Histone deacetylase inhibitors induce attenuation of Wnt signaling and TCF7L2 depletion in colorectal carcinoma cells

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Abstract. Histone deacetylase inhibitors (HDIs) specifically affect cancer cells by inducing cell cycle arrest, activate apoptotic pathways and re-activate epigenetically silenced tumor suppressor genes, but their pleiotropic mode of action is not fully understood. Despite the clinical effects of HDIs in the treatment of hematological malignancies, their potency against solid tumors is still unclear. We investigated the effects and mechanisms of HDI action in colorectal carcinoma cell lines with an activated Wnt signaling pathway, which is implicated in different aspects of tumorigenesis, including cell proliferation, apoptosis, angiogenesis and metastasis. We assessed the effects of HDI treatment in colorectal carcinoma cell lines by measuring histone hyperacetylation, cell viability and expression of Wnt target genes. Upon treatment with HDIs of the hydroxamate class, we found attenuation of Wnt signaling with concomitant induction of apoptosis and colorectal cancer cell death. Strikingly, the effects of HDIs on Wnt signaling were independent of histone hyperacetylation, thus we investigated the role of non-histone target proteins of histone deacetylases (HDACs). The compounds TSA and SAHA induced a rapid proteasome-dependent depletion of the Wnt transcription factor TCF7L2, which may be mediated by inhibition of HDAC 6 and 10. Our findings provide a molecular rationale for the use of HDIs against colorectal carcinomas with activated Wnt signaling.

Introduction

Histone deacetylase inhibitors (HDIs) unfold specific effects against cancer cells. Their pleiotropic mode of action is not fully understood but includes induction of cell cycle arrest, direct activation of apoptotic pathways and reactivation of epigenetically silenced tumor suppressor genes (1,2). However, many genes are downregulated by HDI treatment (3). Often, this downregulation is mediated via many non-histone target proteins of histone deacetylases (HDACs) (4) such as TP53 or RUNX3 (5,6).

Despite the high clinical potential of HDIs against hematological malignancies, their effectiveness against solid tumors has not been proven yet (7,8). Different HDIs were shown to induce *CDKN1A* (encoding the cell cycle kinase inhibitor p21) expression and cell death in colorectal carcinoma cell lines (9,10). In a mouse model of colorectal cancer, inhibition of HDAC2 led to a significant reduction in adenoma formation (11). Yet, clinically proven benefits of HDI treatment are observed in individual cases only (7). However, solid cancer types in clinical studies are diverse and so are the genetic aberrations in these tumors. Hence, we sought to analyze HDI's properties in one specific type of cancer cells, i.e., colorectal carcinoma cell lines characterized by constitutively active Wnt signaling.

Wnt signaling controls many cellular processes during neoplastic transformation (12) and thus represents an important therapeutic target. In a Wnt activated cell, the protein β-catenin, the central player of the pathway, accumulates in the cytoplasm and enters the nucleus. Nuclear β-catenin associates with transcription factors of the TCF/LEF family and recruits transcriptional co-activators and chromatin remodeling complexes which in concert drive the expression of target genes (13). Wnt target genes include *CCND1*, *MMP7*, *VEGF*, *MYC*, *survivin* and also genes involved in feed-back regulation such as *TCF7L2* or *LEF1*. In many epithelial cancers, the Wnt pathway is constitutively active due to mutations in different components of the pathway as for instance almost every colorectal carcinoma harbors either an *APC* or a *β-catenin* mutation (14).

The mode of action of HDIs in respect to Wnt signaling is not well defined. HDIs were shown to downregulate the expression of single endogenous Wnt target genes, e.g., *CCND1*, *VEGF* and *survivin* (15,16). In other studies activation of Wnt signaling due to HDI treatment could be demonstrated (17). We assessed the effects of HDI treatment in colorectal carcinoma cell lines to further elucidate the mode of HDI action.

