Induction of the Transcriptional Repressor ZBTB4 in Prostate Cancer Cells by Drug-Induced Targeting of MicroRNA-17-92/106b-25 Clusters

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Introduction
Prostate cancer is the most common tumor in men, and it is estimated that in 2012, 241,700 men will be diagnosed with prostate cancer and 28,170 will die from this disease (1). Prostate cancer is highly complex, and androgen deprivation therapy by surgical or chemotherapeutic means has been critical for treatment of early and advanced prostate cancers (2–5). Androgen deprivation therapies are highly variable but are primarily focused on inhibition of androgen synthesis that decreases circulating levels of the endogenous hormone or on blocking the androgen receptor signaling with various androgen receptor agonists (2). Not surprisingly, many patients with prostate cancer develop castrate-resistant prostate cancer (CRPC), which was previously referred to as hormone-refractory or androgen-independent (6). Some patients with CRPC may still be responsive to some forms of anti-androgen therapy; however, many new combination therapies often include a cytotoxic drug such as docetaxel or other taxane derivatives plus various agents that block angiogenesis or growth factor receptor signaling pathways in clinical trials (3–5). Development of new therapies for CRPC is also focused on decreasing the toxic side effects and increasing efficacy of these treatments and this includes the use of new mechanism-based drugs that target specific pathways and genes.

The addiction of many tumors to oncogenes such as various kinases including growth factor receptors has led to development of various kinase inhibitors and neutralizing antibodies such as erlotinib (Tarceva) and trastuzumab (Herceptin), which are clinically used for cancer chemotherapy (3–5, 7, 8). It has recently been suggested that nononcogene addiction by cancer cells is also critical for maintaining the tumorigenic state of cells (9–11) and "a large class of nononcogenes (that) are essential for cancer cell survival and present attractive drug targets" (10). Specificity protein (Sp) transcription factors Sp1, Sp3, and Sp4 are overexpressed in multiple cancer cell lines and tumors (12–21). Although these factors are important for embryonic and postnatal development (22, 23), there

Abstract
Androgen-insensitive DU145 and PC3 human prostate cancer cells express high levels of specificity protein (Sp) transcription factors Sp1, Sp3, and Sp4, and treatment of cells with methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (CDODA-Me) inhibited cell growth and downregulated Sp1, Sp3, and Sp4 expression. CDODA-Me (15 mg/kg/d) was a potent inhibitor of tumor growth in a mouse xenograft model (PC3 cells) and also decreased expression of Sp transcription factors in tumors. CDODA-Me–mediated downregulation of Sp1, Sp3, and Sp4 was due to induction of the transcriptional repressor ZBTB4, which competitively binds and displaces Sp transcription factors from GC-rich sites in Sp1-, Sp3-, Sp4-, and Sp-regulated gene promoters. ZBTB4 levels are relatively low in DU145 and PC3 cells due to suppression by miR paralogs that are members of the miR-17-92 (miR-20a/17-5p) and miR-106b-25 (miR-106b/93) clusters. Examination of publically available prostate cancer patient array data showed an inverse relationship between ZBTB4 and miRs-20a/17-5p/106b/93 expression, and increased ZBTB4 in patients with prostate cancer was a prognostic factor for increased survival. CDODA-Me induces ZBTB4 in prostate cancer cells through disruption of miR–ZBTB4 interactions, and this results in downregulation of pro-oncogenic Sp transcription factors and Sp-regulated genes.

Therapeutic Discovery

Molecular Cancer Therapeutics

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is evidence that Sp1 expression decreases with age (24, 25) and, in animal models, minimal to nondetectable levels of Sp proteins are observed (14, 15). Evidence of a significant role for Sp transcription factors in nononcogene addiction pathways in cancer cells is supported by RNA interference (RNAi) studies that show that Sp1, Sp3, and Sp4 regulate pro-oncogenic factors that play a role in cell proliferation, survival and angiogenesis. Sp-regulated genes include EGF receptor (EGFR), hepatocyte growth factor receptor (c-MET), bcl-2, survivin, VEGF, and its receptors (VEGFR1 and VEGFR2), p65NF-κB, and putatary tumor transforming gene 1 (PTTG1; refs. 13, 18–21, 26, 27). Moreover, knockdown of Sp1, Sp3, and Sp4 in cancer cells also inhibits cell proliferation and G0–G1 to S-phase progression and induces apoptosis (15, 18–20, 28).

Methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate (CDODA-Me) is a synthetic triterpenoid methyl ester derived from glycyrrhetinic acid, a major component of licorice extracts (29). In this study, we show that CDODA-Me inhibits androgen-insensitive prostate cancer cell and tumor growth and downregulates Sp1, Sp3, and Sp4 through induction of the Sp repressor ZBTB4 and downregulation of miR-20a and related paralogs that inhibit ZBTB4 expression. MiR-dependent suppression of the Sp repressors ZBTB4 and ZBTB10 are critical for the high expression of Sp transcription factors (30, 31), and disruption of miR-ZBTB4 by CDODA-Me plays an essential role as an inhibitor of androgen-insensitive prostate cancer.

Materials and Methods

Cell lines, antibodies, plasmids, and reagents

The PC3 and DU145 prostate cancer cells were purchased from the American Type Culture Collection. Cells were initially grown and multiple aliquots were stored at −180°C for future use as required. Cells were purchased more than 6 months ago and were not further tested or authenticated by the authors. Cells were grown in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic antimycotic solution (Sigma-Aldrich) and main-chored from the American Type Culture Collection. Cells were purchased from Cell Signaling and Aviva Systems Biology, respectively. Mimics and antisense microRNAs and their counterpart controls were purchased from Dharmaco. siRNAs for Sp1, Sp3, and Sp4 were purchased from Sigma-Aldrich and scrambled control siRNA from Qiagen. Reporter luciferase constructs for pSp1, pSp3, pVEGF, and pSurvivin were previously used in our studies (30, 31).

Invasion and scratch (migration) assay

Invasion assay was conducted as previously described (31) with addition of indicated compound after 18 hours. For scratch assay, after cells were more than 80% confluent in 6-well plates, the scratch was made using a sterile pipette and then treated with control [dimethyl sulfoxide (DMSO)] or compound. Cell migration into the scratch was determined after 18 hours (7–8 determinations/treatment).

Cell proliferation, apoptosis, and fluorescence-activated cell-sorting cell-cycle analysis

PC3 and DU145 prostate cancer cells (2 × 10^4) were plated in 12-well plates, and the next day, fresh medium containing either control (DMSO) or the indicated compound was added. Cells were counted at the indicated times using a Coulter Z1 cell counter (Beckman Coulter). For cell-cycle analysis, cells were treated with staining solution containing propidium iodine (50 μg/mL) after treatment with the compound for 24 hours. Stained cells were analyzed by a FACScalibur Flow Cytometer (Becton Dickinson Systems). Apoptosis was detected using Annexin V-FITC kit (Invitrogen) according to the manufacturer’s protocol.

Quantitative real-time PCR analysis for mRNAs and miRNAs

Total RNA was extracted using the mirVanaRNA extraction kit (Applied Biosystems). The change in expression of miRNAs was quantified using TaqMan miRNA Kit using either RNU6B or U6 small nuclear RNA as control. For mRNA quantification, SYBR real-time PCR was carried out and normalized to TBP as previously described (31). The following primer sets used for quantitative real-time PCR analysis:

- **E2F1** (forward): 5'-ATGTTTTTCCCTGTGCCCCTGAG-3'
- **E2F1** (reverse): 5'-ATCGTGTTGAGGGATGAGG-3'
- **E2F2** (forward): 5'-CTTTGAGGCTACTGACACG-3'
- **E2F2** (reverse): 5'-CCACAGTGTGCCTGTG-3'
- **E2F3** (forward): 5'-CACCCTGGACCTCAAATGTG-3'
- **E2F3** (reverse): 5'-AAGCTTGGAAACCGGTTT-3'
- **TPB** (forward): 5'-TGACAGGGACCAAGTGAATGT-3'
- **TPB** (reverse): 5'-CTCACAGTGGTCCTCACCATG-3'

