

HDAC8 and STAT3 repress *BMF* gene activity in colon cancer cells

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Histone deacetylase (HDAC) inhibitors are undergoing clinical trials as anticancer agents, but some exhibit resistance mechanisms linked to anti-apoptotic Bcl-2 functions, such as BH3-only protein silencing. HDAC inhibitors that reactivate BH3-only family members might offer an improved therapeutic approach. We show here that a novel seleno- α -keto acid triggers global histone acetylation in human colon cancer cells and activates apoptosis in a p21-independent manner. Profiling of multiple survival factors identified a critical role for the BH3-only member Bcl-2-modifying factor (Bmf). On the corresponding *BMF* gene promoter, loss of HDAC8 was associated with signal transducer and activator of transcription 3 (STAT3)/specificity protein 3 (Sp3) transcription factor exchange and recruitment of p300. Treatment with a p300 inhibitor or transient overexpression of exogenous HDAC8 interfered with *BMF* induction, whereas RNAi-mediated silencing of STAT3 activated the target gene. This is the first report to identify a direct target gene of HDAC8 repression, namely, *BMF*. Interestingly, the repressive role of HDAC8 could be uncoupled from HDAC1 to trigger Bmf-mediated apoptosis. These findings have implications for the development of HDAC8-selective inhibitors as therapeutic agents, beyond the reported involvement of HDAC8 in childhood malignancy.

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Histone deacetylase (HDAC) enzymes have been implicated in both normal physiology and pathophysiology, and several HDAC inhibitors have entered clinical trials.^{1–3} These inhibitors include vorinostat, a direct-acting compound that fits the HDAC active site, and romidepsin, a prodrug that through reductive metabolism generates a zinc-binding thiol.⁴ Evaluation of such compounds against solid tumors and hematological malignancies has provided insights into clinical efficacy, as well as resistance mechanisms and off-target effects.^{1–3}

Off-target effects such as cardiotoxicity and thrombocytopenia arise from inhibitors acting on multiple HDACs,^{5,6} whereas resistance has been linked to anti-apoptotic Bcl-2 functions, including BH3-only protein silencing.^{7–9} HDAC inhibitors that reactivate BH3-only family members, such as Bcl-2-modifying factor (Bmf), might offer an improved therapeutic approach for treating cancer and other conditions.

We previously reported on the HDAC inhibitory activity of methylselenopyruvate (MSP), a novel seleno- α -keto acid metabolite generated by transamination of the natural organoselenium compound methylselenocysteine (MSC).^{10–12} MSP

was identified as a competitive inhibitor of HDAC8, and this was supported by molecular modeling studies of MSP/HDAC8 interactions *in silico*.¹⁰

HDAC8 was the first human HDAC to be crystalized with bound inhibitors,^{13–16} but it remains one of the least understood HDACs, with no known direct transcriptional target or corepressor partner.^{17,18} In pursuing the mechanism of action of MSP in human colon cancer cells, we identified a key role for HDAC8 in regulating *BMF* gene activity and Bmf-mediated apoptosis. The work provides support for HDAC8 as a relevant mechanistic target for cancer therapy, beyond its reported involvement in childhood cancer.¹⁹

Results

MSP triggers Bmf-mediated apoptosis independent of p21 induction. Metabolic conversion of MSC to MSP was reported previously.¹¹ An inhibitor of aminotransfer reactions caused a dose-dependent loss of histone acetylation in human colon cancer cells treated with MSC, but not MSP (Figure 1a). This was associated with loss of cleaved poly(ADP-ribose)

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Abbreviations: Bmf/*BMF*, Bcl-2-modifying factor protein/gene; CdLS, Cornelia de Lange syndrome; ChIP, chromatin immunoprecipitation; HAT, histone acetyltransferase; HDAC, histone deacetylase; KMSB, β -keto-methylselenobutyrate; MSC, methylselenocysteine; MSP, methylselenopyruvate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *NIPBL*, nipped-B-like; STAT3, signal transducer and activator of transcription 3; PARP, poly(ADP-ribose)polymerase; RNA Pol II, RNA polymerase II; SAHA, suberoylanilide hydroxamic acid; SM, selenomethionine; Sp1, specificity protein 1; Sp3, specificity protein 3