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Materials and methods

Cell culture and drug treatment. Colorectal carcinoma cell lines SW480 and HCT116 were obtained from the American Type Culture Collection (LGC Promochem, Wesel, Germany). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal bovine serum (FBS), sodium pyruvate, L-glutamine and non-essential amino acids (PAN Biotech, Aidenbach, Germany) at 37˚C in a 7.5% CO_2 humidified chamber. Cells were treated with 1 μM trichostatin A (TSA; Sigma, Munich, Germany) or 5 μM suberoylanilide hydroxamic acid (SAHA; Cayman Chemicals, Ann Arbor, MI, USA) dissolved in ethanol and 1 mM valproic acid (VPA; Sigma) dissolved in PBS. Vehicle treatment with either ethanol or PBS served as internal control. The proteasome inhibitors MG132 and Epoxomicin (both from Calbiochem, Darmstadt, Germany) were dissolved in DMSO.

Cellular assays. Cell growth and viability were evaluated by using bromodeoxyuridine incorporation (BrdU) and methyl thiazol tetrazolium bromide reduction (MTT) assays, respectively. Cells were plated in triplicates in 96-well plates. After treatment with HDIs and control substances for 24, 30 and 48 h or 24 and 48 h after siRNA treatment, MTT or BrdU assay (BrdU cell proliferation ELISA; Roche, Mannheim, Germany) was carried out. The mean absorbance values of HDI-treated cells were normalized to the mean absorbance values of vehicle-treated cells. To detect apoptotic cells, the Apo-One homogeneous Caspase-3/7 assay (Promega, Madison, WI, USA) was carried out according to the manufacturer's instructions.

For cell cycle analysis, cells were trypsinized after 24 h HDI treatment or 48 h after siRNA transfection. The cell cycle was measured via propidium iodide incorporation on a LSRII flow cytometer and analyzed with FACS DiVa software (Becton Dickinson, Franklin Lakes, NJ, USA). Data evaluation was performed with FlowJo software (TreeStar Inc., Ashland, OR, USA).

HDAC knockdown - siRNA transfection. HDAC knockdown was performed with siGENOME SMARTpool siRNA from Dharmacon for the genes HDAC6 (M-003499-00), HDAC10 (M-004072-00) and HDAC11 (M-004258-00). Transfection was carried out with DharmaFECT1 (Dharmacon, Lafayette CO, USA) at a ratio of 1:40 in HCT116 and 1:50 in SW480 cells according to the manufacturer's instructions. Experiments were performed 24 to 72 h after siRNA transfection. Specific target knockdown was measured on mRNA level by quantitative real-time PCR after 24 or 48 h.

Reverse transcription real-time PCR. RNA isolation was carried out with the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany), cDNA synthesis was performed with RevertAID H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany). The ABsolute QPCR SYBR-Green ROX Mix (Abgene, Epsom, UK) was used for quantitative real-time PCRs on a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA, USA). Primer sequences are stated in Table I. Real-time PCR data was analyzed using the ΔΔCt method whereby the relative expression change of a treated sample over a control sample is: fold change = $2^{-\Delta\Delta Ct}$.

Table I. Primer sequences.

Primer name	Sequence 5'→3'
LEF1	
Forward	CTTGGTGAACGAGTCTGAAATC
Reverse	GTGTTCTCTGGCCTTGTCGTG
TCF7L2	
Forward	GGAGCCTCCAGAGTAGACAAG
Reverse	CCACTGGCACTTTGTTAGAGAC
Cyclin D1	
Forward	GCCTGAACCTGAGGAGCCCCA
Reverse	GTCACACTTGATCACTCTGG
c-Myc	
Forward	TCCTGAGACAGATCAGCAACAACCG
Reverse	TCCTCTGGCGCTCCAAGACGTT
VEGF	
Forward	CATGACAGCGCCCCTTCCTGG
Reverse	TGTGAGGACATAGGTCCTTTTAGGCTG
MMP7	
Forward	TGTTGCAGAATACTCACTATTTCC
Reverse	GATCCACTGTAATATGCGGTAAG
GAPDH	
Forward	GCTCTCCAGAACATCATCCCTGCC
Reverse	AGGCCATGCCAGTGAGCTTCC