Western blotting, immunostaining, and luciferase assay

Western blotting was carried out using β-actin as a loading control as previously described (31). Different promoter reporter luciferase constructs, β-galactosidase, ZBTB4 expression plasmid (1 μg per well), or antisense or mimic miRs (150 nmol/L per well) and their corresponding controls (150 nmol/mL) were cotransfected by Lipofectamine 2000 (Invitrogen). The luciferase activities were either normalized to β-galactosidase or protein concentration. For immunostaining, briefly, cells were fixed in 4% paraformaldehyde, permeabilized in PBS with 0.3% Triton X-100 for 10 minutes, and preincubated for 2 hours with 10% normal goat serum for blocking. Cells were then incubated with Sp1 antibody overnight and incubated with fluorescein isothiocyanate (FITC)-conjugated...
secondary antibody. Images were captured with LSM 510 Meta confocal microscope (Carl Zeiss).

**Chromatin immunoprecipitation assay**
Chromatin immunoprecipitation (ChIP) assays were carried out using ChIP-IT Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif) according to manufacturer’s protocol. Briefly, PC3 cells (1 × 10⁶ cells) were treated with DMSO or CDODA-Me (5 μmol/L) for 18 hours, and cells were fixed, sonicated, and co-immunoprecipitated with Sp1 antibody and IgG control. The immunoprecipitated lysates were reversely DNA cross-linked and then DNA was prepared by proteinase K digestion followed by PCR amplification. The ChIP primer sets used were:

- Primer I (forward): 5'-CTTGGAGACAACGAGTCT-3'
- Primer I (reverse): 5'-GGACTCATCCTTACCGCTCA-3'
- Primer II (forward): 5'-CCAGCCTTCTTGGTGTGTTT-3'
- Primer II (reverse): 5'-CTACTCCCAGACGGATCAA-3'

**Cancer patient gene expression data analysis**
Normalized microRNA gene expression data were obtained from Gene Expression Omnibus (accession number GSE23022). Four ZBTB4 prostate cancer patient expression data sets were obtained from the Oncomine database (32–36). For Kaplan–Meier patient survival analysis by ZBTB4 expression level, Dr. Luo in the University of Pittsburgh (Pittsburgh, PA) kindly provided us clinical data for the analysis (37).

**Xenograft studies in athymic mice**
Male athymic nude mice (FornI nu, ages 6–8 weeks) were purchased from Harlan Laboratories. PC3 cells (1 × 10⁶) in 1:2 ratio of Matrigel (BD Bioscience) were injected in the flank of nude mice, and mice were randomly divided into 2 groups of 6 animals each. Either corn oil (control) or CDODA-Me (15 mg/kg/d) was orally administered to each group, respectively. The volume and size of xenografted tumors were measured throughout the study: \( V = LW/2 \), where \( L \) and \( W \) were length and width. The selected tissues were further examined by routine hematoxylin and eosin (H&E) staining and RNA analysis.

**Immunohistochemistry**
Paraffin-embedded tissue sections (5-μm thick) were deparaffinized, and endogenous peroxidase activity was blocked by the use of 2% hydrogen peroxide in PBS for 2 minutes. Antigen retrieval for Sp1 staining was made by heating to 100°C in a steamer followed by cooling to 20°C for 10 to 15 minutes. The slides were incubated with a protein blocking solution (VECTOR-STAINT Elite ABC Kit; Vector Laboratories) and stained by manufacturer’s protocol with 1:100 dilution of Sp1 antibody (VECTORSTAINT Elite ABC Kit). The staining was developed by diaminobenzidine reagent (Vector Laboratories) as a brown color, and the sections were then counterstained with Gill hematoxylin.

