Energy Restriction-mimetic Agents Induce Apoptosis in Prostate Cancer Cells in Part through Epigenetic Activation of KLF6 Tumor Suppressor Gene Expression*1

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Chun-Han Chen, Po-Hsien Huang, Po-Chen Chu, Mei-Chuan Chen, Chih-Chien Chou, Dasheng Wang, Samuel K. Kulp, Che-Ming Teng, Qianben Wang, and Ching-Shih Chen

From the Division of Medicinal Chemistry, College of Pharmacy, and the Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio 43210 and the Department of Pharmacology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan

Although energy restriction has been recognized as an important target for cancer prevention, the mechanism by which energy restriction-mimetic agents (ERMAs) mediate apoptosis remains unclear. By using a novel thiazolidinedione-derived ERMA, CG-12 (Wei, S., Kulp, S. K., and Chen, C. S. (2010) J. Biol. Chem. 285, 9780–9791), vis-à-vis 2-deoxyglucose and glucose deprivation, we obtain evidence that epigenetic activation of the tumor suppressor gene Kruppel-like factor 6 (KLF6) plays a role in ERMA-induced apoptosis in LNCaP prostate cancer cells. KLF6 regulates the expression of many proapoptotic genes, and shRNA-mediated KLF6 knockdown abrogated the ability of ERMA to induce apoptosis. Chromatin immunoprecipitation analysis indicates that this KLF6 transcriptional activation was associated with increased histone H3 acetylation and histone H3 lysine 4 trimethylation occupancy at the promoter region. Several lines of evidence demonstrate that the enhancing effect of ERMA on these active histone marks was mediated through transcriptional repression of histone deacetylases and H3 lysine 4 demethylases by down-regulating Sp1 expression. First, putative Sp1-binding elements are present in the promoters of the affected histone-modifying enzymes, and luciferase reporter assays indicate that site-directed mutagenesis of these Sp1 binding sites significantly diminished the promoter activities. Second, shRNA-mediated knockdown of Sp1 mimicked the repressive effect of energy restriction on these histone-modifying enzymes. Third, ectopic Sp1 expression protected cells from the repressive effect of CG-12 on these histone-modifying enzymes, thereby abolishing the activation of KLF6 expression. Together, these findings underscore the intricate relationship between energy restriction and epigenetic regulation of tumor suppressor gene expression, which has therapeutic relevance to foster novel strategies for prostate cancer therapy.

A hallmark feature of tumorigenesis is the shift of cellular metabolism from oxidative phosphorylation to aerobic glycolysis, the so-called Warburg effect, which provides a growth advantage to cancer cells in the microenvironment (1, 2). Recent evidence indicates that the high rate of glycolysis in tumor cells is attributable to the dysregulation of multiple oncogenic signaling pathways (1), including those mediated by hypoxia-inducible factor 1 (3), Akt (4), c-Myc (5), and p53 (6). This glycolytic shift is considered to be a fundamental property of neoplasia, and the high rate of glucose uptake in glycolytic tumor cells, including those of lung, breast, liver, and colon, is the basis for imaging tumors by [18F]2-fluoro-2-deoxyglucose positron emission tomography (7). Moreover, in light of the in vivo efficacy of dietary caloric restriction and natural product-based energy restriction-mimetic agents (ERMAs)2 such as 2-deoxyglucose (2-DG) and resveratrol in suppressing carcinogenesis in various animal models, targeting aerobic glycolysis represents a therapeutically relevant strategy for cancer prevention and treatment.