Western blot analysis. Total cell lysates were made from HCT116 or SW480 cells after treatment with HDIs for 3, 6 and 16 or 20 h. After SDS gel electrophoresis, western blot analysis was used to determine the relative protein levels of TCF7L2 and β-catenin in total cell lysates. GAPDH was used as an internal control. The antibodies were as follows: TCF7L2 (Cell Signaling Technology, Danvers, MA, USA) 1:2,500 in 5% BSA/TBST; β-catenin (BD Biosciences, Franklin Lakes, NJ, USA) 1:10,000 in 0.5% SlimFast/TBST; GAPDH (Abcam) 1:30,000 in 5% milk/TBST. Band intensities were determined using AIDA software (Raytest, Straubenhardt, Germany). The band intensity (BI) of the analyzed protein was referred to the BI of GAPDH to give the normalized BI. The normalized BI of control treated cells was set to 100%.

Immunoprecipitation. For cytosolic and nuclear fractionation of cells, HCT116 cells were grown in 10-cm dishes and HDI treated for 16 h. Cells were washed with ice-cold PBS, scraped into a microcentrifuge tube and centrifuged for 5 min at 1,600 rpm. The cell pellet was resuspended in 100 μl membrane lysis buffer [10 mM HEPES, pH 8.0; 10 mM KCl; 1.5 mM $MgCl_2$; 1 mM DTT; 1X Complete Protease Inhibitor Cocktail (Roche)] and incubated for 15 min on ice. NP-40 was added to a final concentration of 1%. After vortexing for 10 sec, the sample was centrifuged for 3 min at 13,000 rpm. The supernatant is the cytoplasmic fraction. The pellet containing the nuclei was washed 3 times with 500 μl membrane lysis buffer and centrifuged for 3 min. Then the pellet was resuspended in 100 μl ice cold nuclear enve-

lope lysis buffer [20 mM HEPES, pH 8.0; 1.5 mM $MgCl_2$; 25% glycerol; 0.42 M NaCl; 0.2 mM EDTA; 1 mM DTT; 1X Complete Protease Inhibitor Cocktail (Roche)] and vortexed for 30 sec. After rotation at 4˚C for 30 min, samples were centrifuged for 15 min at 13,000 rpm. The protein concentration of the supernatant was determined by Bradford assay. Concentrations of HDI- and vehicle-treated samples were adjusted to 3 μg/μl. Lysates were used immediately or stored in aliquots at -80˚C until use.

For immunoprecipitation, protein G coupled magnetic beads (Dynabeads, Invitrogen) were used according to the manufacturer's instructions. In brief, 50 μl of beads were loaded with 5 μg of antibody. After washing, the loaded beads were incubated with cellular lysates for 10 to 20 min. After several washing steps, the beads were resuspended in 5X SDS loading buffer and heated to 70˚C for 10 min.

To test the acetylation status of β-catenin, immunoprecipitations were carried out with an anti-β-catenin antibody (BD Biosciences) followed by western blot analysis with an anti-β-catenin antibody or an anti-acetylated β-catenin antibody (Cell Signaling Technology).

Statistical analysis. Statistical calculations were carried out with the GraphPad QuickCalcs online calculator (www.graphpad.com/quickcalcs) and STATISTICA 8 software (StatSoft, Hamburg, Germany) using different t-tests as indicated in the text. A p-value of <0.05 was considered to indicate statistical significance. Significant results are indicated by $^{*}p<0.05$; $^{**}p<0.01$ or $^{***}p<0.001$ in the figures.

Results

The HDIs SAHA and TSA attenuate proliferation and induce apoptosis in Wnt-activated colorectal carcinoma cell lines. We analyzed the effects of the HDIs SAHA, TSA and VPA on the growth of the colorectal carcinoma cell lines HCT116 and SW480. Both cell lines possess an activated Wnt signaling pathway due to mutations in *β-catenin* or *APC*, respectively. SAHA and TSA belong to the chemical class of hydroxamic acids; whereas VPA is a short-chain fatty acid. Treatment with hydroxamic acids led to a strong reduction in the number of viable cells, while VPA had no, or only little effect on viability even after 48 h as measured by MTT assay (Fig. 1A). Furthermore, a BrdU assay revealed a pronounced decrease in the cell proliferation rate of SAHA- or TSA-treated cells as opposed to VPA-treated cells (Fig. 1B). To gain further insights into the underlying mechanisms we carried out an apoptosis detection assay. After treatment with hydroxamic acids for 24 and 30 h, the number of apoptotic cells strongly increased (Fig. 1C). After 48 h, the relative apoptotic rate dropped due to the low number of living cells. In contrast, VPA treatment induced no significant changes in the apoptosis rate. These findings were further underlined by flow cytometric cell cycle analysis revealing an increase in the apoptotic cell fraction and an induction of a G2/M-phase cell cycle arrest in response to TSA and SAHA (Fig. 2). In contrast, treatment with VPA led to little, or no changes in the cell cycle.

