

The interplay between histone deacetylases and c-Myc in the transcriptional suppression of HPP1 in colon cancer

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Abbreviations: HPP1, hyperplastic polyposis protein 1; HDAC, histone deacetylase; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; SB, sodium butyrate; SAHA, vorinostat; TSA, trichostatin A; VPA, valproic acid

HPP1 (hyperplastic polyposis protein 1), a tumor suppressor gene, is downregulated by promoter hypermethylation in a number of tumor types including colon cancer. *c-Myc* is also known to play a role in the suppression of *HPP1* expression via binding to a promoter region cognate E-box site. The contribution of histone deacetylation as an additional epigenetic mechanism and its potential interplay with *c-Myc* in the transcriptional regulation of *HPP1* are unknown. We have shown that the treatment of the *HPP1*-non-expressing colon cancer cell lines, HCT116 and DLD-1 with HDAC inhibitors results in re-expression of *HPP1*. RNAi-mediated knockdown of *c-Myc* as well as of HDAC2 and HDAC3 in HCT116 and of HDAC1 and HDAC3 in DLD-1 also resulted in significant re-expression of *HPP1*. Co-immunoprecipitation (IP), chromatin IP (ChIP), and sequential ChIP experiments demonstrated binding of *c-Myc* to the *HPP1* promoter with recruitment of and direct interaction with HDAC3. In summary, we have demonstrated that *c-Myc* contributes to the epigenetic regulation of *HPP1* via the dominant recruitment of HDAC3. Our findings may lead to a greater biologic understanding for the application of targeted use of HDAC inhibitors for anti-cancer therapy.

Introduction

HPP1, also known as tomoregulin,¹ *TPEF*,² *TMEFF2*,³ and *TENB2*,⁴ is a tumor suppressor gene known to be silenced as a result of promoter methylation⁵ in many cancers including those of the colon, rectum, stomach, esophagus and gallbladder.⁶ *HPP1* is involved in modulating cell growth, maturation, and adhesion and its anti-tumorigenic effects have been demonstrated both in vitro and in vivo in prostate⁷ and colon cancers.⁸ The *HPP1* protein is comprised of an epidermal growth factor (EGF)-like domain and two follistatin-like modules.⁹ The EGF-like domain appears to be a ligand for *c-erbB-4* and may be directly involved in cellular growth signaling.¹

Inactivation of *HPP1* by promoter methylation is an early event in the neoplastic progression of gastrointestinal cancers. We have previously reported that aberrant *HPP1* methylation occurs in 40% and 50% of ulcerative colitis-associated dysplastic lesions and carcinomas, respectively.¹⁰ Furthermore, we have demonstrated that 15 of 32 (47%) gastric cancers demonstrate

HPP1 hypermethylation and that it is strongly associated with concomitant hMLH1 hypermethylation.⁵

Sequence analysis of the *HPP1* promoter region has revealed a suppressive E-box that is recognized by *c-Myc*.¹¹ *c-Myc* is an oncogenic transcription factor that promotes tumorigenesis by activating or repressing its target genes which in turn, can modulate cell growth and proliferation.¹² There is evidence to suggest that *c-Myc*'s transcriptional effects may in part be mediated by an interplay with histone deacetylases. It has been reported that *c-Myc* suppresses the transcription of two of its target genes *Id2* and *Gadd153*, via recruitment of histone deacetylase 3 (HDAC3).¹³ Moreover, N-Myc had been demonstrated to act as a transrepressor by recruiting HDAC1¹⁴ and HDAC2.¹⁵

The contribution of histone deacetylation toward the complex transcriptional regulation of *HPP1* has not been elucidated. Aberrant histone deacetylation, leading to chromatin remodeling and in turn, the functional loss of tumor-suppressor genes and/or activation of oncogenes, has been directly linked to tumorigenesis.¹⁶ Histones are small basic proteins that combine with DNA to form the nucleosome core.¹⁷ Histone acetylation

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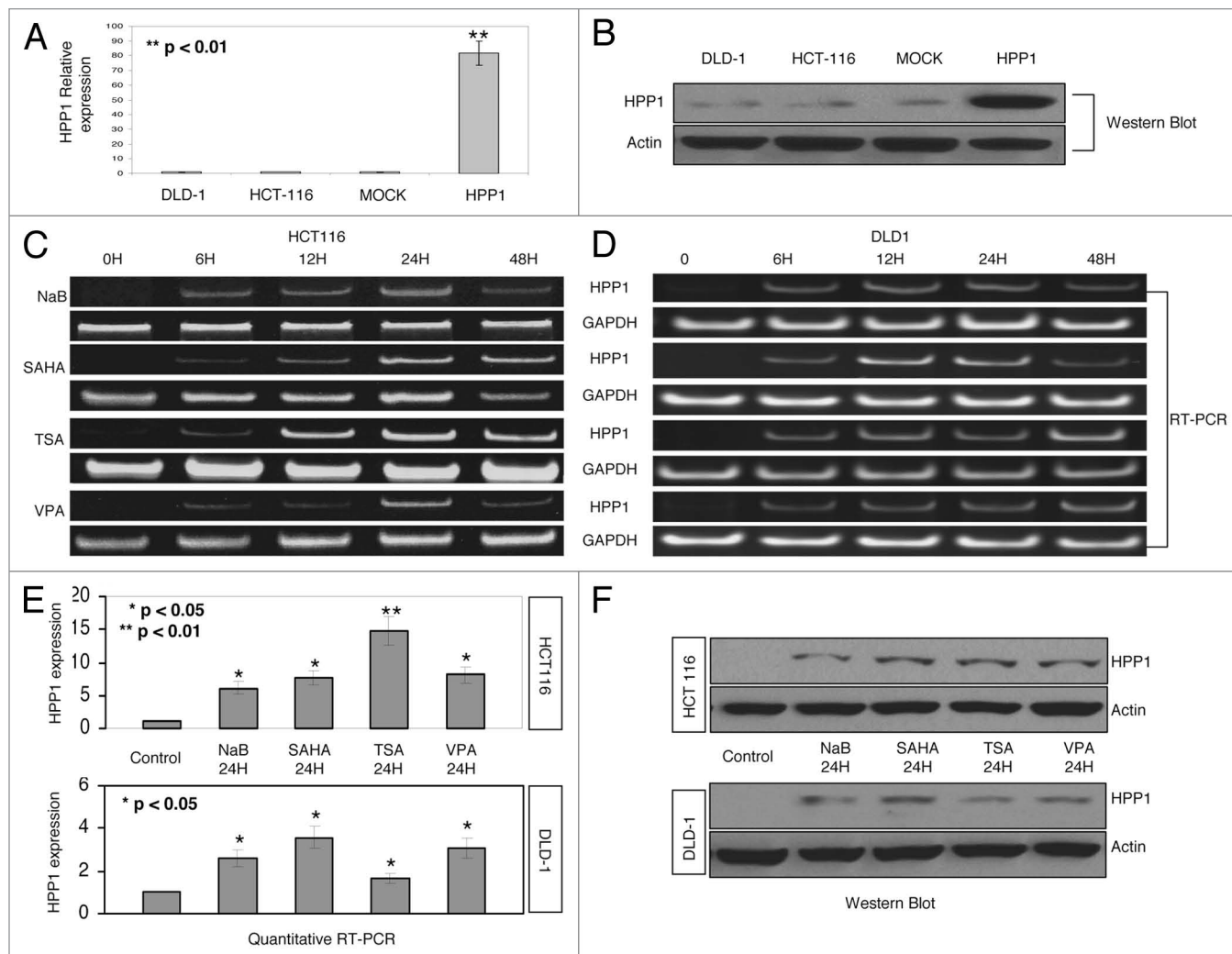