Previously, we obtained evidence that the antitumor effects of the thiazolidinedione peroxisome proliferator-activated receptor γ agonists troglitazone and ciglitazone were, in part, attributable to their ability to mimic glucose starvation to elicit cellular responses characteristic of energy restriction independent of peroxisome proliferator-activated receptor γ (8). These starvation-like responses include reduced glycolytic rates and intracellular levels of NADH and lactate, transient induction of the silent information regulator 1 gene, activation of the intracellular fuel sensor AMP-activated protein kinase, and endoplasmic reticulum stress, the interplay among which culminates in autophagy and apoptosis. On the basis of this finding, we used ciglitazone as a scaffold to develop a novel ERMA, CG-12, whose translational potential is manifested by an antiproliferative potency that is 3 orders of magnitude higher relative to 2-DG. Equally important, CG-12 provides a unique pharmacological tool to study the complex signaling

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2 The abbreviations used are: ERMA, energy restriction-mimetic agent; 2-DG, 2-deoxyglucose; qRT-PCR, quantitative RT-PCR; DMSO, dimethyl sulfoxide; Ac-H3, histone H3 acetylation; HDAC, histone deacetylase; DM, demethylase; HAT, H3 acetyl; H3K4MT, H3 lysine 4 methyltransferase; H3K4DM, H3 lysine 4 demethylase; H3K4Me3, H3 lysine 4 trimethylation; H3K4Me2, H3 lysine 4 dimethylation; H3K4, H3 lysine 4; H3K27Me3, H3 lysine 27 trimethylation.

3 To whom correspondence should be addressed: College of Pharmacy, 336 Parks Hall, The Ohio State University, 500 West 12th Ave., Columbus, OH 43210-1291. Tel.: 614-688-4008; Fax: 614-688-8556; E-mail: chen.844@osu.edu.
network underlying the suppressive effect of energy restriction on cancer cell proliferation. For example, we demonstrated that exposure of cancer cells to CG-12, 2-DG, or glucose-depleted medium triggered an intricate cascade of signaling pathways, leading to autophagic and apoptotic cell death (8).

In this study, we report the first evidence of the epigenetic effect of glucose starvation and ERMs on the transcriptional activation of the tumor suppressor gene Kruppel-like factor 6 (KLF6) through histone modifications. KLF6 plays an important role in suppressing oncogenesis and tumor progression by regulating the transcription of a broad range of genes governing cell cycle progression, apoptosis, and invasive phenotype (9) and is frequently inactivated in many cancer types, including those of the prostate (10, 11), liver (12), lung (13), colon (14), ovary (15), brain (16), stomach (17), and head and neck (18). From a mechanistic perspective, this epigenetic activation of KLF6 gene expression accounts at least in part for the ability of energy restriction to suppress cancer cell proliferation and may foster novel strategies for cancer prevention and therapy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Androgen-responsive LNCaP and androgen-insensitive DU-145 prostate cancer cells were obtained from the ATCC. Cells were maintained in 10% FBS-supplemented RPMI 1640 medium (Invitrogen). CG-12 was synthesized in our laboratory as described previously (19). Glucose-free RPMI 1640 medium was purchased from Invitrogen. 2-DG and actinomycin D were purchased from Sigma. Antibodies used and their sources are as follows: H3K4Me2, H3K27Me3, PLU-1, RBP2, p-Ser 5 RNA polymerase II (Abcam, Inc., Cambridge, MA); ATF-3 (Abnova, Taipei, Taiwan); HDAC4, HDAC5, HDAC7, histone H3, LSD1, and PARP (Cell Signaling Technology, Inc., Beverly, MA); Noxa and DAPK2 (Imgenex, San Diego, CA); β-actin (MP Biomedicals, Irvine, CA); acetyl-histone H3, H3K4Me3, HDAC1, HDAC2, HDAC3, and HDAC8, (Milipore, Billerica, MA); HDAC6, KLF6, Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA); Flag (Sigma-Aldrich); goat anti-rabbit IgG-HRP conjugates, rabbit antimouse IgG-HRP conjugates (Jackson ImmunoResearch Laboratories, West Grove, PA). The sequences of all primers used are listed in **supplemental Table 1**.

**Apoptosis Assay**—Apoptotic cell death was assessed with the Cell Death Detection ELISA kit (Roche Applied Science), which quantitates cytoplasmic histone-associated DNA fragments in the form of mono-/oligonucleosomes. Cells were treated with CG-12 for 48 h in 10% FBS-supplemented RPMI 1640 medium and then analyzed according to the manufacturer’s instructions.