The HDIs SAHA and TSA repress Wnt target genes in colorectal carcinoma cells. In order to explore how HDIs influence Wnt regulated genes we measured the expression of endogenous Wnt target genes by quantitative real-time PCR after treatment with HDIs (Fig. 3). Analyzed Wnt target genes included *MYC*, *CCDN1* (encoding cyclin D1), *VEGF*, *MMP7*, *survivin* and the two transcription factors *LEF1* and *TCF7L2* which are integral parts of the pathway. We observed downregulation of all analyzed genes upon 20 h treatment with hydroxamic acids whereas VPA treatment left the expression of most genes unchanged (Fig. 4). In HCT116 cells, the expression of *CCDN1*, *MYC*, *VEGF*, *MMP7*, *survivin* and *TCF7L2* was significantly downregulated after 16 to 20 h exposure to SAHA or TSA, but not to VPA. In SW480 cells, the expression levels of *CCDN1*, *MYC*, *survivin* and *LEF1* were significantly downregulated by SAHA or TSA, but not by VPA treatment. Only MYC expression, which is known to be regulated by a variety of mechanisms (18), was significantly downregulated by VPA treatment in HCT116.

β-catenin acetylation status remains unchanged in response to TSA treatment. Based on recent findings that the β-catenin protein can be acetylated, we questioned if a modification of this central player of the Wnt pathway may be involved in the transcriptional downregulation of target genes in response to HDI treatment. Immunoprecipitation experiments with anti-acetylated β-catenin antibody and nuclear lysates from HCT116 cells after 16 h TSA treatment revealed no difference in the amount of precipitated acetylated β-catenin between TSA- and vehicle-treated cells (Fig. 5). Similarly, there was no change in the total amount of β-catenin protein in total cell lysates.

The HDIs SAHA and TSA induce TCF7L2 depletion. We investigated if TCF7L2 might be a non-histone target of HDACs by analyzing the relative TCF7L2 protein level in total HCT116 cell lysates after HDI treatment. We found a strong depletion of TCF7L2 protein after 20 h SAHA and TSA, but not after VPA treatment ($p<0.001$) (Fig. 6). This TCF7L2 depletion could not be attributed to transcriptional downregulation alone, as, according to the N-end rule (19), the estimated half-life of the TCF7L2 protein is 30 h. Hence, TCF7L2 depletion might be partly caused by proteasomal degradation. In this case, co-treatment with proteasome inhibitors should attenuate the effect of the HDI treatment. We treated HCT116 cells with TSA for 16 h and added one of the proteasome inhibitors Epoxomicin or MG132 4 or 6 h before the end of the treatment, respectively. As control we treated the cells with the proteasome inhibitors alone. Co-treatment with HDIs and proteasome inhibitors indeed attenuated the downregulation of TCF7L2 protein level (Fig. 6C and D). Treatment with proteasome inhibitors alone did not alter the TCF7L2 level substantially (Fig. 6E), which showed that TCF7L2 was not subjected to constant proteasomal degradation under normal conditions. Thus we demonstrated that HDIs of the hydroxamate class induce a strong proteasome-dependent depletion of TCF7L2.

