HDAC Up-Regulation in Early Colon Field Carcinogenesis Is Involved in Cell Tumorigenicity through Regulation of Chromatin Structure

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Abstract
Normal cell function is dependent on the proper maintenance of chromatin structure. Regulation of chromatin structure is controlled by histone modifications that directly influence chromatin architecture and genome function. Specifically, the histone deacetylase (HDAC) family of proteins modulate chromatin compaction and are commonly dysregulated in many tumors, including colorectal cancer (CRC). However, the role of HDAC proteins in early colorectal carcinogenesis has not been previously reported. We found HDAC1, HDAC2, HDAC3, HDAC5, and HDAC7 all to be up-regulated in the field of human CRC. Furthermore, we observed that HDAC2 up-regulation is one of the earliest events in CRC carcinogenesis and observed this in human field carcinogenesis, the azoxymethane-treated rat model, and in more aggressive colon cancer cell lines. The universality of HDAC2 up-regulation suggests that HDAC2 up-regulation is a novel and important early event in CRC, which may serve as a biomarker. HDAC inhibitors (HDACis) interfere with tumorigenic HDAC activity; however, the precise mechanisms involved in this process remain to be elucidated. We confirmed that HDAC inhibition by valproic acid (VPA) targeted the more aggressive cell line. Using nuclease digestion assays and transmission electron microscopy imaging, we observed that VPA treatment induced greater changes in chromatin structure in the more aggressive cell line. Furthermore, we used the novel imaging technique partial wave spectroscopy (PWS) to quantify nanoscale alterations in chromatin. We noted that the PWS results are consistent with the biological assays, indicating a greater effect of VPA treatment in the more aggressive cell type. Together, these results demonstrate the importance of HDAC activity in early carcinogenic events and the unique role of higher-order chromatin structure in determining cell tumorigenicity.

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Introduction
Higher-order chromatin structure regulates a number of biological processes on different scales of organization. Chromatin modulation is well studied at the nucleosome level, which consists of DNA wrapped tightly around a histone octamer composed of the four core histone proteins (H3, H4, H2A, H2B). The core histones are subject to a variety of post-translational modifications on their N-terminal tails, such as methylation, phosphorylation, and acetylation [1]. Given that chromatin structure plays a significant role in gene transcription, dysregulation of proper chromatin structure is present in many diseases. Changes in chromatin structure orchestrate the alterations in tumor suppressor genes or activation of proto-oncogenes needed for neoplastic progression. Thus, epigenetic regulation of gene expression is emerging as an important facet of carcinogenesis, including colorectal cancer (CRC) [2]. Progression of CRC is coordinated by a series of mutations and chromosomal deletions of key oncogenes (e.g., Kras) or tumor suppressor genes (e.g., p53, APC), or through defects in DNA mismatch repair genes (e.g., hMLH1, hMSH2) [3,4,5]. In recent years, more attention has been drawn to the interplay of mutational and epigenetic events in both the initiation and progression of CRC [6,7]. Many of these genetic/epigenetic events are observed distant from where the actual tumor develops, including global methylation [8,9]. These studies support the concept of field carcinogenesis (also known as field cancerization, field effect, and field defect), which is the proposition that the genetic/environmental milieu that results in a focal neoplastic lesion is present throughout the affected organ [10]. Thus, the genetic and environmental alterations in the diffuse field provide a background on which individual tumors and lesions arise [11]. Our group has recently demonstrated that there are profound nano-scale chromatin alterations in the uninvolved mucosa that may serve as a diagnostic marker of field carcinogenesis.
[12,13,14]. Given the significant and diverse consequences following modifications of chromatin structure and function, it is crucial to identify the underlying physical and biological mechanisms during early stages of field carcinogenesis.

There are several proteins that play a role in determining higher-order chromatin architecture. One group of mediators in chromatin structure is the histone deacetylase (HDAC) family of proteins. The HDAC proteins have important biological function in removing the acetyl group from histones, which in turn promotes compaction of chromatin and influences transcription [15,16]. In addition to deacetylation of core histones, HDACs can also affect protein stability, protein-protein interactions, DNA binding, and protein localization [16]. These crucial biological functions of HDACs have important implications in the pathogenesis of many diseases, especially cancer. Indeed, there are reports supporting significant up-regulation of HDACs in breast, colorectal, and prostate tumors [17,18,19,20]. Despite significant evidence that chromatin modifications play a major role in cancer development, there is limited information on HDAC regulation in early and field carcinogenesis.