**Transient Transfection and Luciferase Assay**—LNCaP cells were transfected by electroporation using Nucleofector kit R (Lonza, Walkersville, MD) according to the manufacturer’s protocol and then cultured in 6-well plates in 10% FBS-supplemented RPMI 1640 medium. Plasmids expressing shRNA for KLF6 and Sp1 were obtained from Origene Technologies (Rockville, MD) and Sigma-Aldrich, respectively. FLAG-Sp1 plasmid was prepared as described previously (20). HDAC1-FLAG (13820) and HDAC4-Flag (13821) plasmids were purchased from Addgene (Cambridge, MA). For the luciferase assay, transfected LNCaP cells were cultured in 6-well plates for 48 h. Luciferase activities were determined with the dual-luciferase system (Promega, Madison, WI), which uses co-transfected herpes simplex virus thymidine kinase promoter-driven Renilla reniformis luciferase as an internal control.

**RT-PCR**—Total RNA was isolated and reverse-transcribed to cDNA using TRizol reagent (Invitrogen) and the iScript cDNA synthesis kit (Bio-Rad), respectively. For semi-quantitative PCR, products were resolved by 1.2% agarose gel electrophoresis and visualized with ethidium bromide. For real-time PCR, cDNAs were amplified in IQ SYBR Green Supermix (Bio-Rad) and detected with the Bio-Rad CFX96 real-time PCR detection system. Relative gene expression was normalized to GAPDH and calculated by using the 2(-ΔΔC(T)) method (21).

**ChIP Assay**—ChIP was performed as reported previously, with modifications (22). After cross-linking and cell lysis, the cross-linked chromatin was sonicated, diluted, precleaved by incubation with protein A/G-agarose beads and normal mouse/rabbit IgG, and then immunoprecipitated with 4 μg of specific antibodies at 4 °C overnight. Protein A/G-agarose beads and yeast tRNA (Invitrogen) were then added and incubated for 1 h at 4 °C. Beads were then washed sequentially with the reported low-salt, high-salt, LiCl, and TE buffers (10 mM Tris, pH 8.0, containing 1 mM EDTA). After elution of the protein-DNA complexes and reversal of cross-linking, DNA fragments were isolated and analyzed by real-time PCR.

**Plasmid Construction and Site-directed Mutagenesis**—The genomic sequences of RBP2 (uc001qie. at chr12:chr12:389223\(-49220)), PLU-1 (uc001gyf.2 at chr1:202696533\(-202778149)), LSD1 (uc001bgj.2 at chr1:23345341–23410184), HDAC1 (uc010odh.1 at chr1:32757108–32793590), HDAC2 (uc003pwd.1 at chr1:32457327–114292954), HDAC3 (uc003llf.2 at chr1:41000443–141017023), and HDAC4 (uc002vk.3 at chr2:239969865–240323243) were obtained from the University of California Santa Cruz genome browser. The primer pairs specific to the promoter fragments containing putative Sp1 consensus sequences of RBP2 (-460 to +60), PLU-1 (-228 to +21), LSD1 (-195 to +167), HDAC1(-310 to +10), HDAC2 (-440 to +10), HDAC3 (-180 to +80), and HDAC4 (-320 to +10) were PCR-amplified from whole genomic DNA isolated from LNCaP cells. The amplified fragments were cloned into KpnI/BglIII sites of the pGL3-Basic vector (Promega) to generate the following constructs: pGL3-RBP2-Luc, pGL3-PLU1-Luc, pGL3-LSD1-Luc, pGL3-HDAC1-Luc, pGL3-HDAC2-Luc, pGL3-HDAC3-Luc, and pGL3-HDAC4-Luc. Reporter plasmids containing mutated Sp1 binding sites (GGGCGG to GTTCGG) were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**Western Blotting**—Western blotting was performed as described previously (8). Densitometric analysis of protein bands was performed by using Gel-Pro Analyzer (V3.1, Media Cybernetics, Bethesda, MD) to determine the relative intensities of drug-treated samples versus those of vehicle-treated controls after normalization to the internal reference protein β-actin.

