# Class I Histone Deacetylase Expression Has Independent Prognostic Impact in Human Colorectal Cancer: Specific Role of Class I Histone Deacetylases *In vitro* and *In vivo*

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**Abstract**

**Purpose:** Recently, several studies reported a strong functional link between histone deacetylases (HDAC) and the development of tumors of the large intestine. However, despite the importance of these molecules, comparably little is known on expression patterns and functions of specific HDAC isoforms in colorectal cancer.

**Experimental Design:** We characterized class I HDAC isoform expression patterns in a cohort of 140 colorectal carcinomas by immunohistochemistry. In addition, effects of HDAC inhibition by valproic acid and suberoylanilide hydroxamic acid, and specific HDAC isoform knockdown by short interfering RNA, were investigated in a cell culture model.

**Results:** We found class I HDACs highly expressed in a subset of colorectal carcinomas with positivity for HDAC1 in 36.4%, HDAC2 in 57.9%, and HDAC3 in 72.9% of cases. Expression was significantly enhanced in strongly proliferating ($P = 0.002$), dedifferentiated ($P = 0.022$) tumors. High HDAC expression levels implicated significantly reduced patient survival ($P = 0.001$), with HDAC2 expression being an independent survival prognosticator (hazard ratio, 2.6; $P = 0.03$). Short interfering RNA–based inhibition of HDAC1 and HDAC2 but not HDAC3 suppressed growth of colon cancer cells *in vitro*, although to a lesser extent than chemical HDAC inhibitors did.

**Conclusions:** The strong prognostic impact of HDAC isoforms in colorectal cancer, the interactions of HDACs with tumor cell proliferation and differentiation *in vivo*, and our finding that HDACs are differentially expressed in colorectal tumors suggest that the evaluation of HDAC expression in clinical trials for HDAC inhibitors might help to identify a patient subgroup who will exceptionally profit from such a treatment.

Posttranslational modifications of core histones by acetylation, methylation, phosphorylation, ubiquitination, and sumoylation are known to elicit profound transcriptional changes by altering the DNA/histone conformation of cells (1). In this regard, changes in the acetylation status of lysine residues within the $NH_2$-terminal tail of core histones are probably one of the most important and best characterized mechanisms of epigenetic transcriptional regulation. Reversible histone acetylation in normal and transformed cells is regulated by histone acetylases and histone deacetylases (HDAC; ref. 2). It has been shown that aberrant deacetylation of histones by enhanced HDAC activity in human tumors leads to conformational changes within the nucleosome, which results in transcriptional repression of genes involved in differentiation and negative regulation of cell proliferation, migration, and metastasis (3). In addition, HDACs are known to deacetylate and thereby change the activity of a large number of tumor-relevant proteins (4) and to play a role in the regulation of vascular tone and angiogenesis (5, 6).

To date, four classes of HDACs comprising >18 isoenzymes have been identified in humans (3). In our study, we focus on $NAD^+$-independent class I isoforms HDAC1, HDAC2, and HDAC3, which are known to represent a target for a growing class of new therapeutics, the HDAC inhibitors (HDI). HDIs have been shown to suppress tumor growth *in vitro* and *in vivo* and some of these substances, including hydroxamic acids such as suberoylanilide hydroxamic acid (SAHA) and short-chain fatty acids such as valproic acid (VPA), have entered late-phase clinical trials for the treatment of a variety of solid and hematologic malignancies including colorectal cancer (1). SAHA was recently approved for the treatment of cutaneous T-cell lymphoma in second- or third-line therapy.

Novel treatment options for colorectal cancer, the third most common cancer in the Western world (7), are still urgently needed. Although this disease is surgically curable at early stages, the tumor frequently does not become symptomatic

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before the metastatic stage, which is associated with high mortality rates. Although changes in the chemotherapeutic regimens used and combination with radiotherapy have considerably improved survival of patients with advanced neoplasms (8), the current therapeutic concepts, especially for late-stage tumors, are far from being optimal. Based on the fact that synergistic effects of HDIs and conventional chemotherapeutics have been proposed (9–11), and that HDIs have been shown to act as radiosensitizers in a variety of cancer cell lines, including colon cancer cells (12, 13), HDIs might be extremely useful for radiochemotherapeutic combination therapies.

The role of HDACs in colorectal carcinogenesis is further emphasized by the fact that short-chain fatty acids such as butyrates, physiologically produced as a consequence of high-fiber diet, are known HDIs (14). This finding provides an interesting possible functional explanation for the long known interlink between nutrition and colorectal cancer formation. Finally, the detection of antibodies directed against HDAC3 has been suggested as a serologic antigen/biomarker for colorectal cancer (15).

Although, lately, several studies reported a functional link between specific class I HDACs and the development of tumors of the large intestine (16–18), it is surprising how little is known on the expression patterns and specific functions of single HDAC isoforms in primary human tumors. However, this information is of utmost importance because pan-HDIs will most likely be used for the treatment of malignant solid human tumors in the near future and it is not yet clear whether selectivity of inhibitors for specific HDAC isoforms might reduce unwanted side effects. Even more important is the fact that selection strategies to prospectively identify patients who might benefit from such a treatment have not been established thus far.

In our study, we addressed this apparent lack of translational information by characterizing specific class I HDAC isoform expression patterns and functions in a large number of colorectal tumor samples and in colon cancer cell culture models. Our goal was to provide information that might help to identify specific HDAC isoforms important in the tumorigenesis of colorectal cancer. In addition, we wanted to test the hypothesis that a stratification of patients according to their HDAC expression patterns might allow for prediction of individual patient prognosis. Our data might be the basis for studies on HDACs as biomarkers for the prediction of response to HDI treatment in future clinical trials.