Figure 1. Expression of *HPP1* in colon cancer cell lines. DLD-1, HCT116, MOCK (HCT116 transfected with empty vector control), and *HPP1* (HCT116 transfected with full-length *HPP1*) by quantitative RT-PCR (A) and western blot (B): *HPP1* is re-expressed in HCT116 (C) and DLD-1 (D) cells treated with HDAC inhibitors (SB 5 mM, SAHA 5 μ M, TSA 200 nM, and VPA 2 mM) when compared with vehicle alone (control) at different time points. The re-expression of *HPP1* at 24 h increased significantly by qRT-PCR in response to HDAC inhibitors (E). A similar trend in protein expression was also observed by western blot analyses (F).

is a posttranslational modification of the core nucleosomal histones that affects chromatin structure and gene expression. The acetylation status of histones is regulated by the opposing activities of the corresponding enzymes, histone acetylases (HATs), and HDACs.¹⁸ Acetylation correlates with remodeling of nucleosomes, resulting in the relaxation of chromatin structure which facilitates the accessibility of a variety of factors to DNA causing transcriptional activation. In contrast, deacetylation of the histone tails induces transcriptional repression through chromatin condensation. Inappropriate transcriptional repression of tumor suppressor genes mediated by HDACs may be a common molecular mechanism associated with tumorigenesis.¹⁹ As such, there has been growing interest in the use of HDAC inhibitors as antineoplastic agents.²⁰ In this study, we have sought to elucidate the contribution of HDAC activity and its potential interplay with c-Myc as it pertains to the transcriptional regulation of *HPP1*.

Results

Treatment with HDAC inhibitors results in the re-expression of *HPP1*

We have previously demonstrated that *HPP1* is highly methylated in colon cancer cell lines and tissues and re-expressed upon exposure to the demethylating agent 5-aza-dC.¹⁰ Another epigenetic mechanism by which gene expression can be repressed involves deacetylation of chromosomal histones. The baseline expression of *HPP1* in DLD-1, HCT116, and Mock (HCT116 cells transfected with empty vector pcDNA 3.0) cells as compared with *HPP1* overexpressing cells is shown in Figure 1A and B. Subsequently, HCT116 and DLD-1 cell lines were treated with multiple HDAC inhibitors including NaB (5 mM), TSA (200 nM), SAHA (5 μ M), and VPA (2 mM). Treatment with all four inhibitors induced the re-expression of *HPP1* with a consistent peak at approximately 24 h by RT-PCR (Fig. 1C and D) and