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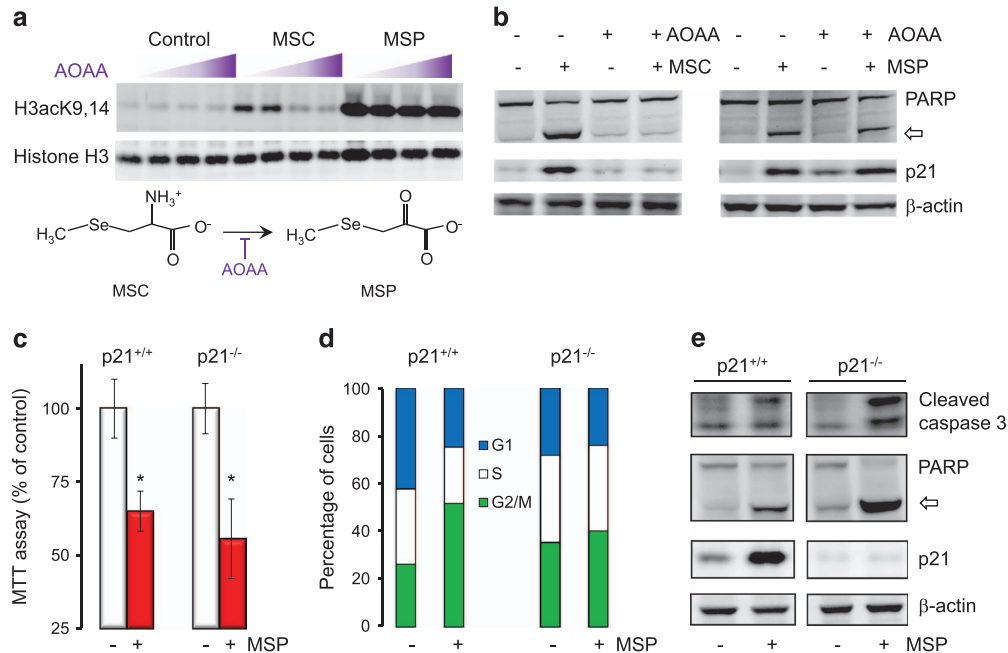


Figure 1 Induction of p21 by MSP is not a prerequisite for apoptosis in colon cancer cells. (a) HCT116 cells were incubated with the transaminase inhibitor aminoxyacetic acid (AOAA) 1 h before treatment with MSP, MSC, or vehicle (control). Whole-cell lysates were immunoblotted 6 h later for histone H3 acetylation. Wedge symbol indicates increasing concentration of AOAA. (b) AOAA pretreatment for 1 h blocked p21 induction by MSC and the cleavage of PARP, whereas MSP was unaffected by AOAA. Open arrow, cleaved PARP. (c) HCT116^{p21+/+} and HCT116^{p21-/-} cells responded similarly to MSP treatment in MTT assays; mean \pm S.D., $n=3$, $*P<0.05$ using Student's *t*-test. (d) G₂/M cell cycle arrest at 48 h required the presence of p21. (e) In the absence of p21, MSP strongly increased cleaved caspase-3 and (open arrow) cleaved PARP. Results in **a–e** are representative findings from two or more independent experiments

polymerase (PARP) and p21 induction by MSC, whereas MSP was effective in the presence or absence of the transaminase inhibitor (Figure 1b). HCT116^{p21+/+} and HCT116^{p21-/-} cells treated with MSP exhibited similar responses in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 1c), but p21^{-/-} cells did not accumulate in the G₂/M phase of the cell cycle, unlike p21^{+/+} cells (Figure 1d). Notably, HCT116^{p21-/-} cells had higher levels of cleaved caspase-3 and cleaved PARP compared with HCT116^{p21+/+} cells exposed to MSP under the same conditions (Figure 1e). These findings suggested that p21 induction is necessary for G₂/M arrest, but is not a prerequisite for apoptosis induction by MSP.

