Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis

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Summary

Inappropriate transcriptional repression involving histone deacetylases (HDACs) is a prominent cause for the development of leukemia. We now identify faulty expression of a specific mediator of transcriptional repression in a solid tumor. Loss of the adenomatosis polyposis coli (APC) tumor suppressor induces HDAC2 expression depending on the Wnt pathway and c-Myc. Increased HDAC2 expression is found in the majority of human colon cancer explants, as well as in intestinal mucosa and polyps of *APC*-deficient mice. HDAC2 is required for, and sufficient on its own to prevent, apoptosis of colonic cancer cells. Interference with HDAC2 by valproic acid largely diminishes adenoma formation in *APC*^{min} mice. These findings point toward HDAC2 as a particularly relevant potential target in cancer therapy.

Introduction

Carcinogenesis is a multistep process during which cells acquire several critical features due to genetic instability and alterations in gene expression (Hanahan and Weinberg, 2000). In promyelocytic leukemia, a prominent cause is the expression of fusion proteins from chromosomal translocations that code for aberrant transcriptional repressors due to inappropriate recruitment of histone deacetylases (HDACs) (Krämer et al., 2001; Melnick and Licht, 2002). The recruitment of histone acetyltransferases and HDACs is considered to be a key element in the dynamic regulation of many genes regulating cellular proliferation and differentiation during normal development and carcinogenesis (Berger, 2002; Glass and Rosenfeld, 2000; Jenuwein and Allis, 2001; Strahl and Allis, 2000). Mammalian histone deacetylases can be divided into three subclasses (Verdin et al., 2003). HDACs 1, 2, 3, and 8, which are homologs of the yeast RPD3 protein, constitute class I. HDACs related to the yeast Hda1 protein form class II, and mammalian homologs of the yeast Sir2 protein constitute the third class. HDACs are usually subunits of multiprotein complexes, for example the transcriptional corepressors mSin3, N-CoR, and SMRT, which recruit HDACs to transcription factors (Glass and Rosenfeld, 2000). HDAC inhibitors are considered as candidate drugs in therapy for leukemia, but apparently also in various other forms of cancer, including those of nonhematopoietic origin (Johnstone, 2002; Krämer et al., 2001; Marks et al., 2001; Melnick and Licht, 2002; Remiszewski, 2002). However, compared to the fusion proteins serving aberrant transcriptional repressor functions in leukemia, little is known about genetic causes for aberrant repression in epithelial forms of cancer.

Colonic cancer is frequently associated with aberrant signaling through the Wnt pathway due to loss of both functional copies of the tumor suppressor *adenomatosis polyposis coli* (*APC*) and/or mutations in the β-*catenin* gene, although additional mutations are required for cancer development (Fodde, 2003; Giles et al., 2003; Kinzler and Vogelstein, 1996). APC is a key component of the β-catenin destruction complex that is required for basal turnover and Wnt-induced stabilization of β-catenin. Wnt signaling prevents GSK-3β-dependent phosphorylation and subsequent APC-dependent degradation of β-catenin. Loss of functional APC leads to reduced turnover

SIGNIFICANCE

HDAC inhibitors are considered promising novel anticancer drugs, and prove useful in preclinical and first clinical trials. Nevertheless, little is known about whether aberrant transcriptional repression plays a role in the development of solid tumors, or which might be the critical components of the transcriptional repression machinery. Knowledge of mechanisms of faulty HDAC-dependent transcriptional repression acquired during the development of solid tumors would allow screening for such forms of cancer that can be expected to respond to therapy with HDAC inhibitors. Also, specific isoenzymes in the family of more than ten HDACs may be identified which appear as particular relevant targets for the development of isoenzyme-specific HDAC inhibitors.

and accumulation of β-catenin protein. Although β-catenin influences a number of cellular processes, the primary function of accumulated β-catenin in intestinal cancer development appears to be mediated by interaction with the Tcf-4/Lef1 transcription factors (Behrens et al., 1996; Molenaar et al., 1996; Morin, 1999). Known target genes are *Cyclin D1* (Shtutman et al., 1999; Tetsu and McCormick, 1999), *c-Myc* (He et al., 1998), and *PPAR*δ (He et al., 1999), among several other target genes of the Wnt pathway (http://www.stanford.edu/rnusse/pathways/targets.html). Only for some of these genes has a link to the transformed phenotype of cancer cells been established.

We now show a link between tumor suppression in colonic cancer and aberrant transcriptional repression. Loss of functional APC leads by a Myc-dependent mechanism to overexpression of an apparently rate-limiting component of the transcriptional repression machinery, HDAC2. High expression of HDAC2 is required and sufficient to maintain a transformed cellular phenotype with respect to the lack of apoptosis in cultured colonic cancer cells. Increased expression of HDAC2 in *APC* mutant mice and severe reduction of adenoma formation upon interference with HDAC2 indicate that HDAC2 is a critical target of APC in vivo that is accessible to pharmacological interventions.