In this study, we analyzed the expression of several HDACs in the uninvolved mucosa (i.e. “field”) of human colon cancer. We then selected HDAC2 as a candidate biomarker, as it had previously been shown to be up-regulated in colorectal carcinoma and it is localized only in the nucleus [21]. To determine if HDAC2 could potentially serve as an early biomarker of colorectal cancer, we also analyzed HDAC2 expression in the azoxymethane (AOM)-injected model that mimics early stages of the adenoma-carcinoma sequence of human CRC. Previous research has indicated that HDAC inhibitors (HDACis) target more malignant cells, although the mechanism of action remains largely unknown. Therefore, to more precisely study the role of HDACs in cancerous transformation, we also treated genetic variants of HT-29 colon cancer cell lines that model varying aggressiveness with a pharmacological HDAC inhibitor (HDACi). HDACs are small molecules that interfere with HDAC activity and are currently being tested in clinical trials for cancer therapy. These inhibitors induce a variety of cellular effects by reactivating suppressed genes involved in tumor-cell growth and apoptosis regulation [22,23,24]. We selected one such agent, valproic acid (VPA), which inhibits Class I and IIA HDACs and is actively studied for cancer therapeutics. We analyzed the effect of VPA on chromatin nano-architecture using micrococcal nuclease (MNase) binding, and protein localization [16]. These crucial biological functions of HDACs have important implications in the pathogenesis of many diseases, especially cancer. Indeed, there are reports supporting significant up-regulation of HDACs in breast, colorectal, and prostate tumors [17,18,19,20]. Despite significant evidence that chromatin modifications play a major role in cancer development, there is limited information on HDAC regulation in early and field carcinogenesis.

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Methods

Tissue Samples and RNA Isolation

Human colon resections and rectal biopsies were obtained from patients at NorthShore University HealthSystem in Evanston, IL, following the Institutional Review Board (IRB) guidelines. Written consent forms were approved by the NorthShore University HealthSystem IRB. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee for NorthShore University HealthSystem. Fisher 344 rats (Harlan, Indianapolis, IN) on a standard AIN76a diet were treated with 2 weekly injections (i.p.) either of 15 mg/kg AOM or saline (Midwest Research Institute, Kansas City, MO). Rats were euthanized after 10 weeks (pre-malignant time point). Total RNA was isolated from tissue using TRIzol reagent ( Molecular Research Center, Inc., Cincinnati, OH) according to manufacturer’s instructions.

Quantitative RT-PCR

Complementary DNA (cDNA) synthesis was performed using a high capacity cDNA synthesis kit (Applied Biosystems by Life Technologies, Carlsbad, CA), following standard protocol. The pre-amplified human cDNA was then diluted with TaqMan Universal Mastermix (Life Technologies, Carlsbad, CA) and loaded on the custom-designed TaqMan Low Density Array (TLDA) card and run on ABI Prism 7900 HT PCR machine. The real time PCR data analysis was done using SDS RQ Manager 1.2. All individual HDAC2 PCR reactions were carried out using 80 nM of the TaqMan probe and PCR Mastermix (Applied Biosystems, Carlsbad by Life Technologies, Carlsbad, CA) in a Cepheid Smart Cycle (Cepheid, Sunnyvale, CA). All samples were normalized to β-actin, and the average fold differences were calculated using the comparative Ct method [25]. Threshold of fold change significance was set as >1.5 (up-regulation) and <0.67 (down-regulation).

Cell Lines and Cell Proliferation Assay

HT-29 cells were grown in McCoy’s 5A medium (ATCC, Manassas, VA) mixed with 10% fetal bovine serum +50 mg/mL penicillin/streptomycin in a 5% CO2 environment at 37°C. C-terminus Src kinase (CSK) shRNA-stably transfected HT-29 cells were selected as a clonal population and grown as previously described [26]. Knockdown of the tumor suppressor CSK results in increased aggressiveness compared to control HT-29 cells, as previously measured by both biological assays and PWS [26,27,28,29]. First, knockdown of CSK in the constructs was confirmed for these experiments using qRT-PCR and Western Blotting methods, compared to controls. Various concentrations of VPA (Sigma, St. Louis, MO) were applied to cells based on previous publications [30]. Additional concentrations of VPA (0.1 mM-2.0 mM) and incubation times (6–48 hours) were tested to minimize cell toxicity in the HT-29 cell lines. Target concentrations and treatment times were then used for subsequent studies. At the end of the incubation with VPA, the media was replaced with fresh media containing WST-1 ([4-(3-[4-iodophenyl]-2-[(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) -reagent (Roche Diagnostics, Indianapolis, IN). After 30 min incubation with WST-1, the absorbance of the plate was read at 440 nm and 600 nm in a Spectramax Plus Spectrophotometer plate reader ( Molecular Devices, Sunnyvale, CA).