To investigate the apoptotic mechanism in more detail, several survival factors were profiled. Induction of pro-apoptotic *APAF1*, *BAK*, *BIM*, and *BMF*, and loss of anti-apoptotic *BCLXL* occurred within 12 h of MSP treatment, in both HCT116 and HT29 colon cancer cell lines (Figure 2a). Induction of *BMF* was particularly striking, and was confirmed both at the mRNA and protein level in time-course studies (Figures 2b and c). Knockdown of *Bmf* using RNAi attenuated the increase in *BMF* mRNA levels following MSP treatment (Figure 2d), and there was a reduction in *Bmf* protein expression (Figure 2e). These changes were associated with reduced PARP cleavage and attenuated levels of cleaved caspases (Figure 2f). Notably, in cells treated with MSP, activated caspases 3, 6, and 9 were reduced to near background levels by *Bmf* knockdown. Cleaved caspase-8 also was detected after MSP treatment and this caspase apparently was unaffected by *Bmf* knockdown (Figure 2f). Further experiments should seek to optimize *Bmf* knockdown

beyond the ~50–60% reduction achieved here (Figures 2d and e), and clarify the relative contributions of internal and external apoptotic pathways. Nonetheless, we conclude that *Bmf* was a key mediator of MSP-induced apoptosis in colon cancer cells.

***BMF* activation by MSP involves HDAC8 and STAT3 de-recruitment.** To prioritize histone marks for *BMF* chromatin immunoprecipitation (ChIP) assays, immunoblotting was first performed on whole-cell lysates of colon cancer cells. At the early time point of 3 h, MSP increased histone H3 acetylation (for example, H3acK9,14, H3acK9, and H3acK18) without dramatic changes in histone H3 methylation or phosphorylation (Supplementary Figure 1a). Histone H4 hyperacetylation also was detected within 3 h of MSP treatment, as evidenced by increased H4acK12 and H4acK5,8,12,16 histone marks (Supplementary Figure 1b). Time-course studies confirmed that histone hyperacetylation was maintained for several hours in response to MSP treatment, and that histone methylation marks associated with gene activation, such as H3K4me3, became prominent after 24 h (Supplementary Figure 1c). These findings indicated that MSP induced global histone acetylation before alterations in other histone marks.

Because global histone acetylation changes preceded alterations in other histone modifications, we conducted ChIP assays in colon cancer cells at an early time point, 4 h. Consistent with the *BMF* induction by MSP (Figure 2b), RNA polymerase II (Pol II) was recruited (Figure 3a) and there was a marked increase in local histone acetylation (Figure 3b). In parallel analyses of *BCLXL* (Supplementary Figure 2), reduced histone acetylation and attenuated Pol II levels were