Results

Wild-type APC represses HDAC2 expression

APC-deficient HT-29 colonic cancer cells fail to grow in the presence of HDAC inhibitors (Göttlicher et al., 2001). Therefore, we tested whether *APC* status affects HDAC expression by using HT-29 cells in which wild-type APC is expressed from a Zn^{2+}-inducible transgene (Morin et al., 1996). Expression of HDAC2 was significantly reduced, both at the mRNA (to 42% at 48 hr) and protein (to 21% from 48 hr onward) levels after induction of wild-type APC (Figures 1A and 1B). Downregulation of *PPAR*δ mRNA (to 21% at 48 hr) served as control for APC reconstitution. Expression of β-Gal instead of APC proved specificity (He et al., 1999). Expression of two other class I HDACs, HDAC1 and 3, was not changed within the range of ±20% of signal intensity, indicating a selective effect on HDAC2 (Figure 1B). As APC promotes β-catenin degradation, we further tested whether β-catenin/Tcf-4/Lef1 dependent transcription is involved in the regulation of HDAC2 expression. GSK-3β phosphorylates β-catenin and consequently promotes β-catenin degradation. To test its role in HDAC2 expression, we inhibited GSK-3β by lithium (15 mM) to allow for a 6.9-fold accumulation of β-catenin protein in HEK293T cells containing wild-type APC (Phiel and Klein, 2001; Stambolic et al., 1996). *HDAC2* mRNA (data not shown) and protein expression was elevated by 4.1-fold upon β-catenin accumulation (Figure 1C). Lithium also induced HDAC2 levels in Zn^{2+}-treated HT29-APC cells, although time courses are complicated due to combined effects of APC induction on apoptosis and GSK-3β inhibition (data not shown). Furthermore, transfection of a dominant-negative mutant of Tcf-4 in APC-deficient HT-29 cells led to a reduction of *HDAC2* mRNA (data not shown) and protein levels (to less than 50%, Figure 1D), while β-catenin levels were unchanged. These data indicate that HDAC2 expression depends on β-catenin and Tcf-4/Lef1, and is increased in cells lacking functional APC. The delay of HDAC2 expression upon LiCl exposure suggested the need for synthesis of intermediary factors such as c-Myc or

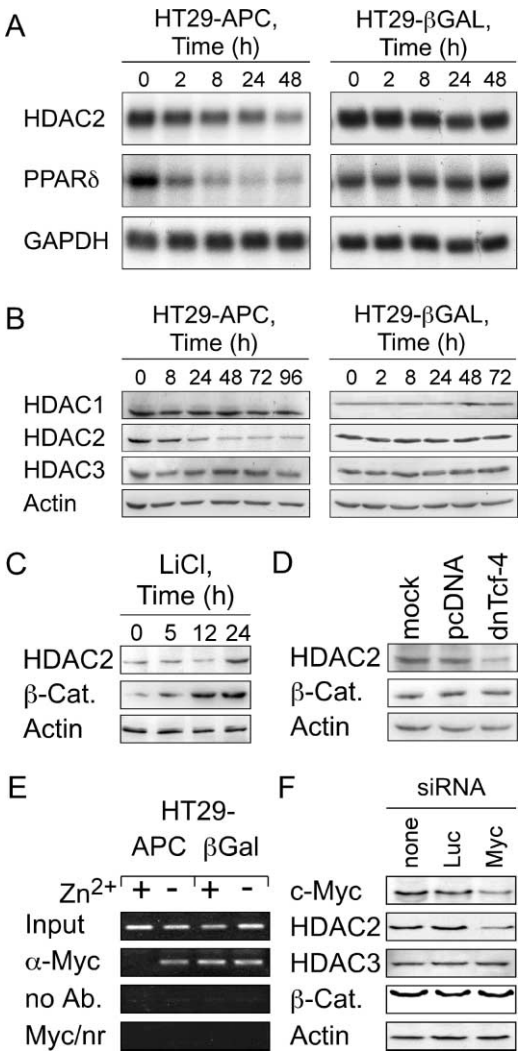

Figure 1. HDAC2 is regulated by the APC/β-catenin pathway

A: *HDAC2* mRNA levels and expression of *PPAR*δ and *GAPDH* for controls were determined by Northern blot hybridization in HT-29 cells expressing APC or β-Gal from a Zn^{2+}-inducible transgene.

B: Downregulation of HDAC2 protein upon restoration of wild-type APC was confirmed by Western blot analysis with HDACs 1 and 3 for comparison.

C: Induction of HDAC2 expression and the expected accumulation of β-catenin were determined upon GSK-3β inhibition by LiCl.

D: The role of Tcf-4/Lef1 for high level HDAC2 expression in APC-deficient HT-29 cells was tested by expressing a dominant-negatively acting mutant form of Tcf-4 at efficiencies of transient transfections greater than 50% as determined by parallel transfection of a GFP expression vector pCMV-GFP. Untransfected cells and transfection of the empty expression vector served as controls.

E: Occupancy of the binding site for c-Myc in the HDAC2 promoter was determined by chromatin immunoprecipitation. Wild-type APC or β-Gal were induced by Zn^{2+} treatment, and presence of the DNA fragment covering the Myc binding site was detected by PCR in input cell lysates (5%) or anti-c-Myc immune precipitates. For controls, precipitates were prepared in the absence of antibody, or specific anti-c-Myc precipitates were subjected to PCR amplification for a nonrelated fragment of the HDAC2 promoter (Myc/nr).

F: The effect of siRNA directed against c-Myc was analyzed by Western blot analysis. Similar results were obtained in 2 or 3 additional experiments (**B**), respectively.

A

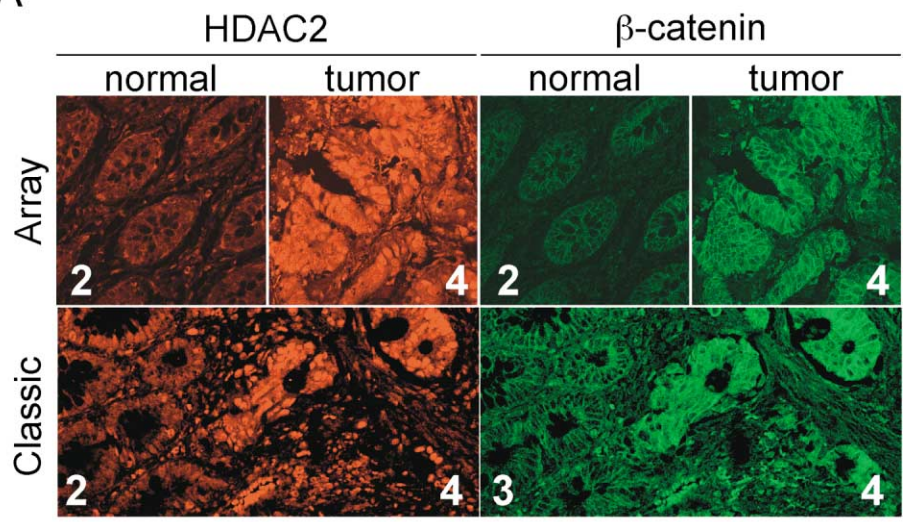

Figure 2. HDAC2 is overexpressed in human colorectal carcinomas

Colonic tumor samples and patient-matched normal tissues were compared for HDAC2 expression and β-catenin levels by immunohistochemistry in the form of 46 spots of tumor and control tissue, respectively, on a multiple tissue array and 11 classical histological sections.