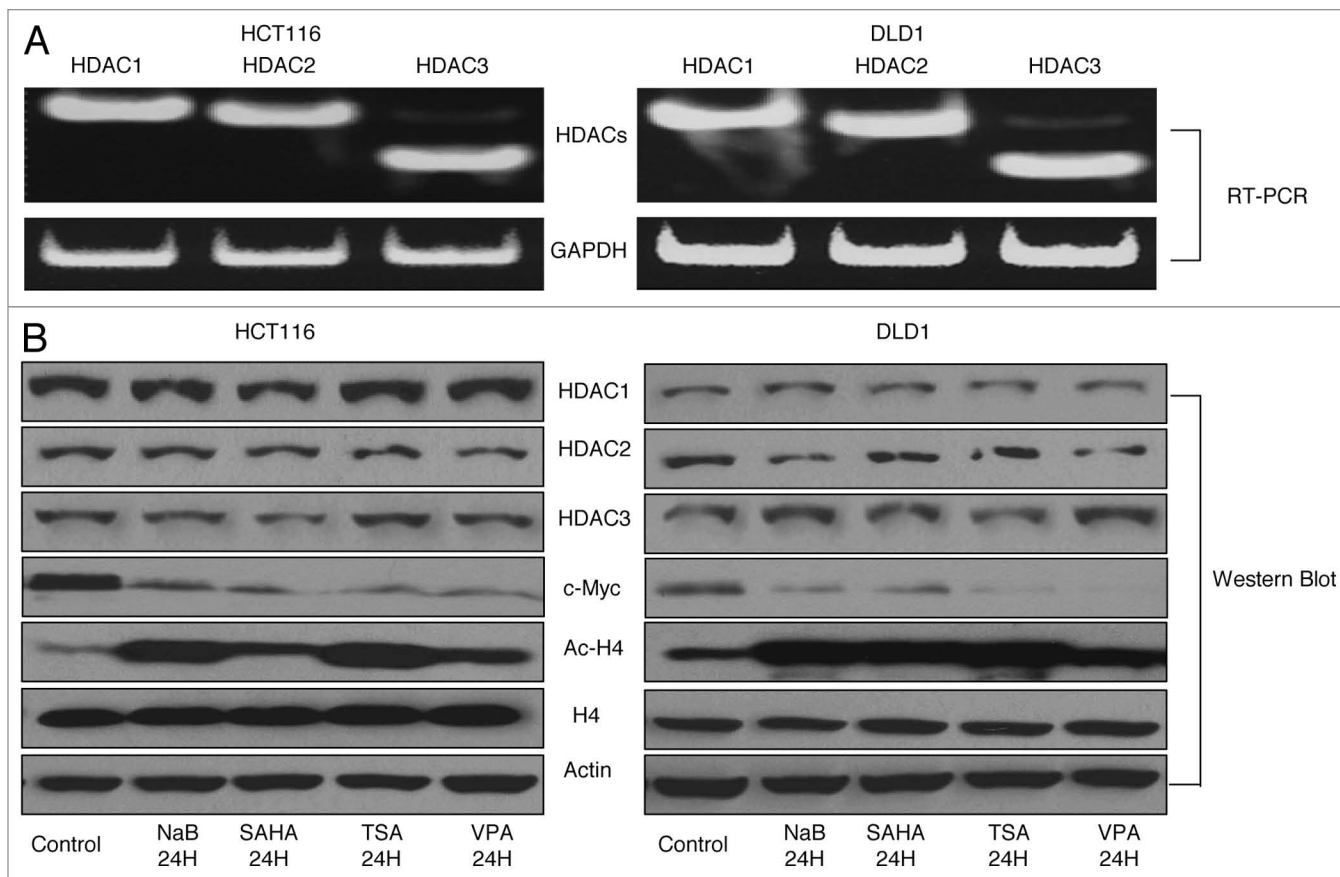


Figure 2. HDAC inhibitors induced accumulation of histone acetylation in association with *HPP1* re-expression and attenuation of c-Myc. HDAC 1, 2, and 3 were highly expressed by conventional RT-PCR in both HCT116 and DLD-1 cells (A). HDAC inhibitors suppressed the expression of c-Myc significantly and increased the accumulation of Ac-H4 but with no effect on the expression of HDAC1, 2, and 3 by western blot analyses (B).

qRT-PCR (Fig. 1E) with confirmation by western blot analysis (Fig. 1F).

HDAC inhibitors induce accumulation of acetylated H4 and downregulation of c-Myc

Due to the non-specific nature of HDAC inhibitors, we subsequently examined the roles of HDAC 1, 2, and 3 on *HPP1* expression. These particular HDACs were selected for further study as they have been widely described as the key regulators of transcriptional suppression.^{13,14} All three HDACs were highly expressed in HCT116 and DLD-1 (Fig. 2A). As expected, treatment with the HDAC inhibitors did not alter the detectable expression of individual HDACs in the cell lines; however the expression of Ac-H4, an activating chromatin mark, increased significantly (Fig. 2B). Concomitantly, the expression of c-Myc, a noted suppressor of *HPP1* expression, was uniformly downregulated in both cell lines in response to HDAC inhibitors. (Fig. 2B)

HPP1 is re-expressed following siRNA-mediated knockdown of HDACs

HDAC-specific siRNA knockdowns of HDACs 1–3 were performed in both HCT116 and DLD-1 cell lines. Dose-related effects of siRNA knockdown of HDAC3 are shown in Figure 3A and B. In HCT116, isolated knockdowns of HDAC2 and -3 resulted in a significant ($P < 0.05$) reversal of *HPP1* suppression

(Fig. 3C), while in DLD-1 and HDAC1 and -3 appeared to be the critical elements (Fig. 3D). This suggests that the contribution of individual HDACs toward the transcriptional regulation of genes such as *HPP1*, may be cell line-dependent. However, in both cell lines, knockdown of HDAC3 was demonstrated to have the most dominant impact on *HPP1* expression. As seen in HCT116, combined knockdown of HDAC2 and -3 resulted in only a slight additive effect on *HPP1* re-expression (Fig. 3E and F). A similar effect was observed following combined knockdown of HDAC1 and -2 in DLD-1 (data not shown)

Simultaneous knockdown of HDAC3 and c-Myc yields an additive effect on *HPP1* re-expression

HPP1 harbors a known cognate repressive c-Myc binding site on its promoter region.¹¹ Accordingly, knockdown of c-Myc by siRNA resulted in a significant upregulation of *HPP1* in both HCT116 and DLD-1 (Fig. 4A and B). As noted, we have shown that HDAC3 was the most influential of the analyzed HDACs in regulating *HPP1* expression in both HCT116 and DLD-1 cell lines. Simultaneous knockdown of c-Myc with HDAC3 resulted in a slight additive effect on *HPP1* expression (Fig. 4C–F). Given c-Myc's recently described ability to recruit HDACs, we sought to further elucidate the interplay between c-Myc and HDACs in the transcriptional regulation of *HPP1*.