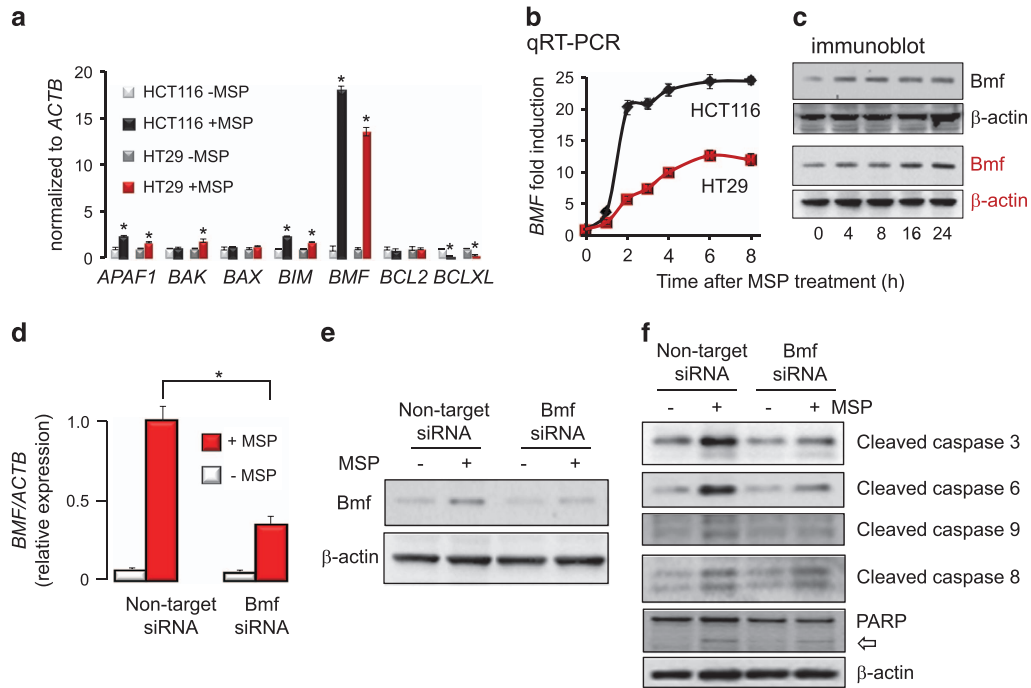


Figure 2 *Bmf* has a pivotal role in the apoptotic mechanism triggered by MSP. (a) Quantitative real-time PCR (qRT-PCR) assays of survival genes normalized to β -actin (*ACTB*), 12 h after treatment of colon cancer cells with MSP (10 μ M) or vehicle. Data indicate mean \pm S.D., $n = 3$, * $P < 0.05$ using Student's *t*-test to compare MSP-treated cells with vehicle controls. (b, c) Time-course for induction of *BMF* mRNA (mean \pm S.D., $n = 3$) and *Bmf* protein expression. (d, e) In HCT116 cells, MSP was significantly less effective at inducing *BMF* mRNA (mean \pm S.D., $n = 3$, * $P < 0.05$) and *Bmf* protein expression following siRNA-mediated knockdown of *Bmf*. (f) Reduced cleavage of caspases and (open arrow) PARP in MSP-treated HCT116 cells following *Bmf* knockdown. Results in a–f are representative findings from two or more experiments

detected on the corresponding gene promoter after MSP treatment, consistent with the observed transcriptional down-regulation of this pro-survival factor (Figure 2a). We did not examine serine 2-phosphorylated Pol II in association with *BCLXL* or *BMF*, but this might be included in future experiments.²⁰

The *BMF* promoter has predicted binding sites for multiple transcription factors, including Sp1 (specificity protein 1)/Sp3 (specificity protein 3) and signal transducer and activator of transcription 3 (STAT3). ChIP assays with the corresponding antibodies failed to identify consistent associations for Sp1, but upstream of the transcriptional start site there was binding of Sp3 and STAT3 (region 'f', Figures 3c and d). MSP treatment enhanced Sp3 and attenuated STAT3 interactions.

A prior report implicated HDAC1 as a repressor of *BMF* transcriptional activity.⁹ We confirmed HDAC1 interactions on the *BMF* promoter, slightly upstream of the transcription start site (peak region 'e', Figure 3e). The interactions of HDAC1 with *BMF* did not appear to be altered by MSP treatment. We also detected HDAC8 associations under constitutive conditions in colon cancer cells (open bars, peak region 'h', Figure 3f). To our knowledge, *BMF* is the first identified direct target gene for HDAC8 repression, none having been reported before.¹⁷ HDAC8 interactions were markedly reduced in response to MSP treatment (red bars, Figure 3f), and this coincided with increased histone acetyltransferase (HAT) p300 interactions (Figure 3g). Immunoblotting of whole-cell lysates showed no specific loss of HDAC8, or other class I HDACs, in response to MSP treatment (Supplementary Figure 3). We concluded that HDAC1 and HDAC8 might cooperate to

maximally repress *BMF*, but HDAC8 de-recruitment alone was sufficient to activate the target gene.

Inhibition of p300 or overexpression of HDAC8 interferes with *BMF* induction. On the basis of the ChIP data (Figures 3f and g), HDAC8 and p300 were examined further as regulators of *BMF* transcription. Treatment of colon cancer cells with a p300 inhibitor attenuated significantly the basal expression levels as well as the dose-dependent induction of *BMF* by MSP (Figure 4a). In transient transfection experiments, forced expression of HDAC8 also interfered with the ability of MSP to induce the target gene (Figure 4b), supporting a reciprocal relationship between p300 and HDAC8 in regulating *BMF* transcriptional activity. Although the results in Figure 4a were optimized for the specific p300 inhibitor selected, incomplete reductions in *BMF* expression were obtained, suggesting the possible involvement of other HATs in regulating *BMF*. Gain- and loss-of-function experiments, including the use of HAT mutants and HDAC8 silencing, might provide additional insights.