PWS System and Analysis

PWS is a novel imaging technique that measures the nanoscale (~20 nm) distribution of mass density. The in-depth explanation of the theory and PWS instrument used for this study has been reported elsewhere [13,27,29]. In brief, a spatially incoherent white light illuminates a specimen and the reflected back-scattered image is projected on to a CCD camera (Princeton Instruments, NJ, USA) through a spectral tunable filter. The spectral fluctuations of the backscattered light (300–700 nm) are analyzed for every pixel of the obtained image. The PWS technique breaks up the 3-dimensional intracellular volume of a sample into several 1-dimensional cylindrical channels; the multiple interference of backscattered light waves from each of those channels results in the PWS signal. The underlying physical phenomenon is that the
optical interference of backscattered light waves is sensitive to the spatial variations of the optical refractive index at sub-diffractional length scales. For biologically relevant macromolecules (DNA, RNA, proteins, lipids, etc.), the refractive index is a linear function of the local macromolecular density [31,32]. Thus, PWS can quantify spatial variations of macromolecular density.

The PWS readout is an image (2-dimensional [2-D] map) of a cell showing the intracellular distribution of a parameter called disorder strength \( L_d \), which captures the spatial fluctuations of macromolecular density. \( L_d \) is defined as: 
\[
L_d = \delta n \times \ell^2
\]
where \( \delta n \) is the standard deviation of the refractive-index (i.e., mass-density) variations, while \( \ell \) is the correlation length of these variations. The coefficient \( \alpha \) depends on the cytology sample preparation (\( \alpha = 1 \) in our case), while \( \beta \) depends on the configuration of the optical set up (~1 for this study). As \( L_d \) is a measure of the spatial variations of macromolecular density, it increases with macromolecular condensation [33]. The precise nature of the compaction depends on the intracellular location where \( L_d \) is increased. We used the 2-D map, \( L_d(x,y) \), of each nuclear region and calculated the mean nuclear \( L_d^{(n)} \) (the average over \( x \) and \( y \) pixels). This \( L_d^{(n)} \) and the standard error calculated from its standard deviation \( \sigma^{(n)} \) are depicted in all bar plots presented in this manuscript. We randomly selected 50–80 cells for each control and treatment depicted in all bar plots presented in this manuscript. We found that each HDAC was significantly up-regulated (\( p<0.001; \) Figure 1A). To confirm the nanoscale alterations in chromatin structure, we compared TEM images of nuclei from control patients (no dysplasia) and patients with an adenoma (pre-cancerous lesion). The electron micrographs revealed significant nanoscale differences in structure, including increased chromatin compaction, presence of multiple nucleoli, and alterations in the distribution of chromatin in the human rectal biopsies with an adenoma present, compared to control (Figure 1B). We then confirmed the expression of HDAC2 in human field CRC using an individual qRT-PCR. HDAC2 was selected as a target biomarker due its nuclear localization, while many other HDAC family members translocate from the nucleus ad have exhibited protein interaction in the cytoplasm. Additionally, it was previously shown that HDAC2 is overexpressed in colorectal carcinomas but not in the field of the tumor [21]. In human resection samples, we found that HDAC2 expression was also about 2-fold higher in patients harboring an adenoma elsewhere in the colon compared to the control (no dysplasia) group (\( p<0.05, n = 12 \) subjects; Figure 1C). These findings suggest that HDAC expression, and especially HDAC2, are important mediators of chromatin alterations observed at a distance from the actual tumor site.