A: Representative double-immunofluorescent stainings of a tissue array sample and a classical tumor sample with normal tissue next to it are shown. Assigned scores are indicated in each frame.

B: The tables summarize scoring results from 57 tumor samples. Distribution of samples over the score classes as well as the average score and statistic evaluation by Student's t test are presented in the upper table. The lower table summarizes the differences between tumor and normal tissue. The number of samples is shown in which all combinations of differences in HDAC2 or β-catenin expression were found. β-Cat, β-catenin; no, normal tissue; tu, tumor.

B

	Frequency of score 1	2	3	4	Avg. score ± S.D.	*p*
β-Cat., norm.	1	50	5	1	2.1 ± 0.4	$<10^{-13}$
β-Cat., tumor	0	11	38	8	2.9 ± 0.6	
HDAC2, no.	9	47	1	0	1.9 ± 0.4	$<10^{-16}$
HDAC2, tu.	0	14	29	14	3.0 ± 0.7	

Comparison of tumors with matched normal tissues		β-Catenin score: equal	β-Catenin score: higher
HDAC2 score	equal	5	5
	higher	11	36

PPARδ. A role of c-Myc is likely, since the promoter region that harbors a binding site for c-Myc at 621 to 616 bp upstream of the HDAC2 start codon is occupied with c-Myc in HT-29 cells as shown by chromatin immunoprecipitation (ChIP) assays. Expression of wild-type APC by $Zn2^{+}$ induction virtually abolished promoter occupancy with c-Myc (Figure 1E). The functional significance of Myc was tested by siRNA transfection. siRNA efficiently knocked down Myc protein expression by 49% and HDAC2 expression by 67%, whereas, as expected, β-catenin levels were not affected (Figure 1F). Downregulation of HDAC2 appears to be even more pronounced than that of c-Myc, which may be due to the timing of the experiment.

Elevated HDAC2 levels in human colonic cancer

We compared 57 samples of mostly moderately differentiated TNM-T3 or -T4 tumors versus patient-matched normal tissues with respect to alterations in HDAC2 and β-catenin levels (Figure 2A). Elevated HDAC2 expression was observed in 47 samples (82%), and more than 1 score point difference between normal and tumor tissue was found on average (Figure 2B). In the vast majority of samples, HDAC2 and β-catenin were regulated in the same way, suggesting that they are, in most cases, related events in the development of colonic cancer (Figure 2B). Only 5 samples did not show elevated HDAC2 levels despite immunologically detectable increased levels of β-catenin, which could be due to additional defects in the β-catenin/Tcf pathway. Elevated HDAC2 expression without apparent increases in β-catenin levels was observed in 11 samples. This may be due to the limited quantitative power of immunohistochemistry, or it may indicate that HDAC2 expression and accumulation of β-catenin are not always associated. Other mechanisms of HDAC2 upregulation, such as *Myc* amplification may exist in addition to APC/β-catenin signaling. The separate analysis of 6 mucinous type, 11 well differentiated, or 2 poorly differentiated samples, respectively, did not yield any significant difference compared to the analysis of all samples. Neither did the analysis