HDAC8 and STAT3 cooperate to repress *BMF* transcription. To clarify the apparent reciprocal relationship between Sp3 and STAT3 in regulating *BMF* (Figures 3c and d), we performed RNAi experiments on the corresponding transcription factors. Knockdown of STAT3 increased the constitutive and MSP-induced levels of *BMF*, supporting a suppressive role for STAT3 on the *BMF* promoter (Figure 5a). Unexpectedly, knockdown of Sp3 did not produce a corresponding decrease in *BMF* expression, nor was there an apparent

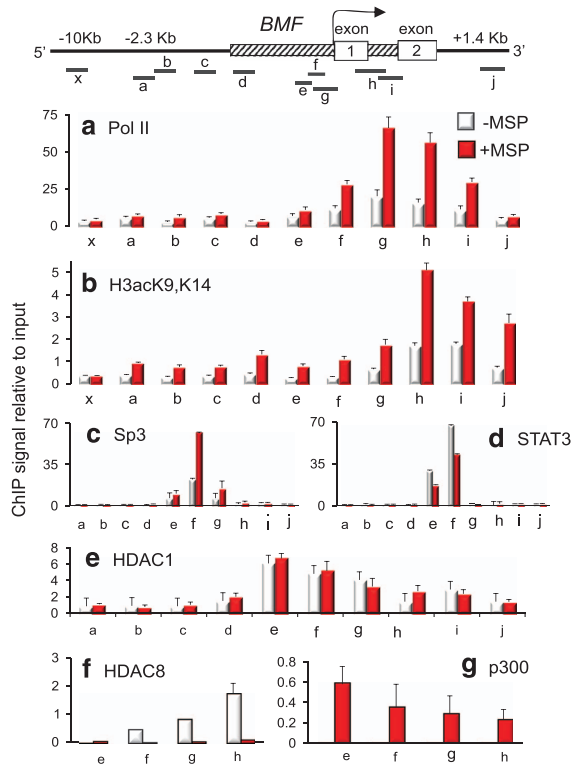


Figure 3 HDAC8 is a repressor of the *BMF* gene. Primers were designed to interrogate different regions of the *BMF* gene, including the promoter (hatched box) and flanking sequences. (a) MSP treatment enhanced RNA Pol II recruitment and (b) local histone acetylation levels. (c) MSP increased Sp3 transcription factor binding, adjacent to the transcription start site (arrow), and (d) reduced STAT3 associations. (e) HDAC1 interactions with the *BMF* promoter were unaffected by MSP treatment. (f) HDAC8 was present on *BMF* under constitutive conditions and was de-recruited following MSP treatment, coincident with increased p300 associations (g). ChIP assays were conducted at 4 h in HCT116 cells treated with MSP (+MSP) or vehicle (–MSP). Data were normalized to input controls; H3acK9,K14 also was normalized to histone H3. Results in a–g indicate mean \pm S.D., $n=3$, from a single experiment that was repeated on three or more separate occasions

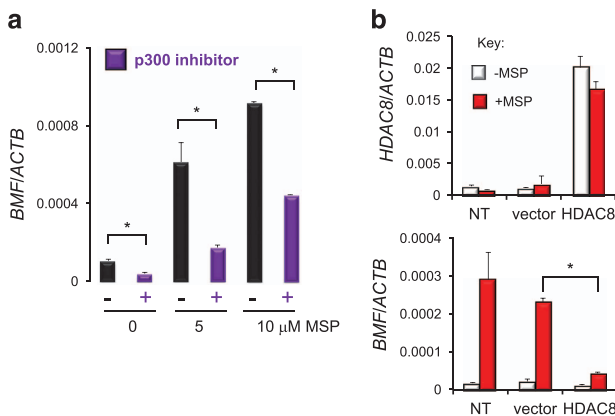


Figure 4 Inhibition of p300 and HDAC8 overexpression interferes with *BMF* transcription. (a) Pretreatment of colon cancer cells with p300 inhibitor C646⁴⁶ (10 μ M) reduced the constitutive levels of *BMF* and interfered with induction of the target gene by MSP. (b) Forced expression of HDAC8 by transient transfection rescues cells from *BMF* induction by MSP. In a, b, bars indicate mean \pm S.D., $n=3$, from a single experiment that was repeated at least twice (* $P<0.05$, by Student's *t*-test)

compensatory mechanism involving Sp1 upregulation after Sp3 silencing (Figure 5a). These results suggested a possible ‘bystander’ effect in which Sp3 interacts with a cognate binding site that becomes unmasked following STAT3 de-recruitment, without necessarily activating the target gene. We conclude that in addition to STAT3 and Sp3, other factors regulate *BMF* transcriptional activity on HDAC8 release, possibly in association with HDAC1.