## Patients, Materials, and Methods

***Patient characteristics.*** Tissue samples from 140 patients (median age 65.0 years) who underwent partial colonectomy in the Charité University Hospital between 1995 and 1999 were enclosed in this study. Only patients with primary tumors and without other known malignancies at the time of diagnosis and at follow-up were included. None of the patients received radiochemotherapy before surgery. Histologic diagnosis was established on standard H&E-stained sections of the respective tumors according to the guidelines of WHO. Tumor differentiation was assessed by applying the standard WHO grading scheme for colorectal carcinomas. Briefly, individual tumor grade was determined by scoring the proportion of glandular and solid growth patterns within the respective tumor (>95% glandular, well differentiated, G1; 50% to 95% glandular, moderately differentiated, G2; >50% solid, poorly differentiated, G3). Patients with nodal positive colon cancer and patients with either nodal positive or locally advanced ($pT_3/pT_4$) rectal cancer received adjuvant chemotherapy with 5-fluorouracil (5-FU) and levamisole or 5-FU alone according to the guidelines of the German Cancer Society from 1994 (19). Clinical follow-up data were available for all patients. The median follow-up time of patients was 64 months. After 5 years of follow-up, 77 patients were still alive and included in the analysis. Distribution of clinicopathologic data in the study cohort is given in Supplementary Table S1.

***Immunohistochemistry.*** For immunohistochemical detection of HDAC isoforms on tissue samples, prediluted polyclonal rabbit IgG antibody directed against HDAC1 (1:11, Abcam), monoclonal mouse IgG antibody directed against HDAC2 (1:5,000, Abcam), and monoclonal mouse IgG antibody directed against HDAC3 (1:500, Becton Dickinson) were used on 5-μm paraffin sections. As there are known structural homologies between the three proteins, antibody specificity was ascertained by selective short interfering RNA (siRNA) inhibition and subsequent Western blotting for all three HDAC isoforms (see below). Immunohistochemistry was done, as previously described (20). Treatment of slides under omission of the primary antibodies served as negative control.

Ki-67 (MIB-1) staining was done with a Ventana Discovery autostainer (Ventana) using a monoclonal mouse IgG antibody (1:50, Dako) under standard conditions.

***Evaluation of staining of tissue slides.*** Nuclear staining of HDAC isoforms was scored by applying a semiquantitative immunoreactivity scoring (IRS) system, as previously described (20). Briefly, intensity of staining (score 0-3) as well as percentage of cells stained (score 0-4) was evaluated separately (for examples of staining, see Supplementary Fig. S1). The IRS for each individual case ranging from 0 to 12 was calculated by multiplication of the intensity and frequency scores. Cases exhibiting an IRS from 0 to 6 were combined in one group (HDAC negative); cases with an IRS higher than 6 were combined in a HDAC-positive group. Staining of tissue slides was evaluated independently by two pathologists (W.W. and C.D.) who were blinded toward patient characteristics and outcome. To test whether our grouping system (negative/positive) gives robust results, we additionally did all statistical analyses (if applicable) with the raw expression scores and with a three-tied grouping system (weak/moderate/strong), which essentially gave the same results as the two-tied system used (data not shown).

Ki-67 index was determined by counting Ki-67–positive tumor cell nuclei per 100 tumor cells in a representative carefully selected tumor area. The invasive front, the tumor surface, and the transitional zone to normal epithelium were avoided, because in these areas proliferative activity was extremely high in almost all tumors. To ascertain the representativeness of the data, overall proliferative activity on whole tumor sections was additionally estimated by view (data not shown). Correlation of both variables resulted in a correlation coefficient of $r = 0.93$ ($P < 0.001$, Spearman's rank order correlation), thus proving the reliability of the proliferation data obtained. The mean proliferative activity of colorectal carcinomas was 36.3% (SD, 12.3%; Supplementary Table S1).

***Statistical analysis.*** Statistical analyses were done with SPSS 13.0 and GraphPad Prism 4.0. The respective statistical tests used are given in Results, and in the table and figure legends.

***Cell culture and treatment with HDIs and 5-FU.*** The CX-2 colon adenocarcinoma cell line was obtained from the German Cancer Research Center. HT-29 and HCT-116 were from the American Type Culture Collection. Cells were cultured in RPMI (CX-2) or DMEM (HT-29, HCT-116) supplemented with 10% fetal bovine serum.

For chemical HDAC inhibition and treatment with 5-FU, cells were seeded into 96-well plates at a density of $4 \times 10^4$/mL, grown for 24 h, and then treated with given concentrations of SAHA (Alexis Biochemicals) and VPA (Sigma) dissolved in methanol and DMSO, respectively. Control experiments confirmed that the respective inhibitor solvents had no effect on cell number (data not shown).