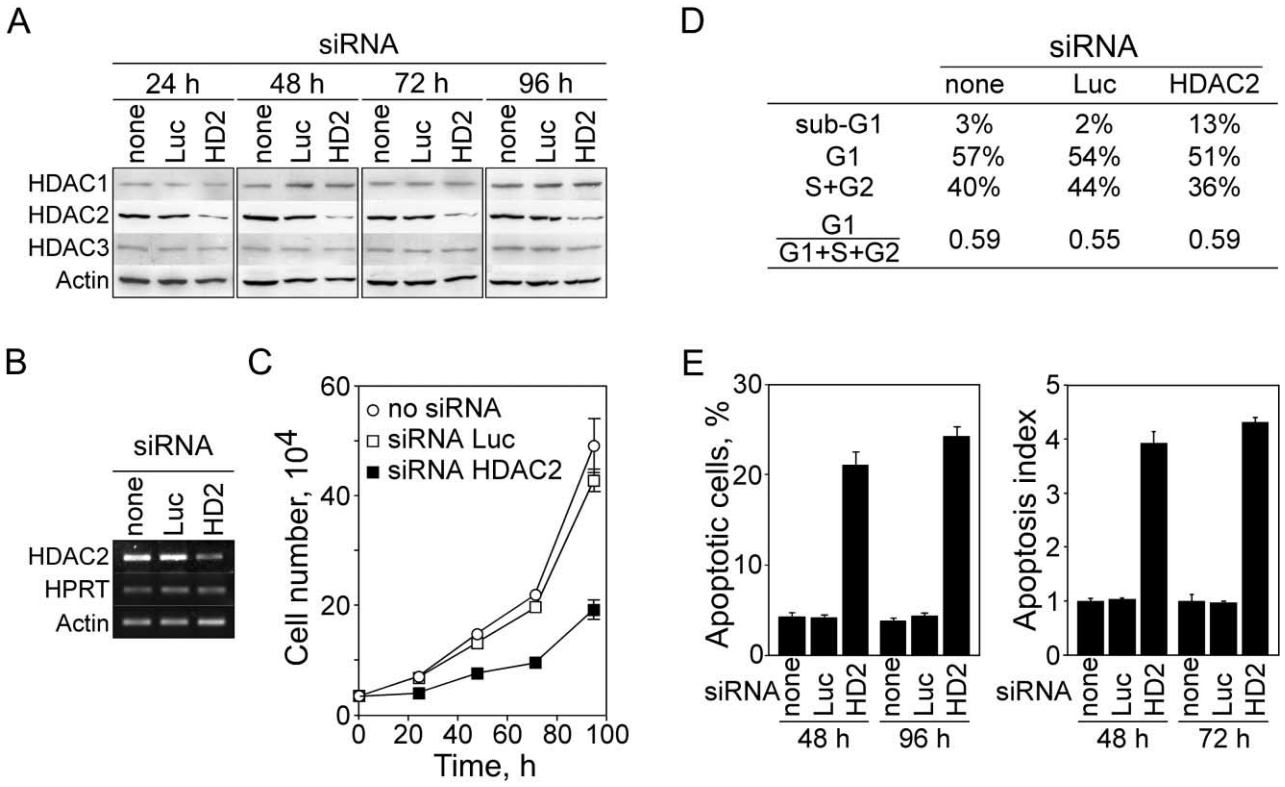

Figure 3. HDAC2 is required for survival of HT-29 cells

HDAC2 was knocked down in HT-29 cells by transfection of a double-stranded siRNA.

A: HDAC2 protein levels were determined at indicated times after siRNA addition by Western blot analysis. HDAC1 and 3 were determined to assess specificity of downmodulation, and Luc siRNA as well as nontransfected cells served as control.

B: Downmodulation at the mRNA level was confirmed 48 hr after siRNA addition by semiquantitative RT-PCR reactions of polyA$^+$-RNA, with two housekeeping genes (hypoxanthine phosphoribosyltransferase [HPRT] and actin) serving as controls.

C: Cell numbers were determined upon HDAC2 downmodulation by counting attached cells per culture at indicated times after siRNA transfection. Values are means ± range of duplicate samples.

D: Control and HDAC2-depleted cells were analyzed 48 hr after transfection for DNA content for determination of cell cycle distribution and assignment of a presumably apoptotic fraction of cells with sub-G1 amounts of DNA.

E: Signs for induction of apoptosis were obtained at indicated times after siRNA addition by collecting attached and floating cells. The relative fraction of cells with fragmented or condensed nuclei (left panel) was assessed microscopically. Quantitative apoptotic indices (right panel) scored cytoplasmic histone-associated DNA fragments generated during the apoptotic process. **A–D** show one representative example out of three sets of experiments with similar results. Values in **E** are means ± range from duplicate experiments.

of the 11 classical tumor sections with tumor and normal tissue present on the same slide differ significantly from the evaluation of all samples together. As a general conclusion from the immunohistochemical analysis, elevated HDAC2 levels are found at an even higher frequency in colonic cancer samples than elevated β-catenin levels in the present or published studies (Hugh et al., 1999; Valizadeh et al., 1997).

HDAC2 is required for HT-29 cell survival

Expression of wild-type APC in HT-29 cells in culture had been shown to reduce cell numbers and to induce apoptosis (Morin et al., 1996, and our unpublished data). To test whether lack of HDAC2 alone would suffice to impair HT-29 cell growth, we knocked down HDAC2 protein levels to less than 30% by transfection of siRNA directed against HDAC2 (Figure 3A). Downmodulation of HDAC2 occurred on the mRNA level, indicating a specific activity of the siRNA (Figure 3B). Cell cultures transfected with HDAC2 siRNA showed significantly reduced growth, with cell counts reduced by more than 50% after 3 days, while control cultures displayed no difference in growth kinetics (Figure 3C). The significant reduction of cell numbers does not appear to be due to lack of proliferation, since the distribution of cells over G1 and S/G2 phases of the cell cycle was not altered (Figure 3D). A substantial fraction of cells with sub-G1 amounts of DNA rather suggests that knockdown of HDAC2 or VPA treatment of HT-29 cells might induce apoptosis. Induction of apoptosis upon HDAC2 knockdown was confirmed by counting cells with fragmented and condensed nuclear DNA and a quantitative assay for cytoplasmic histone-associated DNA fragments (Figure 3E). In summary, the knockdown data suggest that HDAC2 is required for HT-29 cell survival, and that downmodulation to a level comparable to that in APC-proficient cells suffices to induce apoptosis. Similar experiments addressing HDAC3 achieved reduction of protein levels by only 50%, without detectable effects on cell survival (data not shown).

The critical role of HDAC2 in the prevention of apoptosis was confirmed by a second independent approach, which should also clarify whether ectopic expression of HDAC2 alone

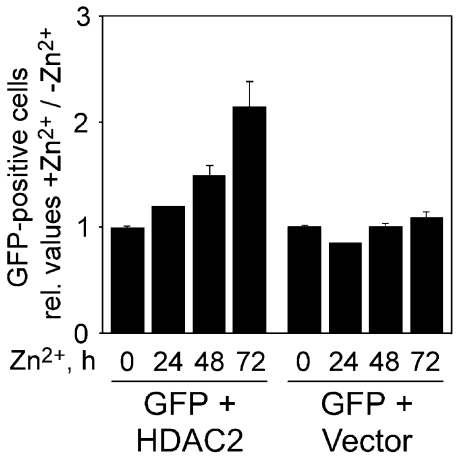

Figure 4. Ectopic expression of HDAC2 antagonizes APC-induced apoptosis

HT29-APC cultures were transfected at low efficiency with an empty expression vector or a vector for HDAC2 to maintain high levels of HDAC2. A GFP expression vector was cotransfected to allow tracing the small fraction of efficiently transfected cells by flow cytometry. Wild-type APC was induced by addition of $ZnCl_2$ in half of the cultures to decrease endogenous HDAC2 levels (and to induce apoptosis). At the indicated time points after addition of Zn^{2+}, attached cells were harvested for detection of GFP-positive cells by FACS. Percentages of GFP-positive cells in cultures without Zn^{2+} were set to 1 at each time point and the relative increase in the percentage of GFP-positive cells in Zn^{2+} treated cultures was calculated as an indicator of preferential loss by apoptosis of those cells that were not transfected. Values are means ± S.D. from two independent experiments with duplicate determinations each. Absolute percentages of GFP-positive cells were in the range of 0.5–4%, depending on time point of analysis.