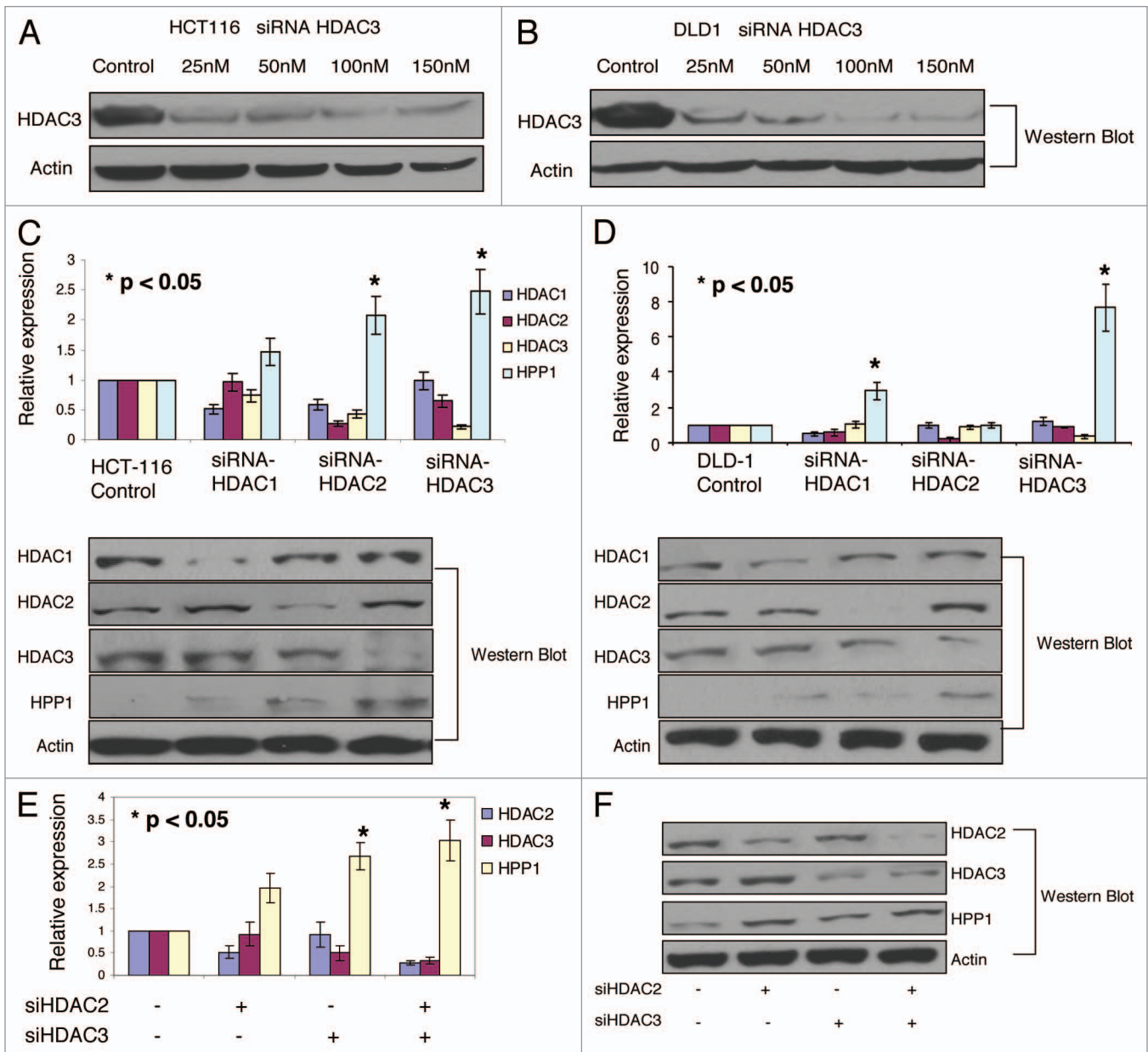


Figure 3. The protein expression of *HPP1* in HCT116 (A) and DLD-1 (B) following the dose related knockdowns of HDAC3. By both qRT-PCR and western blot, there was upregulation of *HPP1* in HCT116 (C) following knockdown of HDAC2 and HDAC3 with similar findings following abrogation of HDAC1 and HDAC3 in the DLD-1 cell line (D). Combined knockdown of both HDAC2 and HDAC3 demonstrated a slight additive effect on *HPP1* re-expression in HCT116 (E and F).

c-Myc and HDACs localize to the *HPP1* promoter

ChIP experiments were performed to determine whether c-Myc and HDAC1, -2, and -3 localize to the promoter region of *HPP1*. Significant enrichment of c-Myc (c-Myc IP) and HDAC3 (HDAC3 IP) chromatin immunoprecipitates was noted in both HCT116 and DLD-1 cell lines (Fig. 5A). Interestingly, we also demonstrated separate enrichment of HDAC2 (HDAC2 IP) in HCT116 and HDAC1 (HDAC1 IP) in DLD1. These findings are in concordance with individual HDAC siRNA knockdown experiments for both of these cell lines as noted above. Conversely, there was only marginal enrichment for HDAC1 (HDAC1 IP)

in HCT116 and HDAC2 (HDAC2 IP) in the DLD-1 cell line (Fig. 5A). These results further suggest that c-Myc binds to the *HPP1* promoter along with a dominant localization of HDAC3 (over HDAC1 and -2) in both cell lines. As such, further investigation of the promoter-based interaction between c-Myc and HDAC3 was performed.

Sequential ChIP and Co-IP demonstrate co-occupancy and interaction of c-Myc and HDAC3 at the *HPP1* promoter

ChIP-re-ChIP experiments were further performed to demonstrate the co-occupancy of c-Myc and HDAC3 on the *HPP1* promoter. ChIP assay lysates were prepared from HCT116 and