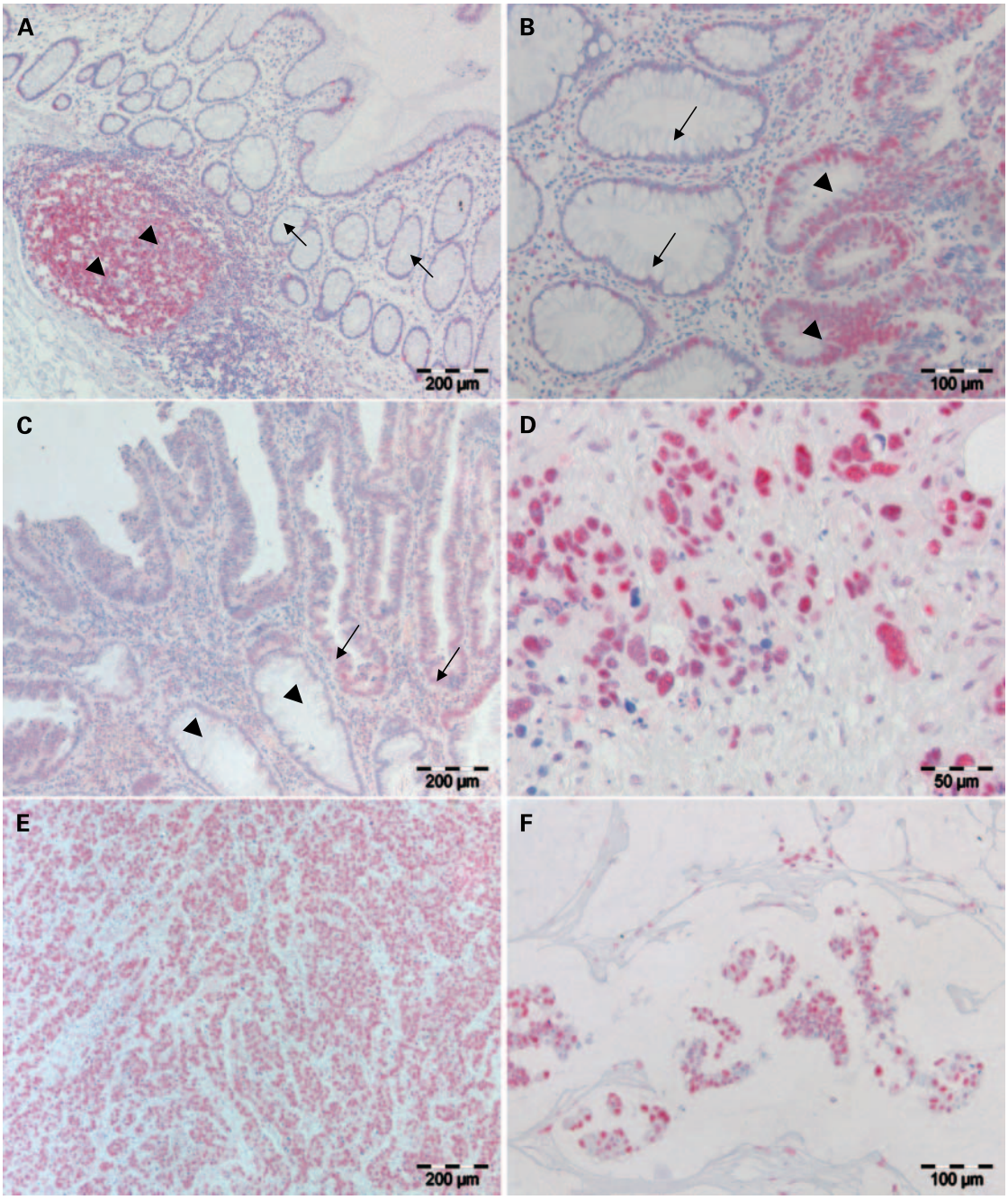


**Fig. 1.** Class I HDAC isoform expression in colorectal tissue. *A,* only focal HDAC1 (as well as HDAC2 and HDAC3, not shown) staining was evident in the nuclei of normal colon epithelial cells (*arrows*). Note the HDAC1 positivity in stroma cells of colon mucosa as well as in the germinal center of an activated mucosal lymphatic follicle (*arrowheads*). *B,* normal colon mucosa (*arrows*) negative for HDAC3 with transition into a colon adenocarcinoma (*arrowheads*) with strong nuclear HDAC3 positivity. *C,* normal colon mucosa (*arrowheads*) with transition into a high-grade adenoma (*arrows*). Neither normal epithelium nor dysplastic epithelium exhibits relevant expression of HDAC2 in this case. *D,* poorly differentiated colorectal adenocarcinoma showing strong nuclear positivity for HDAC1. *E,* low-power magnification of a colon adenocarcinoma with homogenous strong nuclear expression of HDAC2. *F,* mucinous colon adenocarcinoma positive for HDAC3 in the nuclei.

For combined treatment with 5-FU (GRY Pharma), the respective concentrations of the drugs were added at the same time point. Predesigned siRNA duplexes (for sequences, see Supplementary Table S2) were purchased from Qiagen. A nonsilencing siRNA was used as negative control. The day before transfection, cells were seeded into 24-well plates at a density of $4 \times 10^4$/mL. Cells were transfected with 30 pmol siRNA using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. After 72 h, the efficacy of transfection was checked by immunoblotting.

***Immunoblotting and immunofluorescence.*** Immunoblotting of cell extracts was done as previously described (20). The previously mentioned antibodies directed against HDAC1 (1:30), HDAC2 (1:800), and HDAC3 (1:200) were used. All blots were probed for actin (monoclonal mouse IgG, 1:5,000, Chemicon) to ensure equal protein loading.

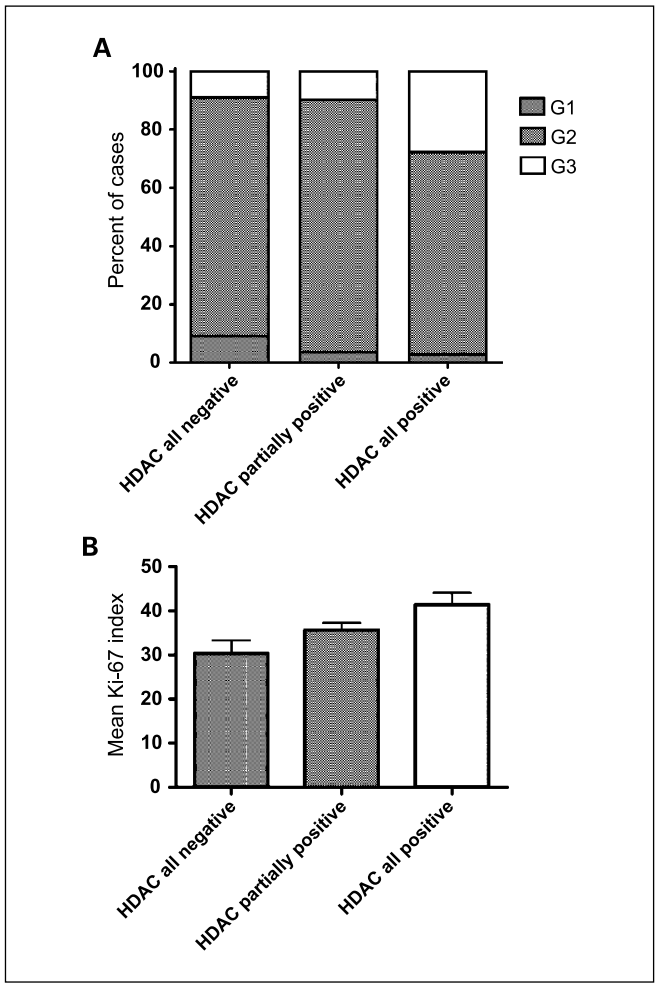


**Fig. 2.** Tumor cell proliferation and tumor differentiation dependent on HDAC isoform expression. Grouped HDAC isoform expression patterns positively correlated with tumor dedifferentiation (*A*; $P = 0.002$) and enhanced tumor cell proliferation (*B*; $P = 0.022$).