Next, we analyzed the HDAC2-mediated effects on chromatin structure at an early time point (10 weeks post initial injection) in the AOM-treated rat model. At this pre-neoplastic time point in the AOM-treated rat model, the colon begins to exhibit the first detectable, pre-neoplastic lesions (such as aberrant crypt foci) [36,37]. TEM micrographs again revealed changes in chromatin compaction in the AOM-treated rat distal colons compared to controls (Figure 1D). Even though the colons did not have any polyps or tumors, HDAC2 was significantly up-regulated (1.5 fold, \( p<0.05 \)) in the AOM-injected animals compared to their age-matched controls by qRT-PCR methods (\( n = 12 \) animals; Figure 1E). These observations support the concept that chromatin rearrangements, through HDAC dysregulation, may be important for colon cancer development and could serve as a marker of field carcinogenesis.

The HDAC Inhibitor VPA Differentially Affects Colon Cancer Cell Lines with Varying Tumorigenicity
To study the role of HDACs on nuclear nano-structure in cancerous cells, we used human colon cancer cell lines, HT-29 and CSK shRNA-transfected HT-29. Modest knockdown (~50%) of the tumor suppressor CSK in HT-29 cells results in a more proliferative phenotype; CSK is altered early in the uninvolved colonic mucosa prior to neoplastic transformation, thus serving as a robust cell culture model of early tumorigenic events [26,29]. In the present study, we observed a 1.5 fold increase in HDAC2 expression in the CSK shRNA knockeddown cells compared to HT-29 control cell lines (\( p<0.05; \) Figure 2A). In agreement with the human and animal data, TEM images showed increased chromatin compaction in the more aggressive (CSK constructs) cell line (Figure 2B).

Chromatin accessibility was evaluated using MNase accessibility assays to compare higher-order chromatin structure in the HT-29 cell line (Figure 2B).
cell lines. HT-29 and CSK shRNA knockdown HT-29 cells were treated with MNase for increasing amounts of time (Figure 2C). In the absence of MNase, the HT-29 cell lines did not exhibit endogenous nuclease activity. The more aggressive CSK shRNA knockdown cells exhibited enhanced resistance to MNase, which indicated a more closed structural state of chromatin [38]. The difference between the cell lines was most apparent at earlier digestion time points.

HDACIs are being studied as a means of inhibiting overexpression of HDACs during carcinogenesis. In order to systematically study the nuclear differences in HT-29 and CSK constructs, we treated the cell lines with an HDAC inhibitor to perturb chromatin architecture. VPA previously has been shown to selectively inhibit Class I and IIA HDACs, as well as HDAC2 expression levels [30,39]. We first determined the appropriate concentration of VPA and treatment time in HT-29 cell lines using the WST-1 cell viability assay. It has been well established that HDACIs induce apoptosis in cancerous cells [16,40]; therefore, we targeted the least toxic concentrations on the cells (Figure 2D). Low concentrations of VPA moderately increased cell proliferation in HT-29 cells but not in the CSK shRNA knockdown cells. Similar to previous reports, higher concentrations of VPA treatment (0.5 mM –1.5 mM) reduced cell viability [30]. However, the effect on viability was more pronounced in the CSK shRNA cell lines as compared to the HT-29 cell lines with the same treatment conditions. These results are consistent with the hypothesis that HDAC dysregulation and chromatin rearrangements are involved in cell tumorigenicity.

In addition to cell viability, treatment with HDACIs alters chromatin condensation and accessibility in cancerous cells [41]. To examine the effect on chromatin following VPA treatment in HT-29 and CSK knockdown cells, we performed MNase digestions of isolated nuclei. In agreement with recent publications, we found that HT-29 cells treated with VPA were more sensitive to MNase compared to untreated samples, indicating a more open chromatin structural state (Figure 3A) [41]. At early digestion time points, the shift in DNA bands suggested that there was an increase in accessibility with VPA treatment. However, at later time points, the higher concentrations of VPA (0.5 and 1.5 mM) displayed greater accessibility compared to the untreated sample. On the other hand, digestion of CSK knockdown samples revealed a large effect on DNA accessibility with increasing VPA concentrations (Figure 3B). This indicated that the HDACIs induced greater chromatin changes in the more aggressive cell lines.
To verify these higher-order chromatin changes in both cell lines, we performed immunoblotting of acetyl-histone H3. The results showed that there was an increased proportion of acetylation in VPA-treated samples in both HT-29 and CSK constructs (Figure 3C). Because the differences among concentrations in the HT-29 cells may have reflected the apoptotic effects of the HDACIs, we also examined the level of apoptosis induced by VPA. Immunoblotting showed an increase in the cleavage of poly(ADP-ribose) polymerase (PARP), a marker for caspase-mediated apoptosis (Figure 3C). Given that chromatin accessibility and acetyl-histone H3 were assessed on entire cell populations, we next performed TEM imaging to assess nano-scale difference in chromatin between individual cells. TEM imaging confirmed less compact chromatin structures in the VPA-treated nuclei compared to their untreated counterparts (Figure 3D). Furthermore, there was an increase in the number of vacuoles in the cytoplasm among VPA-treated cells, indicating early apoptotic effects of drug treatment. Together, these data show that VPA elicits a greater effect in the more aggressive cell line compared to control, which may relate to the HDAC activity in HT-29 cell lines.