**Statistical Analysis**—Data from quantitative qRT-PCR, ChIP-qPCR, cell death ELISA, Western blotting, and luciferase
reporter assays were analyzed using Student’s t test. Differences between group means were considered significant at $p < 0.05$.

RESULTS

Transcriptional Activation of the Tumor Suppressor Gene KLF6 Represents a Cellular Death Response to Energy Restriction—As part of our effort to understand the complex signaling network by which ERMs suppress cancer cell proliferation, we examined the effect of CG-12 on tumor suppressor gene expression in androgen-responsive LNCaP cells. Among various tumor suppressor genes examined, the ability of CG-12 to up-regulate the expression of KLF6, a zinc finger transcription factor involved in prostate tumorogenesis (10), is especially noteworthy. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis indicates that 48-h exposure of LNCaP cells to 10 $\mu M$ CG-12 boosted KLF6 mRNA levels by 25-fold, accompanied by increases in its proapoptotic target genes, including activating transcription factor (ATF) 3 (28-fold), Noxa (16-fold), and death-associated protein kinase (DAPK) 2 (8-fold) (Fig. 1A, upper panel). These changes were also noted in androgen-nonresponsive DU-145 cells (Fig. 1A, lower panel), indicating that the drug effect on KLF6 mRNA expression was not a cell line-specific event and that it was independent of the functional status of androgen receptor in prostate cancer cells. To address the possibility that CG-12-mediated up-regulation of KLF6 mRNA levels was a result of enhanced mRNA stability, we assessed the effect of 5 $\mu M$ CG-12 versus dimethyl sulfoxide (DMSO) vehicle on KLF6 mRNA turnover by co-treatment with 10 $\mu M$ actinomycin D, a transcriptional inhibitor, in LNCaP cells (Fig. 1B). As shown, the disappearance of KLF6 mRNA was nearly completed by 24 h in vehicle-treated cells, whereas CG-12 had no appreciable effect on prolonging its half-life. Together, these findings indicate that CG-12 mediated the up-regulation of KLF6 mRNA expression through transcriptional activation. Moreover, the ability of CG-12 to increase the expression of KLF6 and its target genes at both mRNA and protein levels was shared by 2-DG and glucose deprivation (Fig. 1C) in LNCaP cells, suggesting that transcriptional activation of the KLF6 gene represents an energy restriction-elicted signaling event.

As ATF3, Noxa, and DAPK2 have proapoptotic activities (10, 23, 24), we assessed the role of KLF6 gene activation in CG-12-induced apoptosis in LNCaP cells via shRNA-mediated knockdown of KLF6. Ablation of KLF6 abolished the ability of CG-12 to up-regulate the expression of these three proapoptotic proteins and protected cells from CG-12-induced apoptosis as evidenced by lack of poly(ADP-ribose) polymerase cleavage and nucleosome formation (Fig. 1, D and E). This protective effect was also observed in DU-145 cells, suggesting the
involvement of KLF6 in CG-12-mediated apoptotic death in prostate cancer cells irrespective of the androgen receptor functional status.