For immunofluorescence, $3 \times 10^4$ cells/mL were plated onto Lab-Tek four-well chamber slides (Nalge Nunc). After treatment with SAHA or VPA at the indicated concentrations, slides were washed twice with PBS and fixed with ice-cold methanol for 20 min at -20°C. Fixed cells were blocked with 10% bovine serum albumin and 1% normal goat serum in PBS and incubated with polyclonal goat IgG directed against acetyl H3/H4 (1:100, Santa Cruz Biotechnology) for 90 min. Then, slides were washed thrice with PBS and incubated for 30 min with a biotinylated secondary antibody (rabbit anti goat IgG, 1:5,000, Dako). After treatment with rhodamine (TRITC)–conjugated streptavidin (1:250, Jackson Immunoresearch Laboratories) and 4′,6-diamidino-2-phenylindole (1:1,000, Invitrogen), cells were visualized by confocal microscopy.

***2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay and cell cycle analysis.*** Cell viability was measured after 48 and 72 h of siRNA or drug treatment using a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt cell proliferation kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells were incubated at 37°C for 4 h. Formazan formation was measured at 490 nm. All experiments were done in triplicates.

For cell cycle analysis, cells were treated for 72 h with either HDAC1 or HDAC2 siRNA, VPA, or SAHA as described above. Then, cells were harvested and fixed overnight in 70% ice-cold ethanol at -20°C. The percentage of cells in $G_0$-$G_1$, S, and $G_2$-M phase was quantified by using a propidium iodide–based cellular DNA flow cytometry analysis method. Briefly, cells were rinsed in PBS and 500 μL of diluent buffer (50 μL of Triton X-100 and 250 mg of bovine serum albumin in 50 mL PBS) were added. RNase digestion was carried out by adding 4 μL RNase (10 mg/mL) for 1 h at 37°C. Then, cells were centrifuged and diluted in 500 μL diluent buffer containing 20 μL propidium iodide. Flow cytometry was done with FACSCalibur according to the manufacturer's instructions (BD Biosciences). All experiments were done in triplicates. Analyses of cell cycle data were done with Cylchred and WinMDI (Freeware).

## Results

***Class I HDAC isoform expression in colorectal tissue.*** Colorectal carcinomas displayed high nuclear expression of all three HDAC isoforms in a considerable subset of the tumors investigated (HDAC1, 36.4%; HDAC2, 57.9%; HDAC3, 72.9%; Supplementary Table S1; Fig. 1). Medians of the raw expression scores in tumor tissue were 5 (quartiles 2.25-9) for HDAC1, 9 (quartiles 6-9) for HDAC2, and 9 (quartiles 6-12) for HDAC3. Expression of HDAC isoforms in residual adenomatous tumor tissue present in the vicinity of some invasive carcinomas corresponded to the expression patterns observed in the respective invasive tumors (Fig. 1). Nuclear expression of all HDAC isoforms in carcinomas showed a high degree of concordance (Supplementary Table S1), indicating overlapping regulation mechanisms. Desmoplastic tumor stroma as well as tumor-infiltrating lymphocytes displayed weak to moderate nuclear HDAC1, HDAC2, and HDAC3 positivity.

Occasional moderate nuclear expression of HDAC1, HDAC2, and HDAC3 was observed in normal colonocytes, although a strict restriction of HDAC expression to the proliferative compartment of colonic crypts, as described previously (17), was not noted. Fibroblasts and fibrocytes as well as lymphoid cells of the colorectal mucosa and submucosa usually displayed weak to moderate nuclear staining of all three HDAC isoforms.

***Correlation of HDAC expression with clinicopathologic variables, tumor differentiation, and cell proliferation.*** To gain insight on the biological role of HDACs in colorectal cancer, we investigated the association of HDAC isoform expression with clinicopathologic variables that are used to describe the progression and aggressiveness of a tumor. The expression of all three isoforms correlated statistically significantly with WHO tumor stage, with locally more advanced tumors showing higher expression levels of all three proteins (HDAC1, $P = 0.008$; HDAC2, $P = 0.017$; HDAC3, $P = 0.005$; Supplementary Table S1). The expression of all HDAC isoforms was higher in tumors with distant metastases (HDAC1, $P = 0.037$; HDAC2, $P = 0.045$; HDAC3, $P = 0.062$; Supplementary Table S1). Interestingly, when the patients were grouped according to their overall class I HDAC expression pattern (all three isoforms negative versus one or two isoforms positive versus all three isoforms positive), high HDAC expression levels were associated with enhanced tumor cell proliferation ($P = 0.022$, one-way ANOVA) and with poor tumor differentiation as indicated by a high (G3) tumor grade ($P = 0.002$, $\chi^2$ test for trends; Fig. 2). Correlation of the raw IRS scores with the number of cycling cells revealed a significant positive correlation of weak to moderate strength for HDAC1 ($r = 0.23$, $P = 0.025$) and HDAC2 ($r = 0.29$, $P = 0.005$) but not HDAC3 ($r = 0.03$,

$P$ = 0.808), indicating that some but not all of the variance in the expression of these isoforms is associated with altered proliferative capacity. This finding is supported by the fact that HDAC isoform expression was not restricted to cycling cells alone (Supplementary Fig. S2). These *in vivo* data from human tumor tissue fits well with the observation that HDI treatment leads to differentiation and reduced proliferation of tumor cells *in vitro* (21–24).