suffices to prevent apoptosis. HDAC2 was expressed from a transiently transfected vector, while endogenous HDAC2 was downregulated by Zn^{2+}-induced expression of wild-type APC. Transfected cells were identified by flow cytometry upon coexpression of green fluorescent protein (GFP). If HDAC2 would not affect induction of apoptosis, normal and green fluorescent cells should be eliminated by apoptosis at the same rate, and their ratio should not change. The experiment, however, showed enrichment of fluorescent cells (Figure 4). Reduction of untransfected HT29-APC cell numbers to about 40% by 72 hr exposure to Zn^{2+} led to a more than 2-fold enrichment of HDAC2-transfected cells. This indicates that almost all efficiently transfected cells had to be protected from apoptosis. Control transfection of the empty vector together with GFP did not enrich transfected cells. These data indicate a prominent role of overexpressed HDAC2 in protection of APC-proficient cells from apoptosis. They suggest that elevated HDAC2 expression is a key mechanism in prevention of apoptosis in APC-deficient cells.

The possibility cannot be excluded that APC affects apoptosis also by additional mechanisms. Those, however, appear unlikely to be able to overcome the control mediated by HDAC2 in HT-29 cells. Reconstitution of wild-type APC or transfection of siRNA in APC-deficient cells reduces HDAC2 protein levels to a similar extent. In the latter case, HDAC2 depletion suffices to induce apoptosis despite the lack of functional APC. If it is assumed that reduction of HDAC2 expression upon reconstitution of wild-type APC expression likewise provides a functionally equivalent proapoptotic stimulus, one would not have to postulate the need for additional proapoptotic signals upon reconstitution of wild-type APC. Also, additional antiapoptotic signals in addition to high-level HDAC2 expression in APC-deficient cells do not need to be postulated from the present experiments, since overexpression of HDAC2 alone suffices to substantially protect from initiation of apoptosis upon reconstitution of wild-type APC (Figures 1 and 3). Nevertheless, other mechanisms to control apoptosis may exist downstream of APC that are not apparent in the present experiments but may exist in other cell types or under other experimental conditions.

The APC/β-catenin/HDAC2 pathway in mice

To test whether HDAC2 is a relevant target of the APC/β-catenin pathway in vivo, we analyzed HDAC2 in *APC*^{min} and *Apc*^{1638N} mice (Fodde et al., 1994; Shoemaker et al., 1997; Su et al., 1992). HDAC2 protein levels are elevated in intestinal polyps of *APC*^{min} mice compared to normal tissue (Figure 5A). In wild-type mice, HDAC2 is preferentially detectable in the lower zone of regenerative proliferation (Figure 5A), as described previously for nuclear β-catenin accumulation and expression of Tcf-4 target genes (van de Wetering et al., 2002). Also, in apparently normal tissue, HDAC2 levels were moderately higher in *APC*^{min} mice. Moreover, protein levels did not decrease toward the upper zone of the epithelium (Figure 5A). Differences in HDAC protein expression were confirmed by Western blot analysis of extracts prepared from tumor tissues or scrapings of intestinal mucosa (Figure 5B). HDAC2 expression is higher in normal tissues and more pronounced in tumors from *APC* mutant mice compared to normal tissues of wild-type mice. HDAC3 expression did not differ between wild-type and *APC* mutant mice (Figure 5B and histology not shown), whereas HDAC7 levels rather appeared to be lower in tumors. In contrast to adenomas expressing only mutant APC due to somatic inactivation of the second allele, normal intestinal epithelium of heterozygous *APC*^{min} mice usually expresses wild-type APC protein and APC^{min} protein at similar levels (Shoemaker et al., 1997). Thus, aberrant patterns of HDAC2 expression in apparently normal mucosa of *APC*^{min} mice suggest haploinsufficiency of *APC* with respect to downregulation of HDAC2 toward the upper zone of the intestinal epithelium.

To assess the role of HDAC2 in adenoma formation, we treated *APC*^{min} mice with valproic acid (VPA) to inhibit class I HDACs and to induce degradation of HDAC2 (Göttlicher et al., 2001; Krämer et al., 2003; Phiel et al., 2001). Increased bulk histone H3 acetylation indicated efficiency of HDAC inhibition, and reduction of HDAC2 levels but not of HDAC3 was confirmed by immunohistochemistry (Figure 5C and data not shown). VPA significantly reduced number and average size of adenomas in entire intestinal tracts (Figures 5D and 5E). Particularly, only 10% of the large tumors (diameter $>$ 1 mm) were found after VPA treatment. In summary, the analysis of *APC*^{min} mice supports the conclusion that loss of functional APC also leads to increased HDAC2 expression in vivo, and that aberrant HDAC activity is required for development of *APC*^{min} dependent intestinal adenomas.

Discussion

Elevated expression of HDAC2 upon increased signaling through the APC/β-catenin/c-Myc pathway establishes an example for aberrant function of the HDAC-dependent transcrip-