**CG-12 Activates KLF6 Gene Expression by the Enrichment of Histone H3 Acetylation (Ac-H3) and Histone H3 Lysine 4 Trimethylation (H3K4Me3) in the Promoter Region**—The KLF6 gene can be inactivated in tumor cells through loss of heterozygosity, somatic mutation, or epigenetic silencing (11). As LNCaP cells do not harbor mutations in the KLF6 gene (11), we rationalized that the up-regulation of KLF6 gene expression might be attributable to epigenetic activation. Recent evidence indicates that H3K4Me3 and histone H3 lysine 27 trimethylation (H3K27Me3) act as transcriptional activating and repressive marks, respectively, in regulating the expression of oncogenes and tumor suppressor genes in prostate carcinogenesis (25) and that histone acetylation and histone methylation function cooperatively to prepare chromatin for transcriptional activation (26). Thus, we assessed the effects of CG-12 on the levels of H3K4Me3, H3K27Me3, Ac-H3, and phospho-Ser-5 RNA polymerase II, a key enzyme involved in transcription initiation (27), occupancy at the promoter region of the KLF6 gene in LNCaP cells (Fig. 2A, upper panel, a–f) by ChIP-qPCR analysis. As shown, CG-12 significantly enriched H3K4Me3 and Ac-H3, accompanied by parallel increases in the presence of phospho-Ser-5-Pol II, upstream of the transcription start site, as well as in the downstream transcribed region (Fig. 2A, lower panel). In contrast, no significant changes in H3K27Me3 were noted in response to CG-12, refuting the role of this repressive mark in regulating the transcriptional activation of the KLF6 gene. As promoter hypermethylation has been implicated in the suppression of KLF6 expression in esophageal cancer cell lines (28), we examined whether a change in DNA methylation status played a role in regulating KLF6 gene expression in LNCaP cells. Treatment of LNCaP cells with the DNA demethylating agent 5-aza-2’-deoxycytidine had no effect on KLF6 mRNA expression, whereas p21, a tumor suppressor gene known to be regulated by promoter methylation in LNCaP cells (29), showed a dose-dependent increase in its mRNA level (Fig. 2B). Together, these data suggest that the activation of KLF6 gene expression in CG-12-treated LNCaP cells resulted at least in part from the enrichment for positive histone marks in the promoter region.

Western blot analysis indicates that this enrichment in active histone marks resulted from the ability of CG-12 to enhance global histone H3 acetylation and Lys-4 methylation. CG-12, glucose-depleted medium, and, to a lesser extent, 2-DG substantially increased the levels of Ac-H3 and H3K4Me3 and H3K4Me2 in a dose- and/or time-dependent manner (Fig. 2, C and D). Reminiscent of the time course observed after glucose starvation, these changes occurred as soon as 12 h after CG-12 treatment (Fig. 2D).

**Increased Positive Histone Marks Are Attributable to the Transcriptional Repression of Histone Deacetylases (HDACs) and H3K4 Demethylases (DMs) in Response to Energy Restriction**—Histone modifications like those described above result from a reversible process that is regulated by a dynamic balance between histone acetyl-/methyltransferase and histone deacetylase/demethylase activities. Aside from the complexity of histone-modifying enzymes involved in H3 acetylation, at least ten methyltransferases and five demethylases have been implicated in H3K4 methylation, each of which displays distinct substrate specificity and biological function in chromatin regulation. From a mechanistic perspective, increases in Ac-H3 and H3K4Me3 might arise from the up-regulation of

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**FIGURE 2. CG-12 increases the genomic occupancy of active histone marks at the promoter region of the KLF6 gene in LNCaP cells.** A, effect of CG-12 on the distribution of various histone marks. Upper panel, diagram depicting the locations of the KLF6 gene of amplicons used in the ChIP-qPCR assay. TSS, transcriptional start site. Lower panel, ChIP-qPCR analysis of the effects of CG-12 (10 μM, 24 h) on KLF6 gene occupancy by H3K4Me3, Ac-H3, H3K27Me3, and phospho-Ser-5-Pol II in LNCaP cells. Column, mean (n = 3); error bars, mean ± S.D. B, the DNA demethylating agent 5-aza-2’-deoxycytidine (5-aza-dC) has no effect on KLF6 gene expression as determined by RT-PCR. LNCaP cells were treated with 5-aza-dC for 72 h. Expression of p21 served as a positive control. C and D, global changes in histone H3 acetylation and H3K4 methylation status in energy-restricted LNCaP cells. Cells were treated with CG-12 or 2-DG at the indicated concentrations for 48 h or glucose-depleted medium for the indicated times (C) and 5 μM CG-12 for the indicated times (D). The respective effects on histone acetylation and H3K4 tri- and dimethylation were evaluated by Western blot analysis. The values denote fold changes as determined by the relative intensity of protein bands of treated samples to that of the respective DMSO vehicle-treated control after normalization to the respective internal reference β-actin. Each value represents the average of three independent experiments.
histone H3 acetyl-/Lys-4 methyltransferases (HATs/H3K4MTs) and/or the down-regulation of histone deacetylases/H3K4 demethylases (HDACs/H3K4DMs).