Knockdown of single HDACs cannot mimic the HDI-induced attenuation of cell proliferation. The previous experiments showed that the hydroxamic acids SAHA and TSA, which inhibit all known HDACs, induced apoptotic cell death

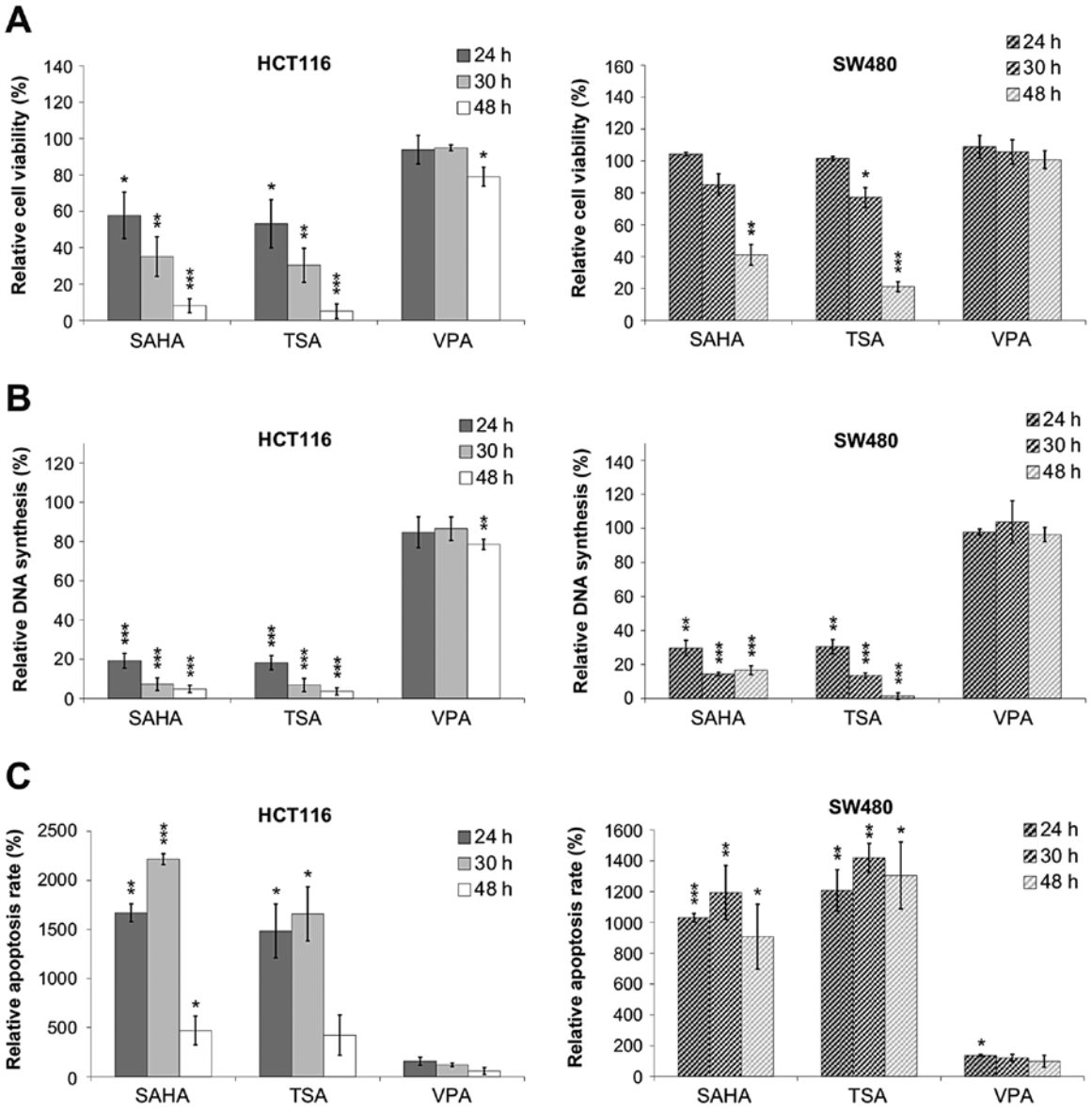

Figure 1. Effects of HDI treatment on colorectal carcinoma cell growth and apoptosis. Cells were treated with 5 μM SAHA, 1 μM TSA or 1 mM VPA for the indicated time spans. The mean values of HDI-treated cells were normalized to the mean values of vehicle-treated cells, which were defined as 100%. Results are means of at least three independent experiments of (A) MTT, (B) BrdU and (C) apoptosis detection assay. Significant results are indicated by *p<0.05; **p<0.01 or ***p<0.001.