Finally, HDAC8 was immunoprecipitated from whole-cell lysates of HCT116 cells and immunoblotted for potential protein partners. No interaction with Sp3 was detected, but a band corresponding to STAT3 was identified (Figure 5b, open arrow). To our knowledge, this is the first evidence reported for a protein partner in the HDAC8 corepressor complex.¹⁷ Notably, the STAT3 band was detected in the presence and absence of MSP treatment (Figure 5b), suggesting that an HDAC8/STAT3 corepressor complex might be released from the *BMF* promoter to recruit p300 and other coactivators (Figure 6).

Discussion

The first X-ray crystallography structures published of a human HDAC, HDAC8,^{13,14} provided valuable insights into the catalytic mechanism and binding characteristics of various inhibitors. A decade later, surprisingly little information was available on the role of HDAC8 in physiology or pathophysiology. HDAC8 associates with α -actin and regulates smooth muscle contractility,²¹ as well as influencing human myometrial activity via the acetylation of heat–shock protein 20.²² Other class I HDACs (HDAC1, HDAC2, and HDAC3) are known to associate with gene promoters in repressor complexes, but promoter interactions with HDAC8 have not been reported, nor have regulatory gene targets of HDAC8 been clearly described.¹⁷ We now report, for the first time, that HDAC8 can associate with the *BMF* gene under constitutive conditions in colon cancer cells, and establish a pro-survival scenario via the repression of *Bmf* protein levels.

The increase in global histone acetylation in colon cancer cells treated with MSP was recapitulated in several other cancer cell lines. For example, time-course studies in BE(2)C neuroblastoma cells revealed a rapid induction of histone acetylation by MSP compared with MSC parent compound (Supplementary Figure 4a). Interestingly, MSC, MSP, and the structural analog β -keto-methylselenobutyrate (KMSB) also induced global histone acetylation in prostate, breast, lung, and leukemia cells, whereas selenomethionine (SM) had no such effect (Supplementary Figure 4b). Unlike MSC, SM is a poor substrate for aminotransferase activity.¹² In a pilot study *in vivo*, we observed that dietary MSC, but not SM, inhibited mouse colon tumor multiplicity, and this was associated with increased *Bmf* expression and histone acetylation in the tumors, without marked changes in overall HDAC8 protein levels (Supplementary Figure 5). We are now seeking to test the *in vivo* efficacy of MSP and KMSB along with the corresponding parent compounds as inducers of *Bmf*. Further insights also might come from ChIP assays following treatment with MSC and a transaminase inhibitor, such as aminooxyacetic acid (AOAA; Figure 1); however, such experiments would have to be optimized to account for the

had less impact on the target gene (Supplementary Figure 9b). Interestingly, in all three cell lines, there was a comparable time-dependent increase in *BMF* expression that plateaued between 6 and 24 h after MSP treatment (Supplementary Figure 9b). We interpret these data as evidence that, beyond STAT3 post-translational modifications, HDAC8 inhibition and de-recruitment likely serves as the primary driver of *BMF* transcriptional activity in response to MSP.

Prior work established that the silencing of *HDAC8* by RNAi activated apoptosis and/or inhibited the growth of human cancer cells.^{25,26} No significant changes in *BMF* mRNA levels were detected after treatment of colon cancer cells with 5-aza-2'-deoxycytidine (Supplementary Figure 10), implying that DNA promoter methylation did not have a critical role in silencing *BMF* transcription under the conditions reported here.