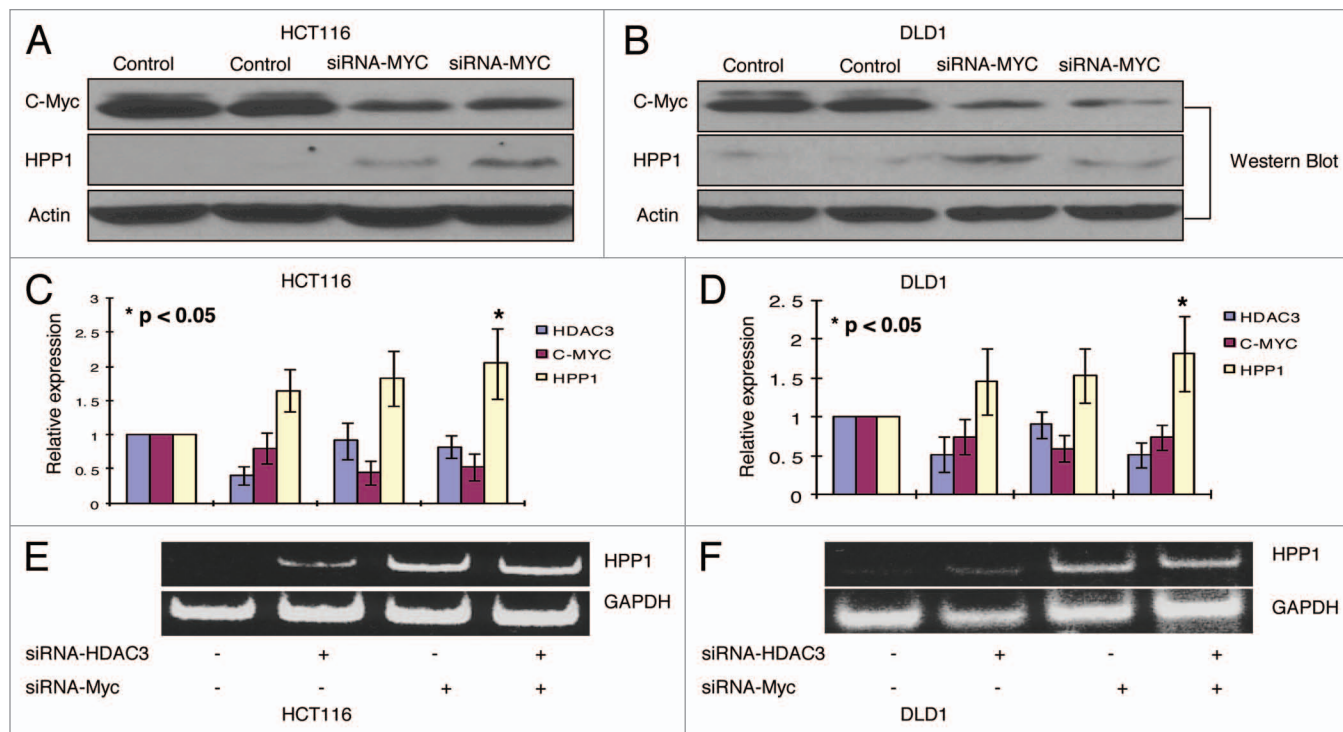


Figure 4. *HPP1* is re-expressed in HCT116 and DLD-1 following knockdown of HDAC3 and/or c-Myc. With c-Myc knockdown, a significantly higher expression of *HPP1* by Western Blot in both HCT116 (A) and DLD-1 (B) was observed as compared with controls. There is a slight additive effect on *HPP1* re-expression by concomitant knockdown of HDAC3 and c-Myc as demonstrated by quantitative real-time (C and D) and conventional RT-PCR (E and F) in both cell lines.

DLD-1 and immunoprecipitated with c-Myc or HDAC3 antibodies to bring down all promoter elements bound by c-Myc or HDAC3. The chromatin complexes were eluted and re-immunoprecipitated with the converse antibody (HDAC3 or c-Myc). PCR assays were performed on the second immunoprecipitated DNA to amplify promoter fragments. There was positive promoter amplification from both cell lines suggesting co-localization of c-Myc and *HPP1* (Fig. 5B). As a negative control, IP was performed using c-Myc or HDAC3 antibody followed by a second IP using anti-rabbit secondary IgG antibody. An unrelated promoter c-Fos was also analyzed to confirm the specificity of the experiment. The direct physical interaction between c-Myc and HDAC3 was further confirmed using co-IP (Fig. 5C).

HDAC3 contributes to H4 acetylation at the *HPP1* promoter

Histone H4 acetylation is a means by which Myc activates transcription.²¹ Myc-dependent repression has been shown to correlate with reduced histone H4 acetylation.¹³ However, the contribution of HDAC3 to H4 acetylation remains largely unknown. We used ChIP to probe for changes in H4 acetylation status at the *HPP1* promoter site at baseline and following knockdown of HDAC3. Knockdown of HDAC3 results in a significant increase in H4 acetylation at the *HPP1* promoter providing additional evidence supporting the role of HDAC3 in regulating *HPP1* expression (Fig. 6).

c-Myc recruits HDAC3 to the *HPP1* promoter

To further confirm that c-Myc primarily binds to the *HPP1* promoter with subsequent recruitment of HDACs, ChIP

experiments were performed in association with knockdowns of c-Myc (Fig. 7A) and HDAC3 (Fig. 8A). With abrogation of c-Myc, significant reduction of HDAC3 binding to the *HPP1* promoter was observed for both cell lines (Fig. 7B). c-Myc knockdown was also associated with reduced enrichment of HDAC2 and -1 chromatin immunoprecipitates in HCT116 and DLD-1 respectively (Fig. 7C). Conversely, HDAC3 knockdown (Fig. 8A) had little effect on the ChIP of c-Myc (Fig. 8B) thus suggesting that c-Myc is the critical binding element and is necessary for the recruitment of HDACs to the *HPP1* promoter.

Discussion

HPP1 is a tumor suppressor gene that is inactivated in over 80% of colorectal cancers and 2/3 of polyps.⁹ There is growing interest in the role of *HPP1* as a serum and stool-based biomarker for colorectal cancer^{22,23} and we have previously described that its tumor suppressive behavior is primarily mediated by STAT1 pathway activation.²⁴ The role of methylation in the transcriptional inactivation of *HPP1* is well described.^{5,11,25} Furthermore, the *HPP1* promoter harbors a cognate repressive E-box c-Myc binding site.¹¹ The contributions of HDACs to the complex transcriptional regulation of *HPP1* and its interplay with c-Myc have not been elucidated.

We have demonstrated, as is the case with a number of epigenetically silenced genes,^{26,27} that the treatment of colon cancer

cell lines with HDAC inhibitors resulted in the re-expression of *HPPI*. From our targeted screening of HDAC candidates, we have demonstrated that HDAC3 makes the most prominent contribution toward the regulation of *HPPI* repression. HDAC3 is a core component of the nuclear receptor co-repressor complexes N-CoR and SMRT^{28,29} and its inhibition induced re-expression of *HPPI* in both HCT116 and DLD-1 cell lines.

Given c-Myc's known role as a suppressive factor in the regulation of *HPPI*, its relationship with HDACs warranted further investigation. c-Myc has traditionally been considered a transcriptional activator, however it is now appreciated that Myc-driven transcriptional repression is also critical for its oncogenic properties.³⁰ Furthermore, there is growing evidence to suggest that histone deacetylation plays a role in these repressive functions.^{13,31}

Herein we have demonstrated a physical interaction between c-Myc and HDAC3 and that they both bind to and co-occupy the key *HPPI* promoter region. It is possible that c-Myc interaction could be direct or indirect through other components of the N-CoR-HDAC3 complex. By sequential ChIP experiments, we have further demonstrated that depletion of c-Myc results in a reduction of HDAC3 association with the *HPPI* promoter. However, in the converse experiment, depletion of HDAC3 did not significantly affect c-Myc binding, thus suggesting that it is c-Myc that is essential for the recruitment of HDAC3 to the *HPPI* promoter. Our findings are similar to those of Kurland et al. in which recruitment of HDAC3 was found to be necessary for the c-Myc suppressive effect on the *Id2* and *Gadd153* genes and that the MbIII co-factor was critical for this relationship.¹³ In similar fashion, we have also observed enhanced H4 acetylation at the *HPPI* promoter region following siRNA-mediated attenuation of HDAC3, adding further evidence to support the active role of functional deacetylation in the regulation of *HPPI*.