***Correlation of HDAC isoform expression and patient survival.*** To test the hypothesis that changes in HDAC expression are relevant for outcome of patients with colorectal cancer, we did a univariate and multivariate survival analysis. Expression of HDAC1 and HDAC2 was significantly associated with reduced patient survival (Table 1; Fig. 3). The 5-year survival in the HDAC1-positive group was 66.7%, whereas the 5-year survival in the HDAC1-negative group was 78.7% ($P$ = 0.02). The 5-year survival in the HDAC2-positive group was diminished to 64.2% when compared with 88.1% in patients with HDAC2-negative tumors ($P$ = 0.001). In contrast, for HDAC3 alone, no statistically significant association with survival was observed. When we did an analysis for combined class I HDAC expression patterns, the survival differences were even more pronounced with 5-year survival rates of 95.5% (all negative), 75.6% (partly positive), and 58.3% (all positive), respectively ($P$ = 0.001, Table 1; Fig. 3).

Multivariate survival analysis was done under inclusion of all factors having impact on patient prognosis in univariate survival analysis and under inclusion of HDAC1 or HDAC2. In this analysis, HDAC2 proved to be an independent prognostic factor ($P$ = 0.03; hazard ratio, 2.6) in colorectal carcinoma (Table 2), whereas HDAC1 had no significant independent prognostic effect (data not shown).

***Hyperacetylation of core histones in colon carcinoma cells by treatment with VPA and SAHA.*** Treatment of CX-2 cells with VPA (2 mmol/L), a HDI discussed to inhibit preferentially class I HDACs (24), and the pan-HDI SAHA (2 μmol/L) for 72 hours led to a significant increase in histone acetylation when compared with control cells as detected by immunofluorescence (Supplementary Fig. S3), which proved the mode-of-action of HDIs in these cells. In addition, VPA treatment, and to a lesser degree SAHA treatment, resulted in the formation of cellular processes and a decrease in nuclear to cytoplasmic ratio of CX-2 cells.

***Inhibition of colon carcinoma cell proliferation by treatment with HDI and isoform-specific siRNA.*** To test whether the effects of chemical HDI inhibition could be contributed to an inhibitory effect on one specific class I HDAC isoform, we compared the effects of HDI treatment with specific class I HDAC isoform knockdown by siRNA. Treatment of colon cancer cells with SAHA (maximum dose 4 μmol/L) revealed a significant dose-dependent reduction in cell number between 69.9% (CX-2) and 39.2% (HT-29) after 72 hours. Treatment with the maximum dose of VPA (4 mmol/L) resulted in a reduction of cell numbers between 77.3% (CX-2) and 35.6% (HT-29) after 72 hours (Fig. 4). After 48 hours, attenuated reductions of cell number were observed in CX-2 (Supplementary Fig. S4).

Treatment of cells with HDAC isoform–specific siRNA resulted in a selective reduction of protein expression of the silenced isoform as determined by Western blot (Fig. 4). Selective knockdown of HDAC2, and to a lesser degree of HDAC1 but not of HDAC3, resulted in a moderate albeit significant reduction of CX-2 cell number after 72 hours (HDAC1, 13.3%; HDAC2, 29.8%; Fig. 4). Selective knockdown of HDAC2 in HT-29 also resulted in a significant reduction in cell number of 21.9% after 72 hours. In HCT-116, the same treatment led to a nonsignificant reduction in cell number of 8.2%; however, this may partly be due to the fact that knockdown of HDAC2 in this cell line, compared with the other two cell lines, was less effective with respect to the reduction of protein expression after 72 hours (Fig. 4). The reduction in cell numbers achieved by selective HDAC isoform inhibition did not reach the effects observed after semiselective and unselective chemical HDI treatment. Forty-eight hours after HDAC isoform siRNA treatment in CX-2, no significant effects on cell number were seen (Supplementary Fig. S4).

***Combined treatment of CX-2 with 5-FU and HDAC inhibitors.*** CX-2 cells were rather resistant to treatment with 5-FU. Low and moderately high doses of 5-FU resulted only in a weak to moderate, albeit significant, reduction of CX-2 cell numbers of up to 26.5% (5-FU, 2 mg/mL) after 72 hours (data not shown). In the low-dose treatment groups (5-FU, 50-200

**Table 1.** Influence of HDAC isoform expression and clinicopathologic variables on patient survival ($N$ = 140)

| | Cases | Events | Mean survival (mo) | SE | Log-rank test $P$ |
|---|---|---|---|---|---|
| Age at diagnosis (y) | | | | | |
| ≤65 | 71 | 15 | 92.3 | 4.6 | |
| >65 | 69 | 24 | 80.1 | 5.5 | 0.082 |
| Dukes stage | | | | | |
| A | 32 | 4 | 88.6 | 4.7 | |
| B | 50 | 5 | 88.6 | 3.1 | |
| C | 48 | 22 | 70.5 | 6.9 | |
| D | 10 | 8 | 23.2 | 8.1 | <0.001 |
| Tumor stage | | | | | |
| $T_1$ | 9 | 0 | — | — | |
| $T_2$ | 33 | 7 | 83.3 | 5.2 | |
| $T_3$ | 85 | 23 | 87.9 | 4.7 | |
| $T_4$ | 13 | 9 | 42.0 | 12.7 | <0.001 |
| Nodal status | | | | | |
| $N_0$ | 85 | 11 | 88.9 | 2.7 | |
| $N_1$ | 27 | 9 | 81.3 | 8.6 | |
| $N_2$ | 28 | 19 | 44.7 | 8.7 | <0.001 |
| Metastasis | | | | | |
| $M_0$ | 130 | 31 | 91.5 | 3.6 | |
| $M_1$ | 10 | 8 | 23.2 | 8.1 | <0.001 |
| Grade | | | | | |
| G1 | 6 | 0 | — | — | |
| G2 | 114 | 31 | 88.4 | 4.0 | |
| G3 | 20 | 8 | 73.1 | 10.9 | 0.157 |
| HDAC1 | | | | | |
| Negative | 89 | 19 | 93.1 | 4.3 | |
| Positive | 51 | 20 | 66.0 | 5.2 | 0.022 |
| HDAC2 | | | | | |
| Negative | 59 | 8 | 100.0 | 4.1 | |
| Positive | 81 | 31 | 76.8 | 5.3 | 0.001 |
| HDAC3 | | | | | |
| Negative | 38 | 6 | 96.5 | 6.0 | |
| Positive | 102 | 33 | 83.4 | 4.4 | 0.072 |
| HDAC groups | | | | | |
| All negative | 22 | 1 | 107.4 | 4.9 | |
| Partially positive | 82 | 21 | 89.8 | 4.6 | |
| All positive | 36 | 17 | 59.1 | 6.5 | 0.001 |