To discern these two possibilities, we used qRT-PCR to assess the effect of CG-12 on the mRNA expression of various histone-modifying enzymes involved in the regulation of H3 acetylation and H3K4 methylation in LNCaP cells, which included HATs (GCN5, PCAF, CBP, p300), H3K4MTs (MLL1, MLL2, MLL3, MLL4, SET1A, ASH1), HDACs (HDAC1–8), and demethylases for H3K4Me3 (RBP-2/JARID1a, PLU-1/JARID1b), and H3K4Me1/2 (LSD1/KDM1). Relative to vehicle control, the mRNA levels of most of the HATs and H3K4MTs examined were significantly decreased after 24-h treatment with 10 μM CG-12, with the exception of the modest (≈20%) but statistically significant increases in the mRNA levels of MLL1 and 3 (Fig. 3A). In contrast, CG-12 suppressed the mRNA levels of HDAC1–4, RBP2, PLU-1, and LSD1 (≥60%), whereas those of HDAC5–8 remained largely unaltered. These findings suggest that the selective repression of HDACs and H3K4DMs played a major role in the observed energy restriction-associated increases in histone H3 acetylation and H3K4 methylation.

Pursuant to these findings, we examined the suppressive effects of CG-12, 2-DG, and glucose starvation on the expression of HDAC isozymes and H3K4DMs by RT-PCR and Western blotting. As shown in Fig. 3B, CG-12, 2-DG, and glu-
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FIGURE 4. Transcriptional repression of H3K4DMs in response to ERMAs and glucose deprivation. LNCaP cells were treated with CG-12 and 2-DG for 48 h or glucose-free medium for the indicated times. The mRNA and protein expression levels of various H3K4DMs were examined by RT-PCR and Western blotting, respectively. The percentages denote the relative intensity of mRNA or protein bands of treated samples to that of the respective DMSO vehicle-treated control after normalization to the respective internal reference β-actin. Each value represents the average of three independent experiments.

Cose starvation suppressed the mRNA and protein levels of HDAC1–4 in a dose- or time-dependent manner without disturbing those of HDAC5–8. From a mechanistic perspective, the concomitant reduction in the expression of HDAC1–4 would lead to increases in H3 acetylation in energy-restricted LNCaP cells. Furthermore, we examined the effect of the transient transfection of LNCaP cells with plasmids encoding HDAC1 and 4 versus PCMV on CG-12-induced KLF6 gene expression. As shown, ectopic expression of either HDAC1 or 4 abrogated the ability of CG-12 to increase KLF6 mRNA expression levels (Fig. 3C), confirming the involvement of these HDAC isoforms in regulating KLF6 mRNA levels in response to CG-12.

These energy-restricted conditions also suppressed the expression of RBP2, PLU-1, and LSD1 at both mRNA and protein levels, in a dose and/or time-dependent fashion (Fig. 4), thereby providing a plausible molecular basis for the accumulation of H3K4Me3 and H3K4Me2 in drug-treated cells. It is interesting to note that, in response to each of these treatments, the extent of down-regulation of these three H3K4DMs paralleled that of HDAC1–4, suggesting that the energy restriction-induced transcriptional repression of these histone-modifying enzymes might share a similar mechanism.

Together, these data suggest that the epigenetic activation of KLF6 gene expression by CG-12 was, in part, attributable to its ability to enhance positive histone marks by suppressing the expression of target HDACs and H3K4DMs.