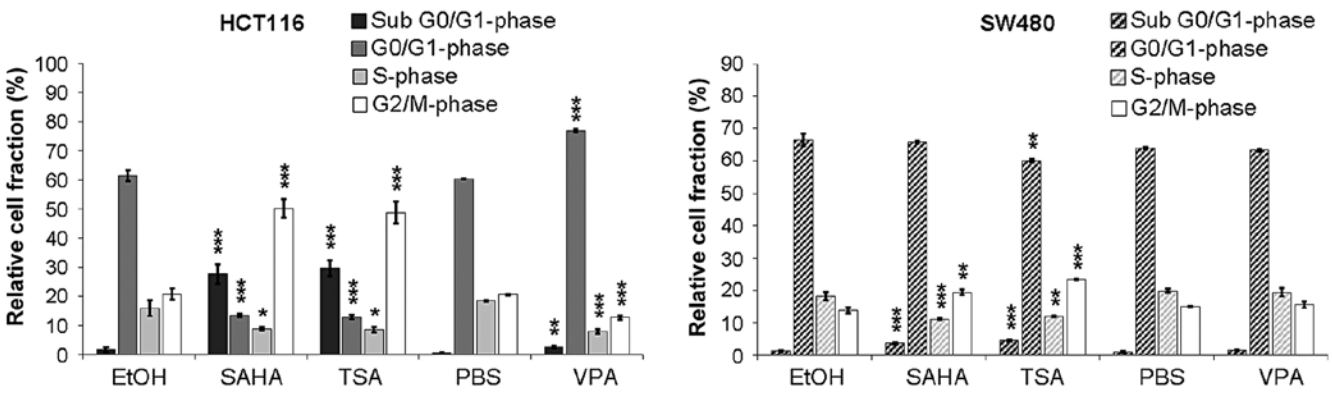

Figure 2. Effect of HDI treatment on the cell cycle. Cell cycle analysis was performed on a BD LSRII flow cytometer after propidium iodide incorporation. HCT116 and SW480 cells were treated with 5 μM SAHA, 1 μM TSA, 1 mM VPA or the buffer substances EtOH or PBS for 24 h before measurement. The diagram shows the relative fraction of cells in the different cell cycle phases. Results are means of three independent experiments. Interestingly SAHA and TSA treatment led to an increase in the G2/M-phase, while VPA induced a cell cycle arrest in G0/G1-phase in HCT116 cells only. Significant results are indicated by *p<0.05; **p<0.01 or ***p<0.001.