Finally, the current findings provide possible new leads for understanding the biological roles of HDAC8 in schistosomiasis,²⁷ chronic obstructive pulmonary disease,^{28,29} and skull morphogenesis.^{30,31} Interestingly, novel mutations affecting *HDAC8* and *EP300* recently were linked to the development of Cornelia de Lange syndrome (CdLS), Rubinstein-Taybi syndrome, and other congenital abnormalities.^{32–35} Loss-of-function *HDAC8* mutations in six CdLS probands increased the acetylation and loading onto chromatin of SMC3, a 'cohesin' that has a role in regulating gene expression.³² Another member of the cohesin complex, *NIPBL* (nipped-B-like), is commonly mutated in patients with CdLS, and the corresponding loss-of-function zebrafish embryos have increased apoptosis in developing neural tissues.³⁴ Chemical activation of the pathway in *nipbl*-loss-of-function embryos rescued the adverse phenotypes and restored the physiological levels of cell death.³⁴ This is consistent with the working model proposed here (Figure 6), in which functional HDAC8 associates with, and represses, pro-apoptotic *BMF*, whereas loss-of-function HDAC8 mutants would aberrantly activate apoptosis and have deleterious consequences during embryo development. On the basis of the results presented here, we speculate that dysregulation of Bmf-mediated apoptosis might have a critical role in CdLS and related syndromes.

Materials and Methods

Cell culture. HCT116^{p21+/+} and HCT116^{p21-/-} cells were provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA). Dr. Zhenghe Wang (Case Western Reserve University, Cleveland, OH, USA) kindly supplied HCT116 cells expressing acetylation and phosphorylation mutants of STAT3.^{23,24} All other cell lines were obtained from ATCC (Manassas, VA, USA) and were cultured under the conditions described elsewhere.^{36–43} Chemicals were from the sources reported^{10,11,43} and were added to cells at the following nominal concentrations, unless stated otherwise: MSP (10 μ M), MSC (50 μ M), KMSB (10 μ M), SM (50 μ M), and SAHA (3 μ M). In some experiments, 0, 0.1, 0.5, or 2 mM concentrations of AOOA (Sigma, St. Louis, MO, USA) were added 1 h before other test compounds to inhibit the transamination reaction.^{44,45} Other experiments used a p300 inhibitor⁴⁶ (10 μ M C646, Sigma), added to cells 30 min before MSP treatment, or DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine, as reported.⁴⁰ Cells were collected at the times indicated in the figures.

Cell viability and cell cycle analyses. MTT assays and fluorescence-activated cell sorting analyses were conducted according to the basic protocols reported elsewhere.^{47,48}

Western blot. Whole-cell lysates were immunoblotted using the methodology described elsewhere.^{36,37} Primary antibodies were to the following acetylated (ac), methylated (me), and phosphorylated (ph) histone marks: H3K9me3, H3K4me2, H4acK12, and H4acK5,K8,K12,K16 (Upstate, Temecula, CA, USA); H3acK9,K14, H3acK9, and H3S10ph (Cell Signaling, Beverly, MA, USA); H3K27me3, H3K4me3, H3S28ph, and H3T3ph (Active Motif, Carlsbad, CA, USA); and H3acK18 (Abcam, San Francisco, CA, USA). Other primary antibodies were to histone H3 and histone H4 (Upstate, Cell Signaling, Active Motif); PARP, p21, Bmf, cleaved caspase-3, cleaved caspase-6, cleaved caspase-8 and cleaved caspase-9 (Cell Signaling); HDAC8 (R&D Systems, Minneapolis, MN, USA); HDAC1 and p300 (Santa Cruz Biotechnology, Dallas, TX, USA); and β -actin (Sigma). Antibodies to p53, acetylated p53, α -tubulin, acetylated α -tubulin, STAT3, Sp1, and Sp3 were as reported.^{10,11,36,37,48–51}

Knockdown and overexpression experiments. HDAC8 overexpression was achieved by transient transfection of 0.5 μ g *HDAC8* construct or empty vector (OriGene, Rockville, MD, USA), using the protocol reported before for *HDAC3* and *HDAC6*.³⁶ In knockdown studies, cells were transfected with Bmf siRNA (SASI_Hs01_00127817, Sigma) or negative control siRNA (Universal Negative control #1, Sigma) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After 48 h, cells were treated with MSP or vehicle and collected at 12 h. RNAi also was used as reported^{48–51} to knockdown Sp1, Sp3, and STAT3.