Figure 2. HDAC inhibition differentially affects cell viability in colon cancer cell line variants. A) TEM micrographs of chromatin structure in the HT-29 colon cancer cell line genetic variants, HT-29 control and CSK knockdown. B) HDAC2 expression is up-regulated in the CSK knockdown cell lines. C) MNase assay on HT-29 (H) and CSK knockdown (C) cells also indicate a more compact chromatin structure present in the CSK constructs. D) HT-29 and CSK knockdown cells in 96-well plates were treated with increasing concentrations of VPA for 24 h and then assayed for proliferation using standard WST-1 assay. Absorbance was measured after 20 min at 37°C. VPA treatment reduced cell viability in both cell lines, while the effect was greater in the CSK constructs. Standard error bars shown with *p<0.05.

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PWS Quantification of Chromatin Architecture in Colon Cancer Cell Lines

Pharmacological HDACIs target a small subset of genes rather than modulating global gene expression [42]. MNase digestions provide structural information of DNA accessibility on a global level over the entire sample while TEM micrographs show these chromatin alterations qualitatively. Therefore, to quantify local, nano-architectural modifications in individual nuclei, we used the PWS technique. This technique measures nanoscale spatial fluctuations of macromolecular concentration, specifically, macromolecular condensation – via a parameter termed disorder strength ($L_d$). In the nucleus, $L_d$ is a measure of chromatin compaction, as has been confirmed by numerical simulations [43]. An increase in $L_d$ occurs in the field of several human cancers and at the earliest stages of carcinogenesis in both clinical patients and animal models [13,29]. Furthermore, previous studies demonstrated that $L_d$ increased for the more aggressive CSK constructs compared to HT-29 control cells [27]. Therefore, the PWS technique was an ideal tool to measure the effect of HDAC inhibition on local chromatin structure and the relationship with...
We hypothesized that HDAC inhibition by VPA opens these local chromatin regions, thus normalizing or decreasing the aggressiveness differences between the cell lines as measured by PWS.

We first determined the effect of VPA on HT-29 and CSK constructs individually. Second, we analyzed the disorder strength difference ($\Delta L_d$) between the two cell lines. Upon treatment with VPA, both the HT-29 and CSK knockout cell lines appeared microscopically similar (Figure 4A). Low concentration of VPA caused $L_d$ to decrease in both the HT-29 cells and CSK knockout cells (28.47% and 39.9%, respectively; Figure 4B). The effect of 0.1 mM VPA on nuclear $L_d$ was greater in CSK constructs, supporting findings from previous research indicating HDACIs target more malignant cells. In both HT-29 and CSK constructs, treatment with 0.5 mM VPA resulted in a slight increase in $L_d$ compared to the lowest concentration, which may be due to the fact that HDACIs induce apoptosis. However, in the CSK constructs this value remained lower than the untreated samples as opposed to the non-significant increase in the HT-29 cells. Treatment with 1.5 mM VPA caused $L_d$ to further decrease in both cell lines (Figure 4B). The differential effect of HDAC inhibition between cell lines may be attributed to the higher level of HDAC2 expression and higher aggressiveness in the CSK constructs. These results are consistent with the biological assays, showing a greater effect of VPA in the more aggressive CSK constructs.

The loss of CSK is an early carcinogenic event, important for Src signaling and cell hyperproliferation, shown in both cell culture models and tissue samples [26,28]. To then understand the relationship of CSK loss with nanoscale chromatin rearrangements, we compared the $\Delta L_d$ between HT-29 and CSK constructs at specific VPA treatments. The CSK constructs exhibited a higher $L_d$ compared to HT-29 control cell lines (55.81% difference; Figure 4C). Low concentrations of VPA (0.1 mM) decreased $L_d$ significantly in both cell lines, but there was still a 24.16% difference between HT-29 and CSK constructs. On the other hand, higher concentrations of VPA (0.5 mM and 1.5 mM) completely normalized the $L_d$ differences in HT-29 and CSK knockout cell lines (~4.74% and ~5.24% difference, respectively; Figure 4C). These results indicate that increasing concentrations of VPA increased chromatin accessibility, which in turn normalized the $\Delta L_d$ between the cancer cell lines. Therefore, in this study, we quantified for the first time the direct relationship between chromatin rearrangements and cellular aggressiveness by comparing nuclear mass-density fluctuations in cancer cell lines.