Sp1 Degradation Underlies the Repressive Effects of CG-12 on Histone-modifying Enzymes—To understand the mechanistic link between energy restriction and histone modifications, we investigated the involvement of the transcription factor Sp1 in CG-12-mediated repression of histone-modifying enzymes, of which the rationale is 2-fold. First, our previous study demonstrated that β-transducin repeat-containing protein (β-TrCP)-dependent proteasomal degradation of Sp1 represents an energy restriction-elicited signaling response (8), which was manifested by the suppressive effect of CG-12, 2-DG, and glucose starvation on the level of Sp1 protein, but not mRNA expression (Fig. 5A). Moreover, the time course of Sp1 protein expression over a 72-h period of treatment with 5 μM CG-12 paralleled that of HDAC1–4, RBP2, PLU-1, and LSD1 at both mRNA and protein levels and showed an inverse relationship with the mRNA and protein expression of KLF6 and its targets ATF3, Noxa, and DAPK2. LNCaP cells were exposed to 5 μM CG-12 for different time periods followed by Western blot analysis and RT-PCR for the aforementioned proteins.

Second, Sp1 has been shown to regulate the expression of HDAC1 (30), HDAC4 (31), and PLU-1 (32), and analysis of the promoter sequences of genes encoding HDAC2–4, RBP2, and LSD1 revealed the presence of one or more putative Sp1 binding elements (GGGCGG) in each promoter (Fig. 6, A and B, left panels).

To establish the functional role of Sp1 in regulating the transcription of these HDAC isozymes and H3K4DMs, we constructed luciferase reporter plasmids harboring the RBP2, PLU-1, LSD1, and HDAC1–4 promoter regions and their respective counterparts containing mutated Sp1 binding sites in which the GGGCGG sequence was replaced with GTTCGG (Fig. 6, A and B, left panels). LNCaP cells were transiently transfected with individual wild-type or mutant reporter plasmids. Relative to the wild-type control, mutation of the Sp1 binding
site significantly diminished the promoter activities of these genes (Fig. 6, A and B, right panels).

Pursuant to this finding, we tested the ability of shRNA-mediated silencing of Sp1 expression to mimic energy restriction-mediated repression of HDAC1–4, RBP2, PLU-1, and LSD1 and the consequent epigenetic changes in LNCaP cells. Western blot analysis indicates that, relative to control shRNA, transfection with Sp1 shRNA reduced the levels of these histone-modifying enzymes in a dose-dependent manner (Fig. 7A). These decreases were accompanied by increases in histone H3 acetylation and H3K4 methylation, and parallel increases in the expression of KLF6 and its target gene products ATF3, Noxa, and DAPK2.

To further validate the role of Sp1 in energy restriction-induced transcriptional activation of KLF6 gene expression, we examined the effect of ectopic Sp1 expression on CG-12-induced epigenetic changes. Overexpression of Sp1 blocked the dose-dependent repressive effects of CG-12 on HDAC1–4, RBP2, PLU-1, and LSD1 expression, thereby abolishing its ability to enhance histone H3 acetylation, H3K4 methylation, and the expression of KLF6 and its target gene products (Fig. 7B).

Together, these findings underscore the important role of Sp1 down-regulation in mediating the epigenetic activation of the KLF6 gene by ERMA and glucose starvation through the repression of histone-modifying enzymes.

**DISCUSSION**

In light of the metabolic adaptation of cancer cells to anaerobic glycolysis to gain growth advantages (1), there is increasing interest in targeting tumor metabolism for cancer therapy. Because chronic energy restriction proves to be difficult to implement as an antitumor strategy, ERMA has received wide attention as they can mimic the beneficial effects of energy restriction through the inhibition of glucose utilization. Previously, we developed CG-12, a thiazolidinedione-based ERMA that exhibits potency 3 orders of magnitude higher than 2-DG in inhibiting cancer cell proliferation through autophagy and apoptosis (8). Although it is well understood that AMPK activation plays a crucial role in ERMA-induced autophagy in cancer cells (33), the underlying mechanism of apoptosis remains unclear. Thus, CG-12 provides a useful pharmacological probe to understand the underlying signaling pathways.