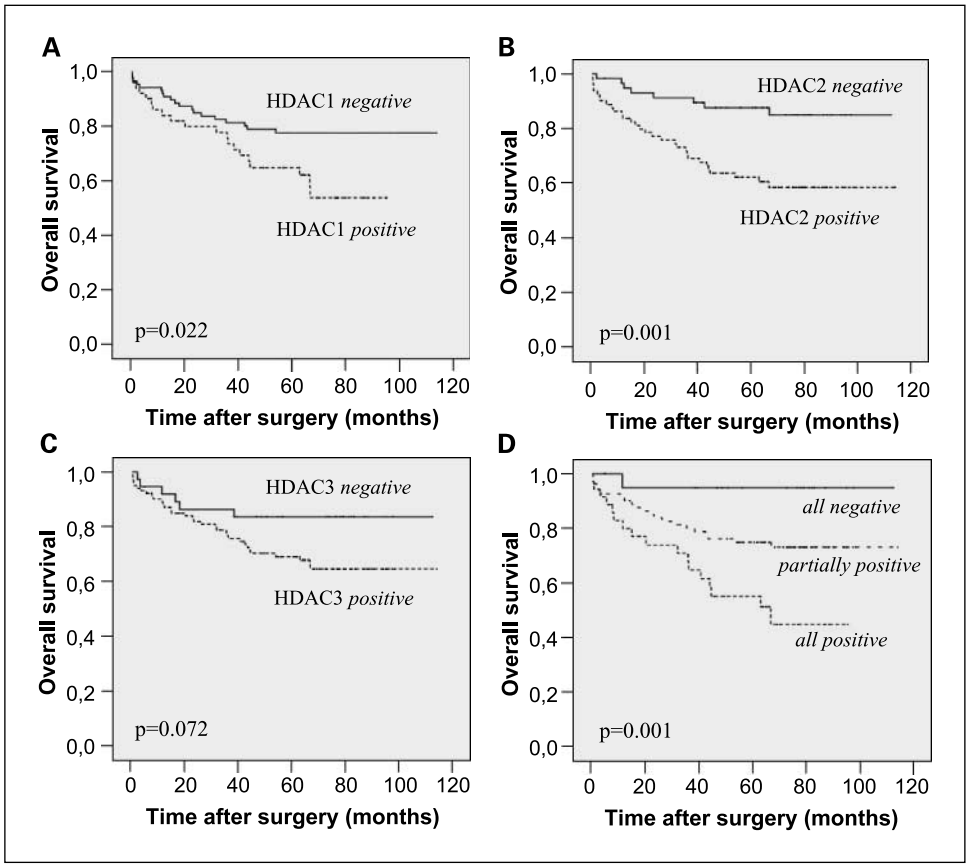


**Fig. 3.** Kaplan-Meier survival curves dependent on HDAC isoform expression patterns. Overall survival dependent on HDAC1 (*A*), HDAC2 (*B*), HDAC3 (*C*), and combined HDAC (*D*) expression. *P* values were calculated with a log-rank test.

μg/mL), the reduction of cell numbers could be slightly enhanced by adding 2 mmol/L VPA or 2 μmol/L SAHA, respectively. However, these differences did not reach statistical significance when compared with treatment with the respective doses of 5-FU alone or treatment with SAHA and VPA alone. In the treatment groups with higher doses of 5-FU (500 μg/mL to 2 mg/mL), no additional reductions in cell number were observed after adding SAHA (2 μmol/L) and VPA (2 mmol/L).

***Effects of HDI treatment on class I HDAC isoform expression in CX-2.*** To assess whether the effects of SAHA and VPA on CX-2 are due to a degradation of specific HDAC isoforms as observed in other cell lines (25), we did Western blots for all three HDAC isoforms after treatment with SAHA (2 μmol/L/4 μmol/L) and VPA (2 mmol/L/4 mmol/L). HDAC1, HDAC2, or HDAC3 expression did not seem to be influenced by treatment with the respective inhibitors after 24 hours, or even after 48 or 72 hours (Supplementary Fig. S4).

***Cell cycle alterations in CX-2 cells after treatment with HDI and isoform-specific siRNA.*** Treatment of CX-2 for 72 hours with 2 mmol/L VPA resulted in an accumulation of cells in $G_0$-$G_1$ phase of the cell cycle, whereas cells in the S and $G_2$-M phases were reduced (Supplementary Table S3; Supplementary Fig. S4). In contrast, SAHA treatment (2 μmol/L, 72 hours) led to an accumulation of cells in $G_2$-M. Specific knockdown of HDAC1 with siRNA produced no discernible effects on the cell cycle after 72 hours. HDAC2 knockdown by siRNA resulted in a weak accumulation of cells in $G_2$-M (Supplementary Table S3; Supplementary Fig. S4). Induction of apoptosis, as indicated by the emergence of a sub-$G_1$ peak, was observed neither after chemical HDAC inhibition (SAHA, 2 μmol/L; VPA, 2 mmol/L) nor after treatment with isoform-specific siRNA in our experimental setting (data not shown). This was confirmed by the absence of a relevant number of apoptotic cells when similarly treated cells were analyzed by fluorescence-activated

**Table 2.** Cox regression analysis under inclusion of clinicopathologic factors and HDAC2 expression ($N$ = 140)

| | Overall survival | |
|---|---|---|
| | **HR (95% CI)** | ***P*** |
| Age at diagnosis | | |
| Per year | 1.035 (1.005-1.065) | 0.021 |
| Tumor stage | | |
| $pT_1/pT_2$ | 1.000 | |
| $pT_3/pT_4$ | 1.071 (0.447-2.568) | 0.877 |
| Lymph node status | | |
| Per positive node | 1.272 (1.139-1.421) | <0.001 |
| Metastasis | | |
| $M_0$ | 1.000 | |
| $M_1$ | 2.864 (1.075-7.627) | 0.035 |
| Grade | | |
| G1/G2 | 1.000 | |
| G3 | 1.900 (0.833-4.337) | 0.127 |
| HDAC2 expression | | |
| Negative | 1.000 | |
| Positive | 2.587 (1.095-6.116) | 0.030 |

Abbreviations: HR, risk ratio; 95% CI, 95% confidence interval.

cell sorting with a modified terminal deoxyribonucleotide transferase–mediated nick-end labeling assay (APO-direct, data not shown).