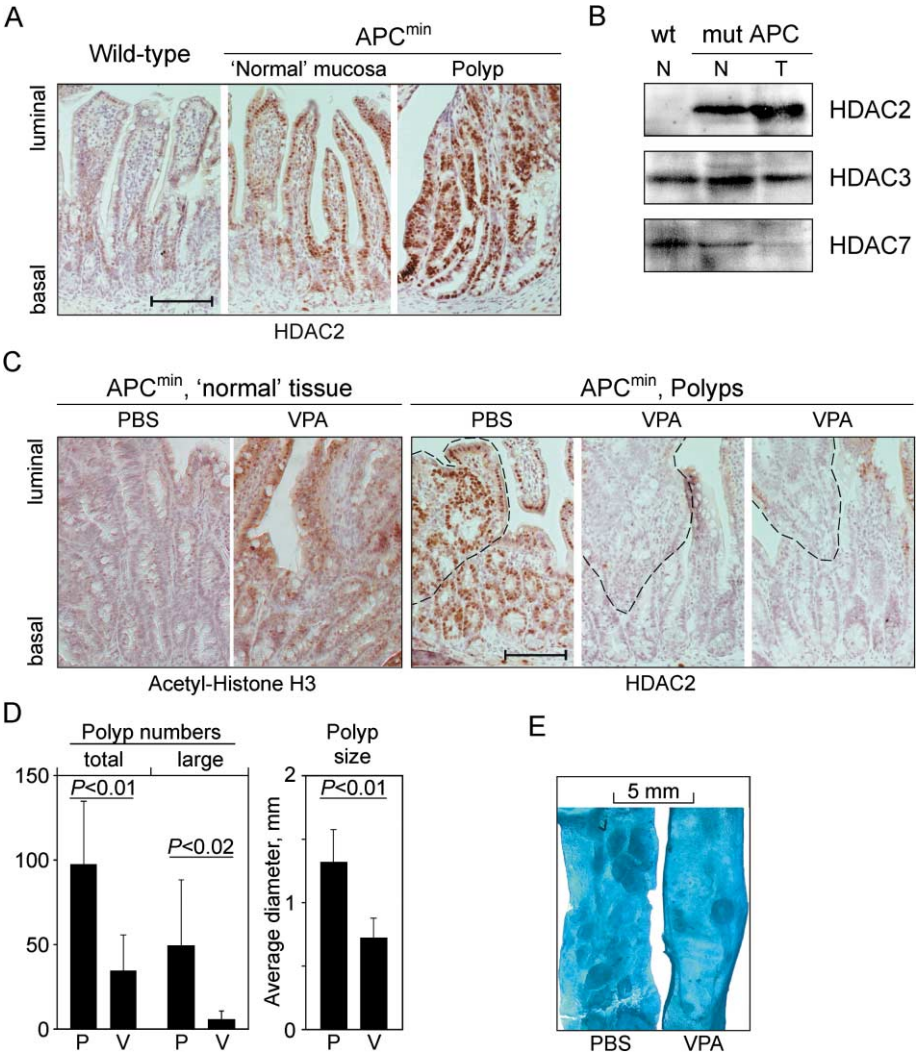

Figure 5. Intestinal HDAC2 expression and adenoma development in VPA-treated *APC*^{min} mice

HDAC2 expression and adenoma formation were analyzed by histological and macroscopic evaluation of guts from heterozygous *APC* mutant mice and litter-matched wild-type animals.

A: Immunohistochemical detection of HDAC2 in the ileum is shown for 12-week-old wild-type mice (normal mucosa) and litter-matched *APC*^{min} mice. Pictures of macroscopically apparently normal mucosa and a representative polyp are taken from the same section. No staining was observed in control experiments without primary antibody. Scale bars indicate 100 μm.

B: Amounts of HDAC proteins were determined by Western blot analysis in equal amounts of soluble protein extracts obtained from scrapings of intestinal mucosa (N) or tumor tissues (T) of wild-type (wt) or Apc^{1638N} (mut APC) mice (Janssen et al., 2002).

C: The degrees of histone acetylation and HDAC2 expression were determined in the ileum of *APC*^{min} mice that had been injected (i.p.) with PBS or VPA for 4 weeks. Adenomas are marked by dashed lines.

D: Adenoma formation on entire intestinal tract was quantitatively evaluated in *APC*^{min} mice that had been injected (i.p.) with PBS (P, n = 4) or VPA (V, n = 5) for 4 weeks. Large polyps were those with a diameter ≥ 1 mm. Values are means ± S.D. P values were calculated by Student's t test.

E: Polyps appear in dark blue circular staining in representative ileum segments from experiments in **D**.

tional repression machinery in nonhematopoietic cancer cells. Elevated HDAC2 expression prevents apoptosis in cultured cells, and interference with HDACs reduces tumor formation in *APC*^{min} mice.

APC regulates HDAC2 most likely at the transcriptional level, because APC does not affect the initial rate of *HDAC2* mRNA degradation after inhibition of de novo synthesis by actinomycin D (data not shown). Interference by a dominant-negative mutant of Tcf suggests that either Tcf-4/Lef1 or a Tcf-4/Lef1-dependently induced protein such as c-Myc or PPARδ induces *HDAC2* transcription (He et al., 1998, 1999). Sequence analysis and EMSA analyses of the *HDAC2* promoter (Zeng et al., 1998) suggested that this could be c-Myc. Experimental evidence for a functional role of c-Myc in expression of HDAC2 is provided here by showing reduction of HDAC2 expression upon knockdown of c-Myc. c-Myc expression is also elevated by protein stabilization upon K-Ras activation (Sears et al., 1999). Therefore, activation of this protooncogene with critical roles in colonic cancer development is likely to increase HDAC2 expression. PPARδ appears to contribute to *HDAC2* regulation in some cell types. Disruption of one *PPAR*δ allele in embryonic stem cells reduces HDAC2 expression by 50% (P.Z. and M.G., unpublished data). Sulindac is thought to interfere with DNA binding of PPARδ (He et al., 1999) and reduces HDAC2 expression by 50% in HCT116, but only marginally in HT-29 cells (P.Z. and M.G., unpublished data). Human xenograft growth in mice substantially depends on PPARδ, whereas adenoma development in *APC*^{min} mice does not (Barak et al., 2002; Park et al., 2001). Thus, different components of the Wnt pathway contribute to HDAC2 expression and Tcfs, c-Myc, occasionally PPARδ, and probably Ras are prominent factors.