Kurland et al. reported that HDAC3 was likely not the only HDAC that contributes to c-Myc's role in transcriptional suppression.¹³ Given that we have demonstrated that non-specific HDAC inhibiting agents resulted in a strong re-expression of *HPPI* and that targeted knockdown of HDAC3 resulted in only a partial restoration of expression, we also felt it likely that the latter was not the sole HDAC relevant for the transcriptional regulation

of *HPPI*. Accordingly, we have demonstrated that HDAC2 and HDAC1 also contribute in part, toward *HPPI* transcriptional repression in the HCT116 and DLD-1 cell lines, respectively. Recent studies have revealed a role for HDAC1 in Myc-dependent repression at the HIV-1³¹ and transglutaminase¹⁴ genes. Similarly, there is growing evidence to suggest an additional role for HDAC2 in mediating c-Myc's transcriptional effects.^{15,32} For example, c-Myc overexpression correlated with HDAC2 upregulation, which resulted in tumor cell proliferation through inhibition of cyclin G₂.¹⁵ Our findings also support the notion of functional redundancy among HDACs.³³ In genome-wide analyses

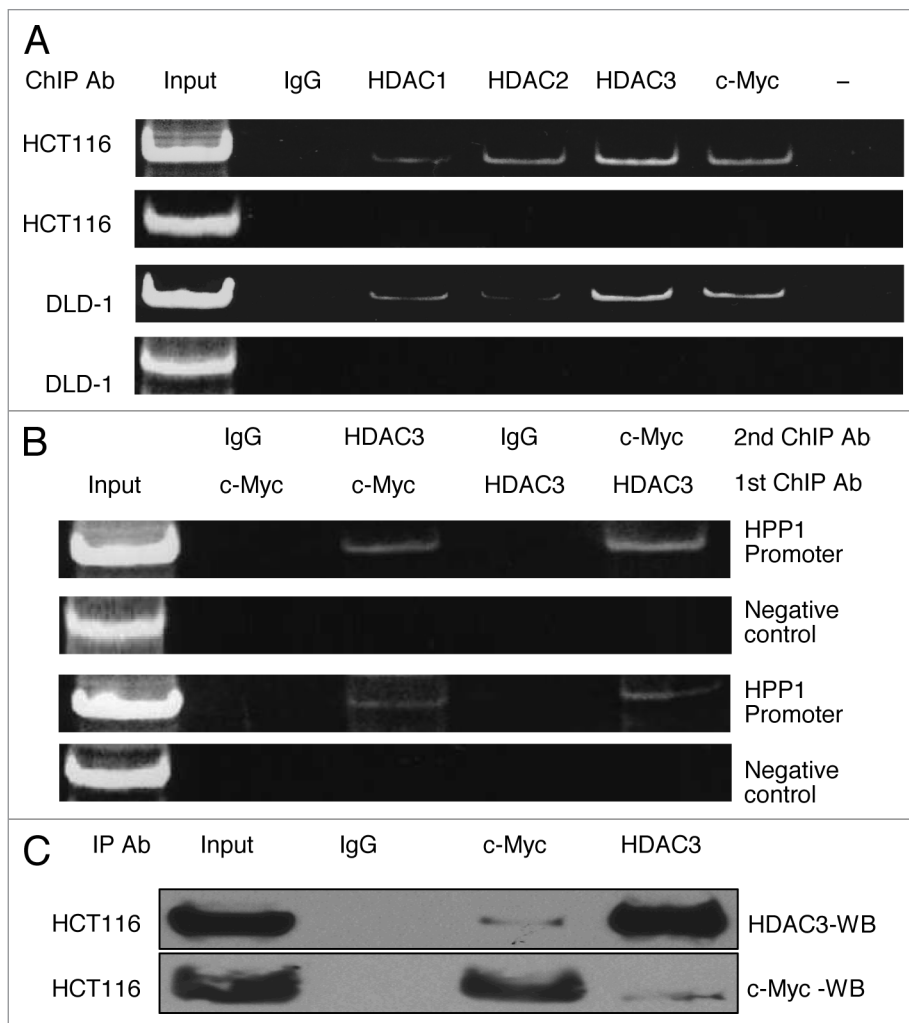


Figure 5. c-Myc-mediated transcriptional repression of *HPPI* in colon cancer is dependent on the dominant recruitment of HDAC3. **(A)** ChIP assays were performed for the *HPPI* promoter in HCT116 and DLD-1 cells using antibodies to HDAC1 (lane 3), HDAC2 (lane 4), HDAC3 (lane 5), and c-Myc (lane 6). Normal rabbit IgG served as a negative control (lane 2), input chromatin (samples without IP) served as a positive control (lane 1). An unrelated promoter of c-Fos was also analyzed to confirm the specificity of the experiment. c-Myc, HDAC1, HDAC2, and HDAC3 were confirmed to bind the *HPPI* promoter. HDAC3 appears to play a more dominant role in the transcriptional regulation of *HPPI*. **(B)** ChIP-re-ChIP experiments further demonstrated the co-occupancy of c-Myc and HDAC3 on the key region of the *HPPI* promoter. Antibodies used for the first IP and second IP are indicated above the lanes. **(C)** Co-IP of c-Myc with HDAC3 from HCT116 extracts demonstrates their physical interaction. Lanes 1 and 2 are input control without IP and negative control respectively. Lanes 3 and 4 are co-immunoprecipitates from c-Myc and HDAC3 respectively.

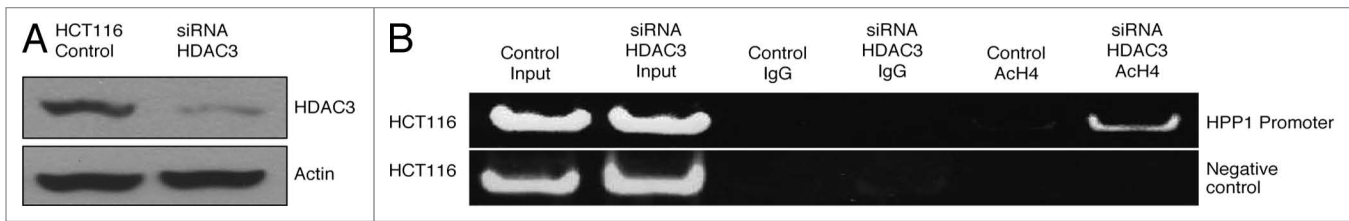


Figure 6. Knockdown of HDAC3 increased the acetylation of H4. ChIP with AcH4 demonstrated a significant increase in H4 acetylation at the *HPP1* promoter following knockdown of HDAC3 in HCT116.