## Discussion

In our study, we found class I HDACs to be highly expressed in some but not all colorectal adenocarcinomas, with HDAC3 showing the highest expression levels followed by HDAC2 and HDAC1. To our knowledge, this is the first study to investigate the expression of the three class I HDAC isoforms (HDAC1, HDAC2, and HDAC3) *in vivo* in colorectal carcinoma, and this is also the first report of HDACs as adverse prognostic factors in this tumor entity.

Our findings are in line with previous studies reporting an elevation of both selected class I HDAC isoform RNA (18) and

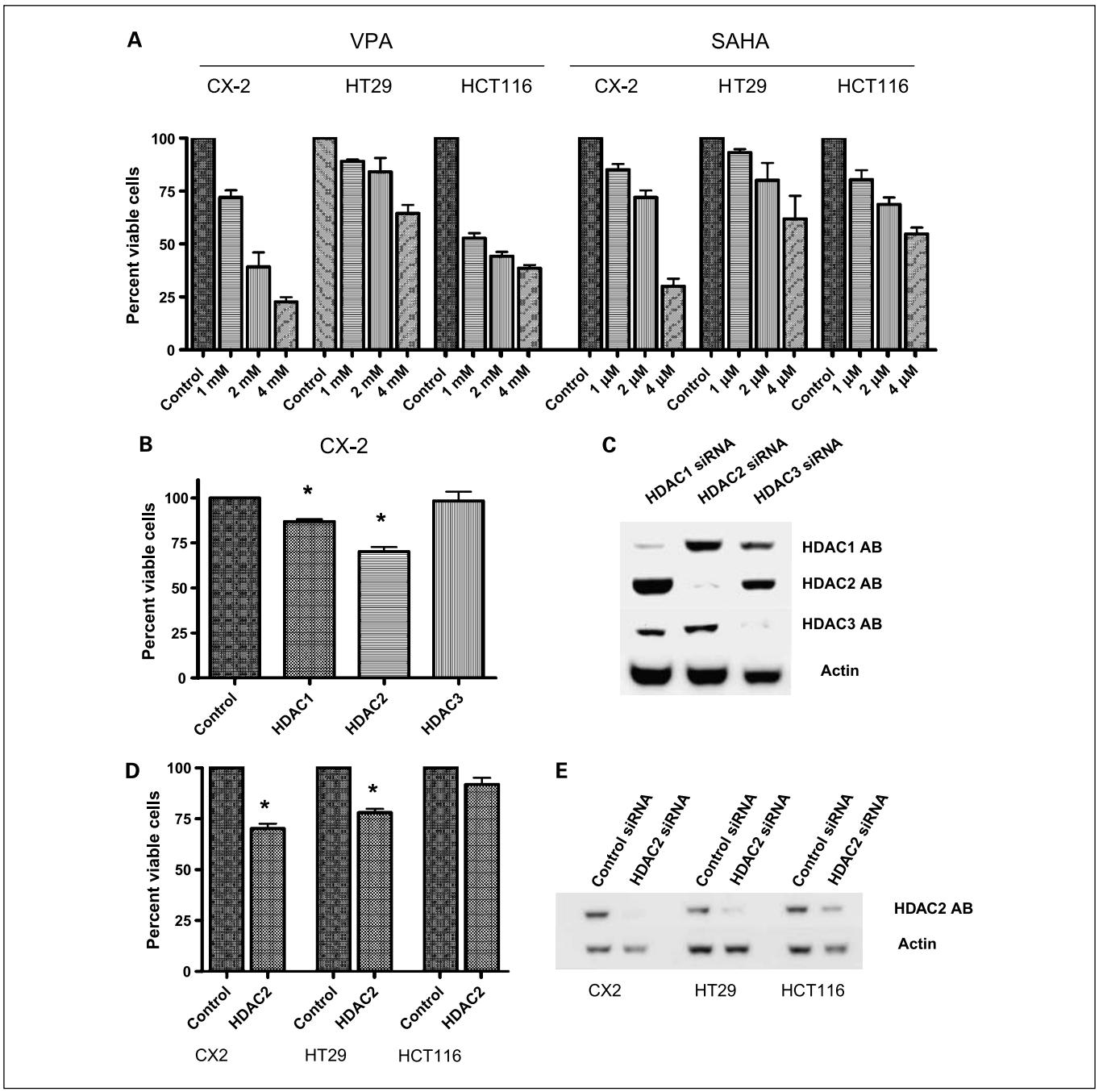


**Fig. 4.** Effects of HDAC inhibition on protein expression and cell number in colon cancer cells. *A,* treatment with either VPA or SAHA led to a significant dose-dependent reduction of colon cancer cell numbers after 72 h. *B,* selective knockdown of HDAC2 and to a lesser extent HDAC1 but not HDAC3 led to a weak to moderate, albeit significant, reduction of CX-2 cell numbers after 72 h. *C,* Western blots showing specific repression of HDAC1, HDAC2, and HDAC3 isoform expression by the corresponding siRNA. Equal protein loading was ascertained by probing against actin. *D,* selective knockdown of HDAC2 in other colon cancer cell lines also led to attenuated, although significant, reductions in cell number. *E,* Western blot showing efficacy of HDAC2 siRNA treatment in CX-2, HT-29, and HCT-116. Statistically significant reductions of treated cells compared with the control are marked by asterisks in *B* and *D* (columnar *t* test).