**Results**
CDODA-Me (Fig. 1A) inhibits LNCaP prostate cancer cell growth (38), and results in Fig. 1A and B show that CDODA-Me also inhibited growth of androgen- insensitive PC3 and DU145 prostate cancer cells, increased the percentage of cells in G0–G1, and decreased the percentage in S- and G2–M phases of the cell cycle. The antiproliferative activity of CDODA-Me was accompanied by induction of both early and late apoptosis, which was determined with an Annexin V-FITC kit (Fig. 1C). We also observed that after treatment of PC3 and DU145 cells with 1.0 or 2.5 μmol/L CDODA-Me for 18 hours, there was a significant decrease in cell invasion using a Boyden chamber assay and in migration using a scratch assay (Fig. 1D).

Previous studies show that CDODA-Me decreased Sp1-, Sp3-, Sp4-, and Sp-regulated gene expression in colon cancer cells (15), and results in Fig. 2A show that CDODA-Me also decreased Sp1, Sp3, and Sp4 protein levels in PC3 and DU145 prostate cancer cells. Using PC3 cells as a model, cells were treated with DMSO or 2.5 μmol/L CDODA-Me for 1 hour and then stained with DAP-1 and Sp1-FITC alone or in combination. There was an overlap between nuclear 4',6-diamidino-2-phenylindole (DAPI) and Sp1 staining in control cells, confirming the nuclear location of Sp1 (Fig. 2B). In cells treated with CDODA-Me, there was a significant decrease in nuclear Sp1 staining. CDODA-Me also decreased expression of several Sp-regulated genes including survivin, VEGF, urokinase plasminogen activator receptor (uPAR), and cyclin D1 in both cell lines (Fig. 2C). The role of Sp1 downregulation by CDODA-Me in modulating cell-cycle progression was confirmed by RNAi, which showed that knockdown of Sp1 (iSp1) increased the percentage of PC3 and DU145 cells in G0–G1 and decreased their percentage in S- and G2–M phases (Fig. 2D), and this was consistent with results obtained for CDODA-Me (Fig. 1B).

Recent studies in breast cancer cells showed that high expression of Sp transcription factors is due to miR-dependent suppression of the transcriptional repressor ZBTB4, which is also a prognostic factor for breast cancer patient survival (31). Figure 3A shows that CDODA-Me induced ZBTB4 protein and mRNA expression in PC3 and DU145 cells; moreover, overexpression of ZBTB4 in these cell lines was accompanied by downregulation of Sp1-, Sp3-, Sp4-, and the Sp-regulated genes VEGF, survivin, and uPAR (Fig. 3B). ZBTB4 overexpression also decreased luciferase activity in PC3 and DU145 cells transfected with constructs containing GC-rich inserts from the Sp1 (pSp1), Sp3 (pSp3), VEGF (pVEGF), and survivin (pSurvivin) gene promoters (Fig. 3B). Like CDODA-Me (Fig. 1B) and iSp1 (Fig. 2D), overexpression of ZBTB4 also increased the percentage of PC3 and DU145 cells in G0–G1 and decreased the percentage of cells in G2–M and...
S (minimal)-phases of the cell cycle (Fig. 3C). The effects of CDODA-Me on binding of Sp1 and ZBTB4 with the GC-rich proximal region of the Sp1 promoter were investigated in a ChIP assay (Fig. 3D). Sp1 but not ZBTB4 was constitutively bound to the GC-rich region and, after treatment with CDODA-Me, occupation of this site by Sp1 was decreased and this was accompanied by increased occupation by ZBTB4 (primer I). In contrast, interactions of Sp1 and ZBTB4 with a distal non–GC-rich region of the Sp promoter were not detected (primer II). These results suggest that the effects of CDODA-Me are consistent with induction of the Sp repressor ZBTB4, which in turn suppresses Sp-regulated genes and responses through competitive displacement of Sp proteins bound to GC-rich sites. Examination of publically available prostate cancer patient array data showed that ZBTB4 is more highly expressed in normal prostate tissue than in prostate carcinomas, and high expression of ZBTB4 is a prognostic factor for increased relapse-free survival (Supplementary Fig. S1A–S1C; refs. 33–36, 38–41), and similar results were reported for patients with breast cancer (31). Future studies will also examine ZBTB4 protein expression in patients with prostate cancer.