of *Drosophila* cells, RNAi-mediated knockdown of HDAC1 or HDAC3 produced overlapping changes in gene expression profiles, suggesting that these two enzymes have partially redundant functions.³²

An additional interesting observation in our study was the significant uniform downregulation of c-Myc in colon cancer cell lines upon exposure to HDAC inhibitors. This suggests that HDAC inhibitors may restore the expression of epigenetically silenced genes such as *HPP1*, not only via the it classical functional repression of HDAC activity but also possibly through the inhibition of c-Myc. Knockdown of HDAC3 in both HCT116 and DLD-1 only slightly downregulated the expression of c-Myc, suggesting that repression may involve additional mechanisms. Takashi et al. reported that SAHA caused both β -catenin accumulation in the nucleus associated with enhanced TCF-mediated transcriptional activities and decreased levels of c-Myc in pancreatic cancer cells (PANC-1), suggesting independence from an activated β -catenin pathway.³⁴ Notably, inactivation of HDAC6 has been shown to inhibit β -catenin nuclear localization which in turn, is important for the induction of c-Myc.³⁵ The exact mechanism by which HDAC inhibitors decrease the expression c-Myc requires further study.

Among the oncogenic mechanisms mediated by c-Myc is the transcriptional repression and downregulation of tumor suppressor genes. We have shown that c-Myc contributes to the complex epigenetic transcriptional regulation of *HPP1* by dominant recruitment of HDAC3. Specific HDAC-directed therapy that reverses the silencing of similarly regulated tumor suppressor genes may represent an area of investigation for future targeted combination cancer treatments.

Experimental Procedures

Antibodies and reagents

Commercially available antibodies were obtained for *HPP1* (Novus Biologicals), HDAC1, -2, -3, -4, Ac-H4, β -actin (Santa Cruz Biotech), and c-Myc (Cell Signaling Technology). Four HDAC inhibitors were utilized and included vorinostat (SAHA; Calbiochem), trichostatin (TSA), sodium butyrate (NaB; Sigma), and valproic acid (VPA; Tocris Bioscience).

Cell culture, treatments, and transfection

The HCT116 and DLD-1 colon cancer cell lines were used to study the epigenetic regulation of *HPP1* as they show negligible

expression. Cells were cultured in RPMI 1640 (Invitrogen) and supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen) at 37 °C in a humidified incubator containing 5% CO₂. Both cell lines were treated with HDAC inhibitors, NaB (5 mM), SAHA (5 μ M), TSA (200 nM), and VPA (2 mM) at different time points (6–48 h). The control cultures were treated with dimethyl sulfoxide (DMSO) or ethanol depending on the solubility of the specific HDAC inhibitor. For siRNA transfection 2 \times 10⁵ cells were seeded into 6-well plates, incubated overnight and then transfected with control or targeted (HDAC 1–3, Thermo Scientific; c-Myc; Dharmacon) siRNAs using Lipofectamine 2000 (Invitrogen) at a final concentration of 100 nM according to the manufacturer's instructions.

RNA extraction and RT-PCR

Total RNA was extracted using RNeasy Mini Kits and QIAshredder columns (Qiagen). According to manufacturer's protocol, 1 μ g of total RNA was converted to DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The Taqman Gene Expression Master Mix and primer/probes for HDAC1 (Assay ID: Hs02621185_s1), HDAC2 (Assay ID: Hs00231032_m1), HDAC3 (Assay ID: Hs00187320_m1), c-Myc (Assay ID: Hs00905030_m1), *HPP1* (Assay ID: Hs00249367_m1), and GAPDH (Assay ID: Hs02758991_g1) were purchased from Applied Biosystems. PCR products were analyzed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions.

Western blot

Western blots were performed as previously described.⁸ The concentrations of primary antibodies were as follows: HDAC1 (1:2000), HDAC2 (1:2000), HDAC3 (1:2000), c-Myc (1:3000), *HPP1* (1:3000), and β -actin (1:2000).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as previously described.³⁶ Coprecipitated DNA was quantified using PCR. The *HPP1* promoter sequence (see below) was obtained from GenBank (NCBI reference sequence NT_005403) and specific primers (underlined) were designed to include the c-Myc E-box binding region (bolded). *HPP1* promoter sequence; TTTG-GAAGCA GCAGGTCCTC AGCCCGCCCG GGGTCACGTG GGAAGAGGCA GTCGGGCTCT GATTGGTGGA GCAGGATGCA GTCCCGGGA GGGAGGGGTC GACGAGGAGG TGCAAGGATG CAAGGAGGAG GCGCCGCGG AAGCCACAGA TGGGCTCGCT CGCCAGGCGC