Induction of HDAC2 upon loss of APC represents a novel mechanism for aberrant function of the HDAC-dependent transcriptional repression machinery upon genetic defects that promote the development of cancer. Such links are sparse in carcinomas in contrast to numerous examples in leukemia (Krämer et al., 2001; Melnick and Licht, 2002). The polycomb group protein enhancer of zeste 2 apparently promotes prostate cancer development by HDAC-dependent transcriptional repression (Varambally et al., 2002). The BRCA tumor suppressors function as both transcriptional activators and repressors. It is, however, not clear if and how this is related to carcinogenesis (Monteiro, 2000; Scully, 2001). A link between tumor development in APC-deficient mice and regulation of gene expression

and chromatin structure had been described in DNA methyltransferase (DNMT)-deficient mice. Genetic inactivation of one of the *DNMT* alleles and chemical inhibition by 5-aza-deoxycytidine severely reduced the numbers of polyps formed (Laird et al., 1995). The concepts of DNA methyltransferase and histone acetylation might address related aspects of the same biological process in colon tumorigenesis, in which histone deacetylation might provide the most dynamic and transient mode of downregulation of critical target genes. At later stages, this downregulation may become more stably established by DNA methylation. The recruitment of individual HDACs and HDAC-containing complexes to critical target genes by transcription factors might provide a level of specificity that is not apparent from alterations in bulk DNA methylation rates. The present data and preliminary gene expression profiles obtained after knockdown of HDAC2 (data not shown) indicate that critical processes during carcinogenesis exist in which levels of HDAC2 are limiting. Other HDACs obviously serve limiting roles in other biological processes, such as control of proliferation during embryogenesis (HDAC1, Lagger et al., 2002) or stress response of the heart (HDAC9, Zhang et al., 2002).

Since the lack of functional APC alone is not sufficient to trigger all the processes critical for tumor development (Giles et al., 2003), it is also unlikely that elevated HDAC2 expression is sufficient. However, HDAC2 appears to be required for some critical steps, since interference with HDAC2 reduces tumor formation in *APC*^{min} mice and elevated HDAC2 levels are found at high frequency in human colon cancer samples. HDAC2, though not necessarily induced by loss of APC, may even be critical in other carcinomas, since HDAC2 levels are elevated in many tumor cell lines compared to normal tissue more pronouncedly than other HDACs (Yang et al., 1997). Furthermore, examples of gastric, breast, and lung carcinoma on a multiple tissue array show increased expression of HDAC2 protein in tumors compared to the corresponding normal tissues included on the same tissue array (data not shown). While prevention of apoptosis appears to be the prominent effect of elevated HDAC2 expression in cell lines derived from colon cancer (HT-29) or melanoma (O. Krämer and T. Heinzel, personal communication), it is not clear whether reduced adenoma formation in *APC*^{min} mice upon HDAC inhibition is also due to apoptosis. Apoptosis rates were below detection limits in wild-type and mutant mice, possibly due to rapid shedding of apoptotic cells from the epithelium (data not shown).

The link between *APC* and *HDAC2*, although depending on additional factors such as c-Myc, provides a reason for the susceptibility of carcinomas of epithelial origin to treatment with HDAC inhibitors. Furthermore, it indicates that HDAC2 might be a particularly suitable target for selective inhibition of HDACs. VPA serves as a model drug. It has been used in therapy of epilepsy for decades and induction of differentiation in cancer cells had been described (Blaheta and Cinatl, 2002). Only recently has it become apparent that VPA preferentially inhibits the catalytic activity of class I HDACs (Göttlicher et al., 2001; Phiel et al., 2001) and, in addition, induces proteasomal degradation of HDAC2 (Krämer et al., 2003). Substantial but incomplete effects of VPA on polyp formation in *APC*^{min} mice may be due to the short half-life of VPA in rodents, which prevents maintenance of efficient serum levels over the day (Göttlicher et al., 2001). VPA treatment is likely to be successful not only during adenoma formation, but also after development of the carcinoma, because HDAC2 expression is elevated in most of the human colonic cancer samples. Furthermore, 4 out of 8 human colonic cancer cell lines showed a strong reduction of cellular biomass upon VPA treatment, while the remaining ones showed a moderate decrease (E.M., unpublished data). Since progression of cancer requires many cellular features, it is likely that combinations of drugs targeting different pathways will be appropriate with HDAC2 inhibitors supporting induction of apoptosis in cancer cells.

Experimental procedures

Cell culture and transfections

Cell culture and transgene induction by 100 μM Zn^{2+} have been described (He et al., 1999; Krämer et al., 2003). Plasmid and siRNA were transfected with Lipofectamine 2000 and Oligofectamine (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Briefly, the *HDAC2* expression vector pCMV-mRPD3-2.1 (Yang et al., 1996) was cotransfected with pCMV-GFP at a ratio of 5:1. Twelve hr after transfection, cells were replated into two samples each before $ZnCl_2$ was added to one of them. Attached cells were harvested 24 to 72 hr later, and the fraction of GFP-positive cells was determined by flow cytometry. pcDNA/Myc-ΔN-hTcf4 (dominant-negative mutant of Tcf-4, Korinek et al., 1997) or empty pcDNA3.1$^{(+)}$ was transiently transfected into HT-29 cells, and parallel cultures were processed 42 hr later for Western and Northern blot analysis. In this experiment, 3×10^5 cells were seeded per well of 6-well plate one day before transfection. Transfections were performed in the absence of FCS and antibiotics for 4 hr, using 3 μg DNA and 8 μl Lipofectamine 2000 per well. Then FCS was added to 10% and cells were cultured until analysis. Transfection efficiencies were monitored by parallel transfection of the GFP expression vector pCMV-GFP. HT-29 cells were transfected in daily intervals with appropriate siRNAs for 48 hr (*c-Myc*) or indicated time periods. siRNAs were 5′-AAGCCUCAUA GAAUCCGCAUG-3′ (*HDAC2*), 5′-AACGUUAGCUUCACCAACAGG-3′ (*c-Myc*) (van de Wetering et al., 2002), and 5′-AACGUACGCGGAAUACUUCGA-3′ (*Luc*) (Elbashir et al., 2001).