Suppression of ZBTB4 in breast cancer cells has been linked to microRNA-17-5p (miR-17), miR-20a, and their paralogs miR-93 and miR-106b, and these miRs were expressed in PC3 and DU145 cells (Fig. 4A). Moreover, the 4 miR paralogs were all more highly expressed in prostate tumors versus nontumor tissues (Fig. 4A), and these results were inversely related to ZBTB4 expression in the same tissues (Supplementary Fig. S1). Treatment of PC3 and DU145 cells with CDODA-Me also decreased expression of the 4 miR paralogs (Fig. 4B), and this was correlated with the induction of ZBTB4 mRNA and
protein observed in these cell lines (Fig. 3A). Moreover, luciferase activity was decreased in PC3 and DU145 cells transfected with constructs containing promoter sequences upstream from miR-93 and miR-106b [pMCM7 (~558)] and miR-17-5p and miR-20a [pmiR-17-92 (pro1353); refs. (39, 40)] and treated with CDODA-Me (Fig. 4C). Because miR-93 and miR-106b are part of the miR-106b-25 cluster located in the 13th intron of the MCM7 gene, we investigated the effects of CDODA-Me on MCM7 and showed that expression of this gene was also decreased in PC3 and DU145 cells. This shows that the miR paralogs are primary targets of CDODA-Me, and the mechanism of drug-induced repression of these miRs is currently being investigated.

Confirmation of the role of the miR paralogs in repressing ZBTB4 and facilitating high expression of Sp1, Sp3, and Sp4 was further investigated in PC3 cells transfected with miR antagomirs and mimics. Figure 5A shows that miR-93 and miR-106b mimics decreased luciferase activity and miR-93 and miR-106b antagomirs increased luciferase activity in PC3 and DU145 cells transfected with the ZBTB4-3′-UTR (2,559 bp) construct, which contains the 3′-untranslated region (UTR) sequence from the ZBTB4 gene with binding sites for the 4 miR paralogs (31). Moreover, transfection of the PC3 cells with miR paralog antagomirs also induced ZBTB4 mRNA expression (Fig. 5B). Using miR-106b and miR-93 antagomirs (As-miR-106b and As-miR-93) as models, we also show that transfection of both antagomirs decreased luciferase activity in PC3 and DU145 cells cotransfected with pSp1, pSp3, pVEGF, and pSurvivin promoter–reporter constructs (Fig. 5C) and also downregulated expression of Sp1, Sp3, Sp4, VEGF, uPAR, and survivin proteins (Fig. 5D).

Both the miR-17-92 and miR-106b-25 clusters are regulated by E2F transcription factors (39–41), and downregulation of the 4 miR paralogs by CDODA-Me (Fig. 4B) could also be due to either direct or indirect effects on E2Fs. Supplementary Figure S2A and S2B show that CDODA-Me decreased expression of E2F1 and E2F2 but not E2F3 protein and mRNA levels, respectively, in PC3 and DU145 cells. Because CDODA-Me downregulates the 4 miR paralogs (Fig. 4B) and induces the transcriptional repressor ZBTB4, we overexpressed ZBTB4 in PC3 and DU145 cells and showed that ZBTB4 decreased expression of E2F1 and E2F2 but not E2F3 (Supplementary Fig. S2C). This suggests that CDODA-Me indirectly suppresses E2F1 and E2F2 through induction of ZBTB4. Moreover, knockdown of Sp1, Sp3, and Sp4 by RNAi also showed that E2F1 and E2F2 were Sp-regulated genes in PC3 cells (Supplementary Fig. S2D), and similar data were observed in DU145 cells (Supplementary Fig. S2E). CDODA-Me–induced suppression of E2F1 and E2F2 is due to disruption of miR–ZBTB4 interactions and results in downregulation of Sp1, Sp3, and Sp4. Previous reports show that drug-induced downregulation of Sp proteins can be proteasome- or ROS-dependent (12, 15, 28, 42); however, glutathione and proteasome inhibitors did not affect CDODA-Me–induced downregulation of Sp1, Sp3,
and Sp4 (Supplementary Fig. S3), and we are currently investigating the mechanisms of CDODA-Me–induced effects on the miR paralogs:ZBTB4-Sp protein axis.