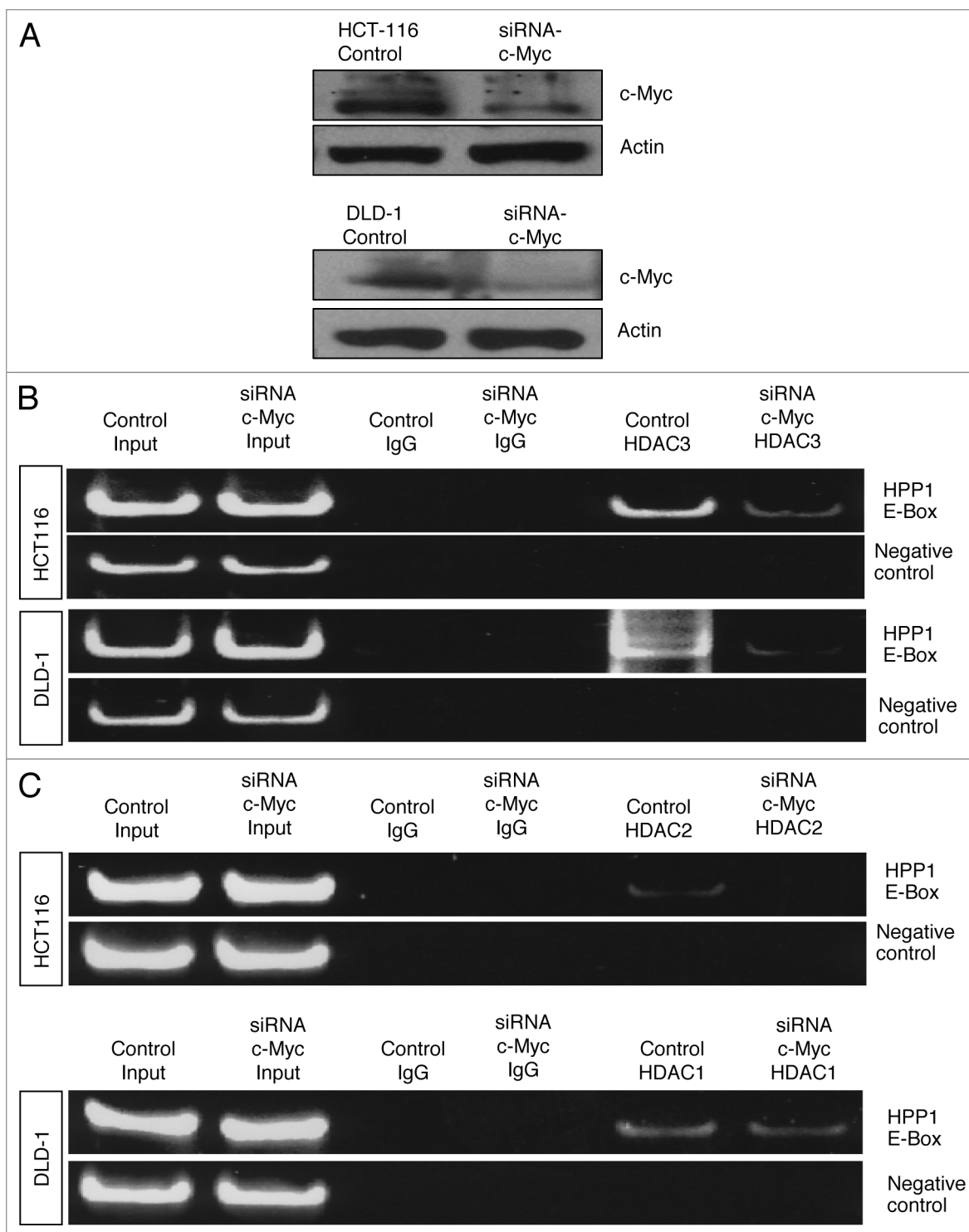


Figure 7. Impact of c-Myc knockdown on HDAC3 binding to the promoter region of *HPP1*: As a consequence of c-Myc attenuation in HCT116 and DLD-1 (A), significantly less enrichment of HDAC3 (HDAC3 IP) chromatin immunoprecipitates was noted in both HCT116 and DLD-1 cell lines (B) when compared with control. There was a loss of detectable enrichment of HDAC2 in HCT116 and minimal reduction in HDAC1 enrichment in DLD1 (C).

TGGCCCGAGT GGGGCTAGGC GGGGATGGCT
CAAATGAGAA. The 152-bp amplicon products were electrophoresed in 8% native polyacrylamide gels and signals were quantitated on a PhosphorImager using IMAGEQUANT software (GE Healthcare Biosciences).

Sequential ChIP

ChIP-re-ChIP assays were performed as described by Reid et al.³⁷ Briefly, the primary immunoprecipitation (IP) was performed using anti-c-Myc or anti-HDAC3 antibodies. The immunoprecipitated complexes were eluted with re-ChIP buffer.

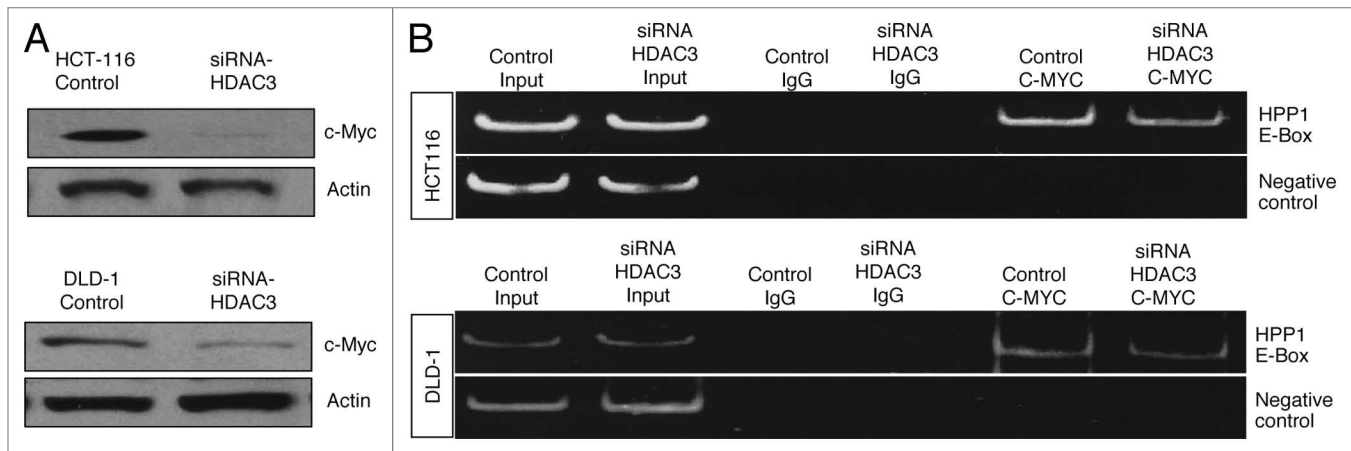


Figure 8. Impact of HDAC3 knockdown on c-Myc binding to the promoter region of *HPP1*: Attenuation of HDAC3 had negligible impact on the enrichment of c-Myc chromatin immunoprecipitates in both HCT116 and DLD1 cell lines (A and B).

The elutions from the c-Myc or HDAC3 IPs were then re-immunoprecipitated with the converse HDAC3 or c-Myc antibody. The resulting re-ChIP immunoprecipitates were examined for the presence of the promoter sequences as described above for the one step ChIP.

Co-immunoprecipitation and western blot analysis

The Pierce Co-Immunoprecipitation (Co-IP) Kit (26149) was used to analyze the interaction between c-Myc and HDAC3. Equal cell numbers (20×10^6 /sample) were lysed in $10\times$ cell volumes of Co-IP buffer and pre-cleared with amine-reactive resin. Samples were then incubated with a 1:50 dilution of anti-c-Myc (C33) and anti-HDAC3 antibodies. Precipitated proteins were

washed three times in $10\times$ cell volumes of Co-IP buffer, released by boiling in SDS sample buffer and separated by SDS-PAGE. Gels were transferred and western blot analysis was performed as noted in section 4.4.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by all authors in this manuscript.

Acknowledgments

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