Real-time RT-PCR and qPCR. Methodologies for RNA extraction, cDNA synthesis, and qPCR were reported before.^{10,40,41} PCR reactions were performed on a Roche Light Cycle 480 II instrument (Roche, Indianapolis, IN, USA). Primers were to the following targets: *BMF*, *BCL2*, *BCLXL*, *BAX*, *BAK*, *BIM*, *APAF1*, and *P21WAF1*. Targets were normalized to internal controls indicated in the figures.

Chromatin immunoprecipitation. After treatment with MSP or vehicle for 4 h, cells were incubated with 1% formaldehyde in cell culture medium for 10 min at room temperature, and then the reaction was stopped with 0.12 M glycine for 5 min. DNA shearing and ChIP used the ChIP-IT Express Enzymatic (Active Motif) kit, following the recommended protocol. In the IP step, anti-histone H3, anti-H3acK9, K14 (Cell Signaling), and anti-RNA polymerase II (Active Motif) were incubated overnight at 4 °C. Additional IP experiments used antibodies to Sp1, Sp3, STAT3, HDAC8, HDAC1, and p300 (from sources listed above). After eluting pull-down DNA from magnetic beads, DNA purification used the QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA). Quantification of immunoprecipitated and input (control) DNA was carried out by qPCR on a Light Cycler 480 II (Roche), with Light Cycle 480 SYBR Green I Kit master (Roche). The primer sequences are available on request.

Molecular modeling, docking, and pocket binding prediction.

The 3D coordinates of the human catalytic domain of HDAC8 in the inhibited conformation and HDAC1 in the active conformation were retrieved from the Protein Data Bank (PDB 1T67 and 4BKX, respectively). The human catalytic domain of HDAC1 in the inhibited conformation was built through homology modeling using PDB 1767 as a starting template (Molsoft ICM 3.7.2-d, Molsoft, San Diego, CA, USA).⁵² Models were energetically refined in the internal coordinate space with Molsoft ICM.^{36,53} Five types of interaction potential were considered: (i) van der Waals potential for a hydrogen atom probe; (ii) van der Waals potential for a heavy-atom probe (generic carbon of 1.7 Å radius); (iii) optimized electrostatic term; (iv) hydrophobic terms; and (v) loan pair-based potential, which reflects directional preferences in hydrogen bonding. Energy terms were based on the Merck molecular force field to account for solvation free energy and entropic contribution. Modified intermolecular terms, such as soft van der Waals, hydrogen bonding, and hydrophobic terms, were included in the analyses. Conformational sampling is based on the biased probability Monte Carlo procedure, which randomly selects a conformation in the internal coordinate space and then moves randomly to a new position independent of the previous one, according to a predefined continuous probability distribution. After each random step, full local minimization improves the efficiency of the procedure. In the ICM-VLS (Molsoft ICM) screening procedure, ligand scoring is optimized to obtain maximal separation between binders and non-binders. Ligand was assigned a score according to its fit within the protein, accounting for electrostatic, hydrophobicity, and entropy parameters.^{36,53} The protocol was first validated by docking trichostatin A into the binding site and

reproducing the crystallographic pose in HDAC8 (PDB 1T64). Binding-pocket volumes were predicted using Pocket Finder (Molsoft ICM 3.7-d).^{54,55}

Statistical analyses. Unless indicated otherwise data were expressed as mean \pm S.D. ($n=3$). Paired comparisons were made using Student's *t*-test. A *P*-value <0.05 was considered as statistically significant, and indicated as such with an asterisk in the corresponding figures.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We dedicate this work to the memory of lifelong selenophile Dr. John A. Milner. Lydia Petell performed initial work-up of the ChIP assays. Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) kindly supplied HCT116^{p21+/+} and HCT116^{p21-/-} cells, whereas Dr. Zhenghe Wang (Case Western Reserve University, Cleveland, OH) provided HCT116 cells expressing STAT3 knock-in mutants. This work was supported by NIH grants CA090890, CA122959, CA90176, CA111842, P30 ES00210, P30 ES023512, and a Chancellor's Research Initiative from Texas A&M University.

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