The PWS results support our hypothesis that HDACIs open local cell tumorigenicity. We hypothesized that HDAC inhibition by VPA opens these local chromatin regions, thus normalizing or decreasing the aggressiveness differences between the cell lines as measured by PWS.

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Proper regulation of higher-order chromatin structures is essential for normal cell function. Abnormal alterations in genome organization leads to aberrant gene expression and has been described in a variety of diseases, including neurological disorders and many cancers [44,45,46]. These conditions have prompted increased efforts to understand and determine important histone modifications through histone deacetylase/acetylase activity and mutations during neoplastic development and progression. Microscopically observable changes in nuclear morphology also serve as a marker of carcinogenesis and are also observed in cells within the field of the tumor [12,47]. Field carcinogenesis is the concept that the genetic/environmental milieu that results in a focal tumor exists not only at that particular location, but affects the organ diffusely. Thus, field carcinogenesis represents the earliest stage of malignancy [11]. However, the cause and mechanisms governing chromatin changes during early stages of field cancerization are largely unknown. For this reason, in this study, we examined how changes in chromatin nano-structure through HDAC regulation are involved in determining the aggressiveness of cells using a multidisciplinary approach. First, we determined the expression of several important HDAC proteins in field colon carcinogenesis using human rectal biopsies. To our knowledge, this is the first study demonstrating that HDAC1, HDAC2, HDAC3, HDAC5, and HDAC7 are up-regulated in the field of human colon cancer. Secondly, we analyzed TEM images of nuclei from these samples to visualize chromatin modifications in the field of CRC. We then validated the expression of a nuclear localized HDAC family member, HDAC2, in colonic resections as a candidate for further study. HDAC2 was significantly increased in the adjacent mucosa obtained from patients harboring an adenomatous polyp (pre-cancerous lesion) compared to non-dysplasia (control) patients using qRT-PCR methods, suggesting that chromatin modifications through HDAC regulation extend throughout the entire field of carcinogenesis. While the up-regulation of HDAC2 has previously been shown by immunohistochemistry in several cancers [19,21], HDAC2 expression has never been described in early field carcinogenesis.

Increased chromatin compaction and other nuclear abnormalities have long served as a marker of dysplasia. Consistent with these later stage events of carcinogenesis, we found increased chromatin condensation and changes in chromatin distribution in

![Image of chromatin regions, normalizing the aggressiveness between the cell lines.](image)

**Figure 4. Changes in nuclear disorder strength (L_d) following VPA treatment.**

- **A:** Representative pseudocolor PWS images from nuclei of HT-29 and CSK constructs untreated or treated with 0.5 mM VPA. Color shows the magnitude of the L_d in an individual nucleus.
- **B:** Percent difference in combined nuclear L_d over experimental repeats in HT-29 and CSK knockdown cells. Nuclear L_d mostly decreased following VPA treatment in each cell line and to a greater extent in the CSK constructs.
- **C:** Percent difference in nuclear disorder strength between HT-29 and CSK constructs after each treatment. Treatment with higher concentrations of VPA (0.5 mM and 1.5 mM) nullified the nuclear L_d differences between the cell lines.

**Table 1: VPA treatment effects on nuclear disorder strength (L_d)**

| Concentration of VPA | HT-29 Cells | CSK Knockdown |
|----------------------|-------------|---------------|
| +0.1mM               | +0.5mM      | +1.5mM        |
| (% Difference)       | -28.47      | 8.09          |
|                      | -23.44      | -39.90        |
| p-value              | < 0.001     | < 0.001       |

**Table 2: VPA treatment effects on nuclear disorder strength (L_d)**

| Concentration of VPA | Control | +0.1mM | +0.5mM | +1.5mM |
|----------------------|---------|--------|--------|--------|
| ΔL_d (% Difference)  | 55.81   | 24.16  | -4.74  | -5.24  |
| p-value              | < 0.001 | < 0.001| ns     | ns     |

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human field carcinogenesis using TEM. Given that these chromatin alterations occur in the diffuse field are known to occur in the tumor, we then assessed the expression of HDAC2 and chromatin nano-structure using TEM in the azoxymethane (AOM)-treated rat model for early CRC. We again observed an increase in chromatin compaction by TEM and found that HDAC2 is also up-regulated at an early, premalignant time point in the AOM-injected rat model, further supporting that concept that chromatin modifications represent early neoplastic events. Together, these results in both the animal model and human patients support the use of HDAC2 as an early biomarker of colon carcinogenesis.