Treatment of male athymic nude mice bearing PC3 cells as xenografts with CDODA-Me (15 mg/kg/d) decreased tumor cell growth and weight (Fig. 6A) and nuclear Sp1, Sp3, and Sp4 staining in tumors (Fig. 6B). Moreover, H&E staining showed a more disorganized and disrupted pattern in treated versus control tumors. Examination of the tumors showed that CDODA-Me decreased expression of miR-106b, miR-93, miR-17, and miR-20a (Fig. 6C). Figure 6D summarizes the pathways activated by CDODA-Me in ZBTB4 induction and function. A, induction of ZBTB4 mRNA and protein. Cells were treated with CDODA-Me for the indicated times, and mRNA and protein levels were determined by real-time PCR and Western blotting, respectively, as outlined in Materials and Methods. B, effects of ZBTB4 overexpression on Sp-regulated proteins and constructs. ZBTB4 (1 μg per well) was overexpressed alone in PC3 and DU145 cells or in combination with several GC-rich Sp-regulated constructs and proteins, and luciferase (Luc) activity was overexpressed alone in PC3 and DU145 cells or in combination with several GC-rich Sp-regulated constructs and proteins, and luciferase (Luc) activity was determined as described in Materials and Methods. ZBTB4 overexpression modulates the cell cycle (C) and Sp1 binding to the GC-rich Sp1 promoter in a ChIP assay (D). Cells were transfected with ZBTB4, and after 24 hours (C) or 18 hours (D), cells were analyzed by fluorescence-activated cell-sorting or in a ChIP assay as described in Materials and Methods. Results are expressed as means ± SE for at least 3 replication determinations, and significantly (P < 0.05) increased (*) or decreased (**) responses are indicated. RLU, relative luciferase units.

Figure 3. ZBTB4 induction and function. A, induction of ZBTB4 mRNA and protein. Cells were treated with CDODA-Me for the indicated times, and mRNA and protein levels were determined by real-time PCR and Western blotting, respectively, as outlined in Materials and Methods. B, effects of ZBTB4 overexpression on Sp-regulated proteins and constructs. ZBTB4 (1 μg per well) was overexpressed alone in PC3 and DU145 cells or in combination with several GC-rich Sp-regulated constructs and proteins, and luciferase (Luc) activity was determined as described in Materials and Methods. ZBTB4 overexpression modulates the cell cycle (C) and Sp1 binding to the GC-rich Sp1 promoter in a ChIP assay (D). Cells were transfected with ZBTB4, and after 24 hours (C) or 18 hours (D), cells were analyzed by fluorescence-activated cell-sorting or in a ChIP assay as described in Materials and Methods. Results are expressed as means ± SE for at least 3 replication determinations, and significantly (P < 0.05) increased (*) or decreased (**) responses are indicated. RLU, relative luciferase units.
which the primary targets are the miR paralogs that serve to suppress ZBTB4 in prostate cancer cells. CDODA-Me induces downregulation of the miRs and induction of ZBTB4, which represses expression Sp transcription factors and the pro-oncogenic Sp-regulated genes and their downstream responses.
Discussion