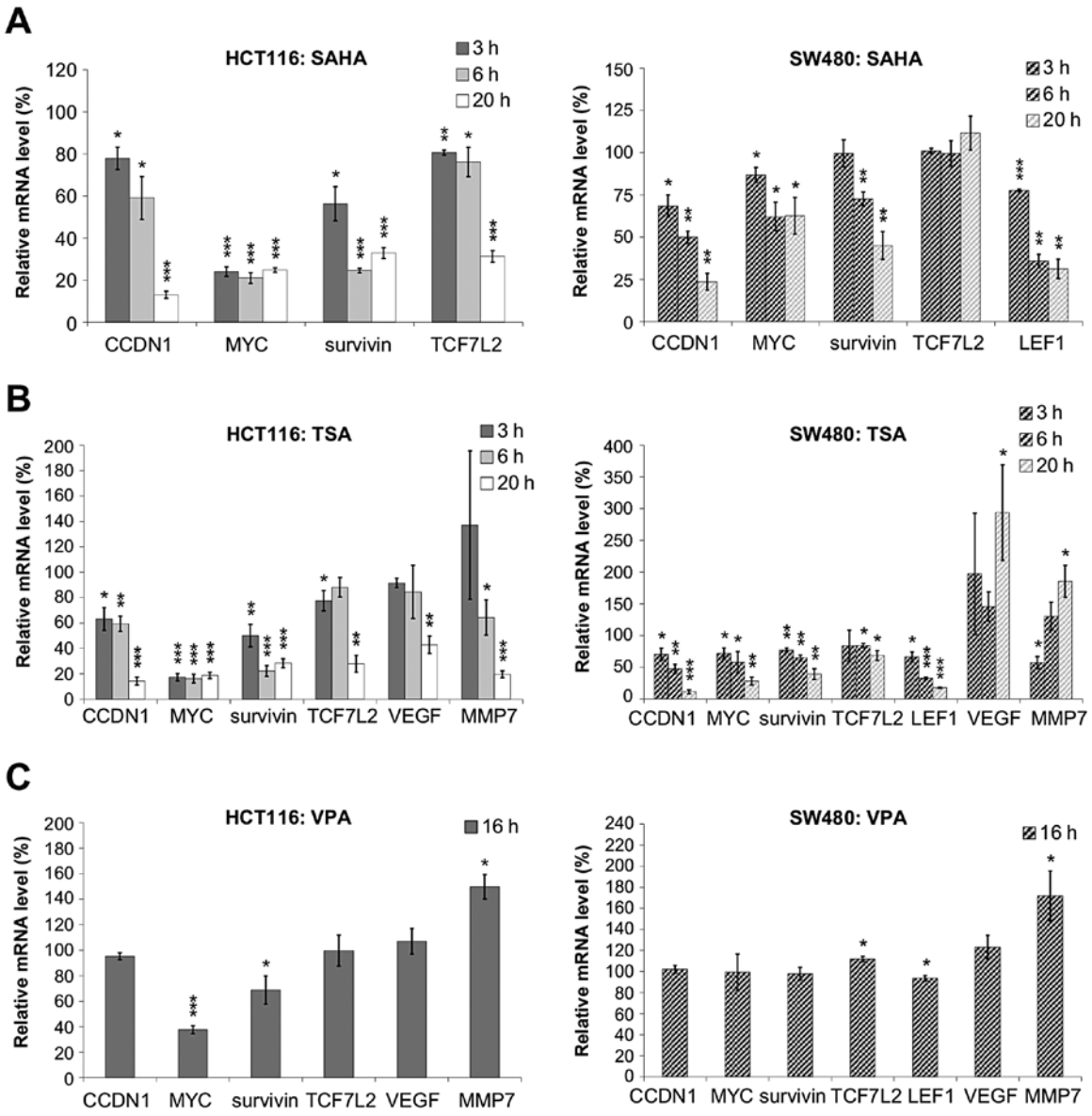

Figure 3. Effect of HDI treatment on Wnt target gene expression at different time points. Wnt target gene expression was analyzed by quantitative real-time PCR using the ΔΔCt method. Cells were treated with (A) 5 μM SAHA, (B) 1 μM TSA or (C) 1 mM VPA for 3, 6 or 16-20 h. Results are means of three independent experiments. The diagrams show the relative expression of the genes *CCND1*, *MYC*, *survivin*, *TCF7L2*, *LEF1*, *VEGF* and *MMP7* in the cell lines HCT116 and SW480 in comparison to vehicle-treated cells (100% relative expression). Wnt target gene expression of VPA treated cells was determined after 16 h. The *VEGF* and *MMP7* expression was analyzed after TSA and VPA treatment. Significant results are indicated by $^{*}p<0.05$; $^{**}p<0.01$ or $^{***}p<0.001$.

