-time PCR with Taqman probes. All results are the mean of at least three separate experiments. Results are presented as *BAG-1* relative RNA levels, wherein 1.0 represents *BAG-1* RNA level in control HCT116 cells. All experiments were normalized to *GAPDH*. Error bars represent 1 SD about the arithmetic mean. For ChIP experiments (**B** and **C**), results are presented as fold enrichment relative units calculated as the mean $\pm$ SD fold enrichment of immunoprecipitated DNA relative to IgG control, which represents ChIP specificity, done in duplicate for each experiment. For all experiments, error bars around data points represent 1 SD about the arithmetic mean and statistical significance was established by Student's *t* test compared with transfected wild-type HCT116 cells. *, $P < 0.05$.

Figure 6.

CTCF decreases and BORIS increases BAG-1 promoter activity in transient assays in HCT116 cells. Cotransfection of either the pBAG-1-Luc (A) or 3×-CTCF-luciferase (B) reporter plasmids with a CTCF expression plasmid (pCMV-CTCF). Cotransfection of either the pBAG-1-Luc (C) or 3×-CTCF-luciferase (D) reporter plasmid transfected into HCT116 cells with a BORIS expression plasmid (pCMV-BORIS). Cells were also transfected with CMV-β-gal, and results are presented as luciferase/β-galactosidase relative units as the relative fold change compared with sham-treated HCT116 control; 1 μg (+) and 2 μg (++) of CMV-CTCF or CMV-BORIS expression plasmid. The total amount of pCMV-based expression vector DNA was held constant at 6.0 μg per transfection. The results are presented as relative fold induction over the sham-treated control. All results are the mean of at least three separate experiments, all done in duplicate. The results of individual transfections varied by <25%. Error bars around data points represent 1 SD about the arithmetic mean. Statistical significance was established by Student's t test compared with transfected wild-type HCT116 cells. *, $P < 0.05$.

Table 1
Expression of the *BAG* family genes in cells lacking *DNMT* genes

Cell lines						
	HCT116	DNMT ^{-/-}	DNMT3B ^{-/-}	DKO	DNMT1 ⁺	DNMT3B ⁺
BAG-1	1.0	0.5	0.7	0.2	2.2	1.8
BAG-3	1.0	0.5	0.6	0.2	2.1	1.6
BAG-4	1.0	0.4	0.5	0.1	2.0	1.7

NOTE: Values are stated as relative to the parental HCT116 cells that are assigned 1.0.