Sp1, Sp3, and Sp4 transcription factors are prototypical nononcogenes that are overexpressed in cancer cells and tumors and regulate a battery of genes that play a role in cancer cell growth, survival, angiogenesis, and metastasis. Research in this laboratory has identified several anticancer agents that target downregulation of Sp1-, Sp3-, Sp4-, and Sp-regulated genes in cancer cells and these include arsenic trioxide, curcumin, nonsteroidal anti-inflammatory drugs (NSAID), and several triterpenoids including betulinic acid, CDODA-Me, and the structurally related methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me; refs. 12–21, 28, 29, 42). The mechanisms associated with drug-induced Sp downregulation are both proteasome-dependent and -independent and also vary with cell context. For example, betulinic acid induces proteasome-dependent downregulation of Sp1, Sp3, and Sp4 in androgen-sensitive LNCaP prostate cancer cells (38), whereas in colon cancer cells, this response is ROS-dependent and proteasome-independent (14). Cell context–dependent differences in pathways leading to Sp downregulation by curcumin and tolfenamic acid have also been observed (16, 17, 19, 21, 43).

CDODA-Me Disrupts miR-17-92/106b-25–ZBTB4 Interactions

Figure 5. MiR mimics and antagonists and their effects on ZBTB4 and Sp-regulated genes/reporter genes. Interactions with the 3’-UTR of ZBTB4 (A) and induction of ZBTB4 mRNA (B). PC3 cells were transfected with the ZBTB4-3’-UTR-luciferase construct containing the CACUUUA miR-binding site and cotransfected with indicated miR mimics (A) or antagonists (A and B). Luciferase activity or ZBTB4 mRNA expression were determined as outlined in Materials and Methods. MiR antagonists decrease luciferase activity (C) and downregulate Sp proteins and Sp-regulated genes (D). Cells were transfected with the antagonists and, in C, were cotransfected with GC-rich constructs. Luciferase activity or protein expression was determined as outlined in Materials and Methods. Results (A–C) are expressed as means ± SE for at least 3 replicate determinations, and significantly (P < 0.05) induced (†) or decreased (∗) responses are indicated. RLU, relative luciferase units.
this compound in LNCaP cells is PPARγ-independent (38). Like betulinic acid (12), CDODA-Me induces proteasome-dependent degradation of Sp1, Sp3, and Sp4 in LNCaP cells (unpublished results); however, in androgen-insensitive PC3 and DU145 cells, CDODA-Me decreased expression of Sp1-, Sp3-, Sp4-, and Sp-regulated genes, and this response was proteasome-independent (Supplementary Fig. S3). The effects of CDODA-Me on inhibition of cell growth, invasion, and migration, induction of apoptosis, and G0–G1 to S-phase arrest (Figs. 1 and 2) were consistent with results obtained by knockdown of Sp transcription factors (Fig. 2D; refs. 15, 18–20, 28). These in vitro results are complemented by in vivo studies showing that CDODA-Me (15 mg/kg) also decreased tumor growth in a PC3 cell xenograft study and downregulated expression of Sp1 (Fig. 6).

A possible explanation for the high expression of Sp1, Sp3, and Sp4 in cancer cell lines is miR-dependent inhibition of the transcriptional repressors ZBTB10 and ZBTB4, which can act as Sp repressors by competitively binding GC-rich promoters, resulting in the loss of Sp proteins from these sites (30, 31). We first characterized miR-27a–mediated suppression of ZBTB10 in breast cancer cells showing that both antisense miR-27a and ZBTB10 overexpression repressed expression of Sp1, Sp3, and Sp4 (30). Recent studies also show that miR-20a and related paralogs that are part of the miR-17-92 (miR-20a and miR-17-5p) and miR-106b-25 (miR-106b and miR-93) clusters inhibit ZBTB4 expression (31). MiR-27a inhibits expression of Myt-1, a kinase that arrests cells in G2–M phase, and As-miR-27a or downregulation of miR-27a by CDODA-Me in colon cancer cells arrest cells in G2–M