Here, we report evidence that the epigenetic up-regulation of tumor suppressor gene KLF6 expression represents a key signaling mechanism by which energy restriction mediates apoptosis (Fig. 8). Previously, we reported that CG-12, 2-DG, and glucose starvation facilitated β-TrCP-mediated proteasomal degradation of Sp1 and other target proteins via a Sirt1-dependent mechanism in LNCaP cells (8, 20), and that this β-TrCP-dependent proteolysis plays an integral role in ERMA-induced apoptosis since dominant-negative inhibition of β-TrCP protected cells against apoptosis (8). In this study, we extended our understanding of the cellular response to energy restriction by defining the epigenetic activation of KLF6 gene expression as a key mediator of energy restriction-induced apoptosis.
KLF6 plays a crucial role in tumor suppression by modulating the expression of a broad range of genes governing biological functions associated with cell growth, cell differentiation, cell adhesion, and endothelial motility (9), among which ATF3 has been shown to represent a major mediator of KLF6-induced apoptosis in prostate cancer cells (24). In this study, we demonstrated the concomitant up-regulation of ATF3, Noxa, and DAPK2 with KLF6 in LNCaP and DU-145 cells treated with ERMA or glucose-depleted medium (Fig. 1). Conceivably, the concerted effort of these proapoptotic proteins underlies the key role of KLF6 in mediating energy restriction-induced apoptosis.

Evidence suggests that the transcriptional activation of KLF6 gene expression resulted from enhanced active histone marks, i.e. H3K4Me3 and Ac-H3, in the promoter region (Fig. 2). Our data also indicate that increased H3 acetylation and H3K4 methylation were attributable to transcriptional repression of HDACs (Fig. 3) and H3K4DMs (Fig. 4) because of reduced Sp1 expression (Fig. 5). Pursuant to previous findings that Sp1 regulates the transcription of HDAC1 and 4 and PLU-1 (30–32), we identified HDAC2 and 3, RPB2, and LSD1 as additional Sp1 target genes through mutational analysis (Fig. 6). The functional role of Sp1 in mediating these epigenetic changes leading to KLF6 gene activation was further confirmed by using shRNA-mediated knockdown and ectopic expression to observe the consequent effects on relevant biomarkers (Fig. 7).

Substantial evidence points to Sp1 as a relevant target for cancer therapy in light of its pivotal role in regulating the expression of a host of key effectors of signaling pathways governing cell cycle progression, cell proliferation, angiogenesis, apoptosis, and metastasis (34–36). In this study, we demonstrated the ability of Sp1 to modulate the expression of multiple histone-modifying enzymes, leading to the transcriptional activation of the KLF6 gene by increasing active histone marks. From a mechanistic perspective, the ability of Sp1 to effect epigenetic changes by modifying histones adds a layer of complexity to its functional role in regulating gene expression, which warrants further investigations.

It is noteworthy that CG-12 also significantly down-regulated (>50%) the mRNA expression of a number of the HATs

![Diagram depicting the mode of action by which energy restriction activates KLF6 tumor suppressor gene expression.](image_url)
and H3K4MTs examined, including GCN5, MLL2, and SET1A (Fig. 3). As none of these three genes contains any Sp1-binding site in their promoters, other mechanisms are involved in the transcriptional regulation of these genes. For example, GCN5 is known to be a direct target gene of Myc (37), of which the mRNA expression was also significantly reduced by CG-12. However, the transcription factors that regulate the gene expression of MLL2 and SET1A remain to be investigated.

In summary, our data identify the epigenetic activation of KLF6 tumor suppressor gene expression as a critical event leading to apoptosis in energy-restricted cancer cells. This causal relationship between energy restriction and the epigenetic modulation of KLF6 gene expression underscores the complexity of the signaling network involved in the apoptotic response to energy restriction. As KLF6 represents a new molecular marker candidate for tumor prognosis and a potential target for cancer therapy (38), our findings are of potential translational value for the development of therapeutic strategies involving new-generation ERMAs such as CG-12.

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3 C. Chen, unpublished data.