Dysregulation of HDACs causes aberrant gene expression, thus promoting tumor development [23]. For this reason, the HDAC family is being studied as a target for cancer therapy. On a molecular level, HDACIs have been linked to cell cycle regulation and induction of apoptosis through regulation of key cell growth genes like Rb (retinoblastoma) and p21\(^{WAF1}\) [48,49]. Through such pathways, HDACIs selectively target more tumorigenic cells, both in vitro and in vivo [15,35]. Our results of VPA treatment on colon cancer lines also show that increasing concentrations of VPA induced higher apoptosis. Similar to the chromatin changes observed in human and animal tissue, we found increased HDAC2 expression, changes in higher-order chromatin structure, histone acetylation, and altered DNA accessibility in the more aggressive CSK constructs compared to the HT-29 control cells. Moreover, increasing concentrations of VPA treatment reduced cell viability to a greater extent in CSK knockout cells compared to HT-29 control cells, supporting previous research that indicated HDACIs target more tumorigenic cell types.

To quantify chromatin modifications at the nanoscale level, we used a novel imaging technique (PWS) to assess mass-density fluctuations in individual nuclei. Unlike traditional microscopy, PWS has been shown to successfully differentiate genetically altered human colon cancer cell lines and animal cancer cells that appear microscopically similar [29]. The PWS technique has also demonstrated exquisite sensitivity to detecting early nano-architectural changes in the field of carcinization of lung, pancreas, and colon cancers [13,14,29,50]. On a cellular level, \(L_d\) likely correlates to chromatin modifications in the nucleus [31]. Indeed, we found that by reducing chromatin compaction through VPA, nuclear \(L_d\) was reduced in both HT-29 and CSK construct cell lines. The decrease in \(L_d\) was more significant in the CSK constructs, suggesting that chromatin rearrangements play an important role in the mechanism of HDAC inhibition. The PWS results are consistent with the cell viability assays, MNase digestions, TEM, and immunoblotting of histone acetylation. Treatment with high concentrations of VPA normalized the \(L_d\) differences between the two cell lines, thus eliminating their differences in cellular aggressiveness. Taken together, these results support our hypothesis that HDACIs target more cancerous cell types and that rearrangements in higher-order chromatin structure influences the activity of the HDACIs.

These results are relevant to diverse applications: i) HDAC2 as a novel biomarker of the field effect in early colorectal carcinogenesis, ii) the support of HDAC inhibitors and other epigenetic therapies for cancer, and iii) the use of PWS to quantify changes in chromatin architecture of cancer cells. This comprehensive approach of biological assays, microscopy, and spectroscopy demonstrates that nuclear nanoscale alterations are important in the field during early carcinogenesis related to structural mechanisms of biological events. Previous reports demonstrate that \(L_d\) is increased in colon, lung, and pancreatic cancers [14,50]. Therefore, early HDAC dysregulation is likely a universal event in other cancers. Additional research on the mechanisms of HDAC2 and other nanoscale higher-order chromatin modifications will lead to a greater understanding of the earliest stages of carcinogenesis. In this way, PWS can serve as an important tool in addressing questions in cancer biology by assessing chromatin remodeling and other nanoscale structural alterations of chromatin. While we found HDACs to be important in early stages of carcinogenesis, there are a number of other mediators of chromatin alterations that may also play a role (e.g., the SWI/SNF family of proteins). Moreover, future studies on patient samples will verify the ability of PWS to address other clinically relevant epigenetic events, such as methylation and chromoresistance.

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Author Contributions

Conceived and designed the experiments: YSC DD HKR VB. Performed the experiments: YSC DD DPK MDC HS. Analyzed the data: YSC DD DPK HS. Contributed reagents/materials/analysis tools: DPK MDC HS. Wrote the paper: YSC DD HKR VB.

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