Figure 6. In vivo studies. Inhibition of tumor size and weights (A), H&E and Sp1, Sp3, and Sp4 staining (B), and miR-106b/miR-92 levels (C) in mice treated with CDODA-Me. Athymic nude mice bearing PC3 cells as xenografts were treated with CDODA-Me (15 mg/kg/d) over a period of 21 days, and tumor volumes were estimated. Tumor weights, H&E staining, and miR levels were determined as outlined in Materials and Methods. D, mode of action for CDODA-Me. Proposed pathways triggered by CDODA-Me in prostate cancer cells/tumors that lead to downregulation of Sp1-, Sp3-, Sp4-, and Sp-regulated genes. Results are expressed as means ± SE for at least 3 replication determinations, and significantly (P < 0.05) decreased (↓) responses are indicated.
phase of the cell cycle (15). In contrast, both CDODA-Me and Sp1 knockdown inhibited G2→G1 to S-phase progression of PC3 and DU145 cells (Figs. 1C and 2D). These results, coupled with relatively low expression of miR-27a, prompted us to examine the effects of CDODA-Me on miR-mediated inhibition of ZBTB4 in prostate cancer cells. ZBTB4 was clearly induced by CDODA-Me in PC3 and DU145 cells (Fig. 3A), and overexpression of ZBTB4 repressed Sp1-, Sp3-, Sp4-, and Sp-regulated genes and reporter genes (Figs. 3B and C). CDODA-Me also decreased expression of the 4 miR paralogs and induced reporter genes (Figs. 4 and 5) as previously reported for miR-20a and miR-17-5p (31). Previous studies show that E2F transcription factors regulate expression of both miR-20a and miR-17-5p (31). Previous studies show that E2F transcription factors regulate expression of both miR-17-92 and miR-106b-25 clusters (39–41). CDODA-Me decreased expression of E2F1 and E2F2 in DU145 and PC3 cells; however, the effects of CDODA-Me were not upstream from the miR cluster (Fig. 6D) because knockdown of Sp1, Sp3, and Sp4 by RNAi showed that E2F1 and E2F2 were also Sp-regulated genes (Supplementary Fig. S2D and S2E). Drug-induced ROS and ROS-mediated disruption of miR-27a/ZBTB10 has also been linked to Sp downstream regulation, and these effects are reversed after cotreatment with antioxidants (14, 19, 28, 42). In contrast, glutathione did not affect downregulation of Sp1, Sp3, and Sp4 by CDODA-Me (Supplementary Fig. S3), and the mechanisms associated with the effects of CDODA-Me on miR-ZBTB4 regulation and expression of Sp transcription factors is currently being investigated.

In summary, our results show that CDODA-Me–mediated suppression of Sp1-, Sp3-, Sp4-, and Sp-regulated genes is due to disruption of miR-20a/17-5p/106b/93–dependent regulation of ZBTB4 in androgen-insensitive prostate cancer cells, and this pathway contributes to the antitumorigenic activity of this drug. Interestingly, ZBTB4 was more highly expressed in nontumor versus prostate tumor tissue (Supplementary Fig. S1A and S1B; refs. 33–36), whereas the inverse expression of the miRs was observed (Fig. 4A), and ZBTB4 overexpression was a prognostic factor for increased prostate cancer patient survival (Supplementary Fig. 1C). These results suggest that “addiction” to nononcogenic Sp transcription factors by prostate cancer cells is due to miR-dependent suppression of ZBTB4, suggesting that the miRs are important targets for activating downregulation of Sp1, Sp3, and Sp4 (Fig. 6D). Current studies are focused on identifying other agents that target miR-20a and related paralogs. We are also investigating the mechanism of drug–miR interactions that result in the induction of the transcriptional repressor ZBTB4, which has both prognostic and functional significance in prostate cancer and serves to downregulate “non-oncogenic” Sp transcription factors that contribute to the prostate tumor phenotype.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: K. Kim, S. Safe
Development of methodology: K. Kim, G. Chadalapaka, S. Chintharlapalli, S. Safe
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Kim, S.K. Pathi, U.-H. Jin, S. Chintharlapalli, S. Safe
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Induction of the Transcriptional Repressor ZBTB4 in Prostate Cancer Cells by Drug-Induced Targeting of MicroRNA-17-92/106b-25 Clusters

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