Western and Northern blot

Antibodies for Western blot analysis of whole cell extracts were (Santa Cruz Biotechnology, California): HDAC1, sc-6298; HDAC2, sc-9959; HDAC3, sc-8138; HDAC7, sc-11489; β-catenin, sc-1496; actin, sc-1616; c-Myc, sc-40. Actin was determined in all Western blots to ensure equal loading of lanes. Probes for Northern hybridizations (5 μg poly[A]$^+$ RNA per lane) corresponded to nucleotides 1336–1647 of *mHDAC2* coding region and complete coding sequences of *mPPARδ* and *rGAPDH*. Films were quantitatively evaluated using the Aida software (Berthold, Germany).

RT-PCR

First-strand cDNA was generated using the RNeasy and Oligotex mRNA mini kits (Qiagen, Hilden, Germany) and Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Five μl of first-strand cDNA preparations were used in PCR reactions with 30, 32, and 34 (*HDAC2*) or 25 and 27 cycles (*HPRT* and *Actin*) to ensure that amplification was in the linear range. Primers were: *HDAC2*, 5′-GCTATTCCAGAAGATGCTGTTC-3′ and 5′-GTTGCTGAGCTGTTCTGATTTG-3′; *HPRT*, 5′-CCAAAGATGGTCAAG GTC-3′ and 5′-CTGCTGACAAAGATTCACTGG-3′; *Actin*, 5′-TCACCCACAC TGTGCCCAT-3′ and 5′-CTCTTGCTCGAAGTCCAGGG-3′.

Chromatin immunoprecipitation

HT29-APC and HT29-βGAL cells were treated with 100 μM $ZnCl_2$ for 18 hr as indicated. Cells were fixed in 1% formaldehyde and harvested for preparing nuclei in lysis buffer (50 mM Hepes [pH 8], 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 10% glycerol, 0.5% NP40, 0.2% Triton X-100, protease inhibitors cocktail [Roche, Mannheim, Germany]). Washed nuclei were lysed in RIPA buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, protease inhibitors cocktail). Chromatin was broken into 500–800 bp DNA fragments by sonication. Following preclear by incubating 1 ml lysates with 25 μl of protein A sepharose CL-4B and 5 μg sheared salmon sperm DNA for 2 hr, immunoprecipitation was performed with 5 μg of a specific anti-c-Myc anti-

body (sc-764, Santa Cruz Biotechnology, California) overnight at 4°C. Protein A Sepharose (25 μl) and 10 μg/ml salmon sperm DNA were added for another 1 hr. Precipitates were washed sequentially in RIPA buffer, RIPA buffer with 10 μg/ml salmon sperm DNA, RIPA buffer with 10 μg/ml salmon sperm DNA and 500 mM NaCl, and RIPA. Precipitate-associated DNA was eluted with 50 mM Tris-HCl (pH 8), 1 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 μg/ml proteinase K for 3 hr at 55°C and then overnight at 65°C, and purified with a QIAquick Spin Kit (Qiagen, Hilden, Germany). DNA was used for PCR detection with the following primers: c-Myc biding site flanking primers: forward (−731) CGA CAA ACG AGA AAA CTA GGC; reverse (−360) TTA GGG TGA ACA ACC TGC GCA; control primers for a nonrelated region of the HDAC2 promoter: forward (−1788) CGT ACA TGG CAT GGA GAA CAG; reverse (−1635) CTT GGA AGA TTC TGA GAA CAG. Numbers indicate upstream positions relative to the start codon of HDAC2.

Cell cycle and apoptosis assays

Adherent and floating cells were collected for fixation (4% formaldehyde), permeabilization (0.5% Nonidet P-40), staining with Hoechst H33258, and analysis of cell cycle profiles by flow cytometry. Apoptosis was analyzed by scoring 200 Hoechst stained cells per sample for the presence of condensed or fragmented nuclei. In addition, the Cell Death Detection ELISA^{PLUS} Kit (Roche, Mannheim, Germany) was used to quantitatively determine cytoplasmic histone-associated DNA fragments.

Animal experiments

Ten- to sixteen-week-old age- and sex-matched heterozygous or wild-type C57BL/6J-*APC*^{min} mice (Jackson Laboratories, Bar Harbor, Maine) were injected (i.p.) with PBS or VPA (2 × 400 mg/kg/day) as isotonic aequous solution of the sodium salt for four weeks. Upon necropsy, entire intestinal tracts were opened longitudinally and fixed in 10% phosphate buffered formaldehyde for 24 hr. Polyp contrast was improved by 1 min staining in 0.1% methylene blue prior to determination of polyp numbers and sizes under a dissecting microscope by two independent observers unaware of the treatment that the mice had received.

Immunohistochemistry

Paraffin sections or human colorectal cancer tissue arrays (46 intact samples of mostly moderately differentiated T3 and T4 carcinomas on CD1 and CDN1 arrays, BioCAT, Heidelberg, Germany, www.biocat.de) or classical sections (11 tumor samples of all TNM-T1 to T4 stages from the Robert-Rössle-Klinik, Berlin, Germany) were stained with primary antibodies against HDAC2 (#51-5100, 1:30 dilution), β-catenin (#13-8400, 1:100) from Zymed (San Francisco, California), acetylated histone H3 (06-599, 1:50, Upstate, Lake Placid, New York) or HDAC3 (sc-8138, 1:50, Santa Cruz Biotechnologies) (Krämer et al., 2003) and Cy3- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania). Almost no fluorescence and no specific patterns were observed in control slides prepared without primary antibodies. Staining was scored in a 4-step scale independently for HDAC2 and β-catenin with fixed instrument settings. Slides were evaluated independently by three investigators without knowledge of sample identity. Score one was designated as no detectable staining above background with no difference between nuclear and cytosolic signal. Score two corresponded to weak staining with a clear difference between nuclear (prominent for HDAC2) and cytosolic (prominent for β-catenin) signals. Score three was strong staining, and score 4 was considered as very strong staining that reached saturation in the chosen sensitivity setting for screen presentation. Data sets were combined after completion of scoring. Horseradish peroxidase staining was performed using biotin-conjugated secondary antibodies (Dako, Glostrup, Denmark) together with the VECTASTAIN ABC and NovaRED kits (Vector laboratories, Burlingame, California).

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