protein (17). In contrast, some studies have reported a loss (26) of class I HDAC isoforms in small sets of colorectal tumors when compared with normal mucosa. However, such an analysis of HDAC mRNA as well as protein expression in extracts of tumor tissue must be interpreted with caution because not only tumor epithelium but also normal and tumor stroma as well as inflammatory cells express relevant amounts of all three isoforms, as we were able to show. HDAC1 (18) and HDAC2 (16, 18) were confirmed to be overexpressed in colorectal cancer by exploratory tissue-based expression analysis on small sets of tumors. In contrast, in one study, Ropero and coworkers (27) reported truncating mutations of the *HDAC2* gene in 20.4% of microsatellite unstable (MSI-H) sporadic colorectal cancers, which led to a loss of expression of the protein. However, as ~20% of sporadic colorectal cancers are MSI-H (28), this affects only ~4% of sporadic tumors. Nevertheless, because MSI-H tumors are said to have slight survival advantages, this may contribute marginally to the survival differences seen in our cohort. Occasional mutational inactivation of histone acetylases has also been described for colorectal cancer (29, 30). Functional data on the regulation of HDAC expression are very sparse. There is some evidence that the expression of class I HDAC isoforms is under shared control of specific growth factors. In addition, an autoregulatory feedback loop controlling expression of class I HDACs has been suggested (31, 32). This fits well with our observation of a coordinate regulation of class I HDAC isoform expression in colorectal cancer tissue.

Based on the results of cell culture studies, it has been proposed that HDI treatment leads to reduced tumor cell proliferation and initiates differentiation. Our finding of a direct correlation of elevated class I HDAC expression levels with tumor dedifferentiation and enhanced tumor proliferation is the first report strongly suggesting that this interaction is also relevant for progression of primary human tumors.

There are few reports on the expression patterns of distinct HDAC isoforms in other tumor entities; however, most of the cohorts investigated thus far were of very small sample size. Moreover, most of the authors did not test for correlations with clinicopathologic data and patient prognosis. Our study, to the best of our knowledge, is the first report on an adverse independent prognostic impact of HDAC isoform expression in human cancer.

Suppressive effects of various HDIs on colon cancer cell growth *in vitro* and *in vivo* have been proven by several groups (33–38). The results of inhibitor-specific cell cycle alterations and cell reduction observed in colon cancer cells fits well with the results of these previous studies on other human colon cancer cell lines. However, degradation of class I HDAC isoforms, which was observed in other cancer cell lines as a consequence of HDI treatment (25), could not be seen in CX-2.

Combined treatment of CX-2 cells with 5-FU and VPA or SAHA did not result in additive reductions in cell number in our experimental setting, which is in line with findings of other authors in other cancer cell lines (10). However, synergistic effects of HDI other than the ones we used and 5-FU have also been described for colorectal cancer cell lines (11). Effects may be largely dependent on the cell lines used and the inhibitor concentrations applied.

Data on the cellular effects of a specific HDAC isoform knockdown are sparse. In one study, Wilson and colleagues (17) reported a reduction in cell number of up to 20% after knockdown of each HDAC1, HDAC2, and HDAC3 by siRNA treatment in the colon carcinoma cell line HCT-116 with an especially prominent effect for HDAC3. In addition, they observed induction of apoptosis after HDAC2 and HDAC3 but not after HDAC1 knockdown. In another study on HeLa cells, specific knockdown of HDAC1 and HDAC3 by siRNA led to a concentration-dependent inhibition of proliferation of up to 50% (39). In our study on CX-2, the effects of HDAC isoform inhibition on cell number were especially pronounced for HDAC2, whereas no effect was observed for HDAC3. The inhibitory effects of specific HDAC2 knockdown on cell number could be confirmed in HT-29 but not in HCT-116 colon cancer cells, which is in line with previous findings (16, 17). These differences may be explained by the fact that different cancer cell lines may need different HDAC isoforms for maintaining cellular proliferation and survival.

However, when compared with the effects of either nonselective (SAHA) or putative semiselective class I (VPA) synthetic small-molecule HDIs, selective HDAC inhibition by siRNA, albeit effectively reducing protein expression, only produced modest alterations with respect to the mentioned cellular readouts, which indicates that a comparably broad therapeutic strategy targeting different HDACs with one therapeutic substance may be more promising than a specific inhibition of single HDAC isoforms. This is in line with the results of Wilson et al. (17) in HCT-116.

An outstanding role of HDAC2 in colon carcinogenesis has been suggested lately in a comprehensive work by the group of Martin Göttlicher, in which the authors showed that loss of the adenomatosis polyposis coli tumor suppressor induced HDAC2 expression depending on the Wnt pathway and c-Myc in the colon carcinoma cell line HT-29 (16). In addition, they showed that HDAC2 expression is required for and sufficient on its own to prevent apoptosis in this cell line. These findings fit well with our *in vitro* results and are also perfectly in line with our clinical findings. Our observation that HDAC2 expression is an independent prognosticator in colorectal cancer further emphasizes the role of this isoform in colorectal carcinogenesis and tumor progression. However, based on our data, it is likely that some but not all of the effects of HDI can be contributed to an inhibition of HDAC2, as HDAC2 knockdown alone only shows moderate cellular effects *in vitro* when compared with HDI treatment.

In conclusion, our results emphasize the important role of class I HDACs, especially HDAC2, in colon cancer biology. Patients showing an overexpression of class I isoforms had dramatically reduced survival times, which hint on a potential use of these markers to clarify individual patient prognosis. Even more important, the prognostic impact of HDAC isoforms, together with our confirmation of the proposed interactions of HDACs with tumor cell proliferation and differentiation *in vivo*, strongly suggests that the evaluation of HDAC expression profiles before HDI treatment might help to identify patient populations who will exceptionally benefit from such a treatment. This should be considered when clinical trials for HDIs are planned.

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