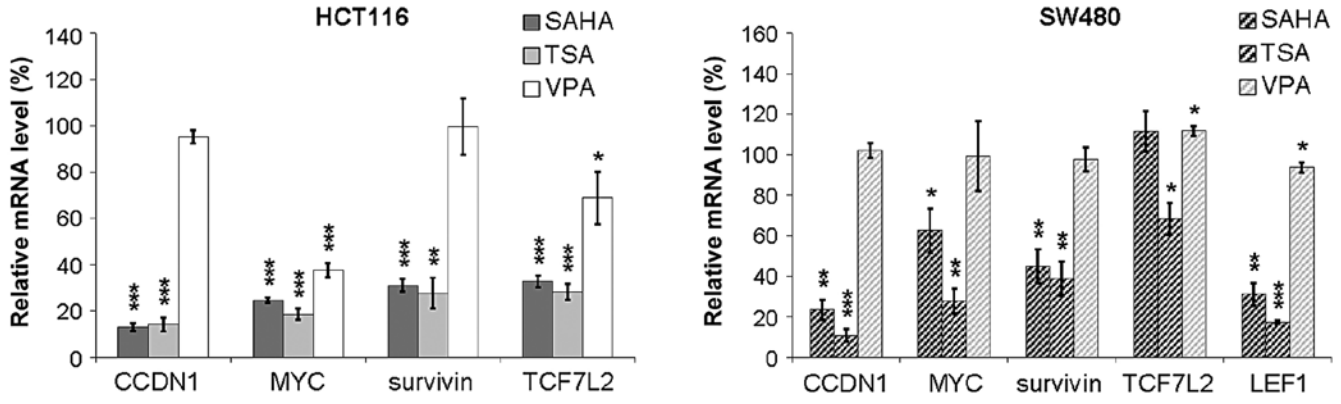

Figure 4. Effect of HDI treatment on Wnt target gene expression. Wnt target gene expression was analyzed by quantitative real-time PCR after treatment with 5 μM SAHA, 1 μM TSA or 1 mM VPA for 16 to 20 h. Results are means of three independent experiments. The relative expression of the genes *CCND1*, *MYC*, *survivin*, *TCF7L2* and *LEF1* are shown for the cell lines HCT116 and SW480. Significant results are indicated by $^{*}p<0.05$; $^{**}p<0.01$ or $^{***}p<0.001$.

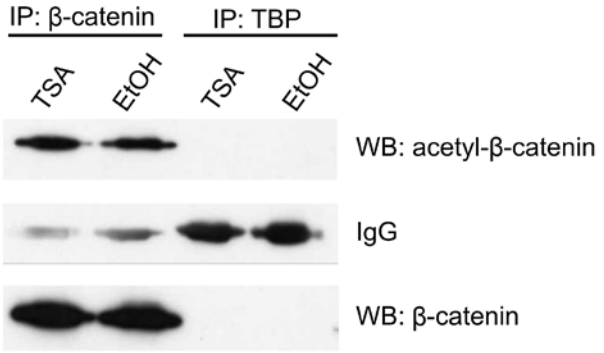

Figure 5. TSA treatment does not alter the acetylation of nuclear β-catenin in HCT116 cells. β-catenin was immunoprecipitated from nuclear lysates of HCT116 cells after 16 h treatment with TSA or EtOH. An anti-TBP antibody was used as unrelated internal control (TBP, tata-binding protein). The precipitate was probed with an anti-acetylated-β-catenin antibody and an anti-β-catenin antibody. IgG bands indicated equal loading of the different IP lysates.

of colon carcinoma cells and attenuation of Wnt signaling. In contrast, treatment with VPA, which inhibits only some HDAC classes, led to little, or no change, in the colon carcinoma cell lines. We reasoned that the different effects of the HDIs might be caused by the differences in their HDAC inhibition profiles. Therefore, we carried out siRNA experiments to analyze the effects of the knockdown of HDACs 6, 10 and 11, which are inhibited by hydroxamic acids but not by VPA, on Wnt signaling and colon cancer cell proliferation.

We controlled for a proper gene knockdown by measuring the mRNA expression level of the three HDACs 24 and 48 h after siRNA transfection. All three HDAC siRNAs reduced the respective HDAC expression to at least 40% after 48 h treatment. By using MTT, BrdU, apoptosis assay and flow cytometric analysis, we found that none of the analyzed HDAC siRNAs induced any significant changes in proliferation or apoptosis of HCT116 and SW480 cells. This indicated that the knockdown of a single HDAC is not sufficient to induce major changes in cell proliferation of the analyzed colon carcinoma cell lines.

Knockdown of HDAC 6, 10 and 11 affects Wnt target gene expression. Next we examined whether siRNA mediated HDAC knockdown had an effect on Wnt target gene expression. Therefore, we performed quantitative real-time PCR of

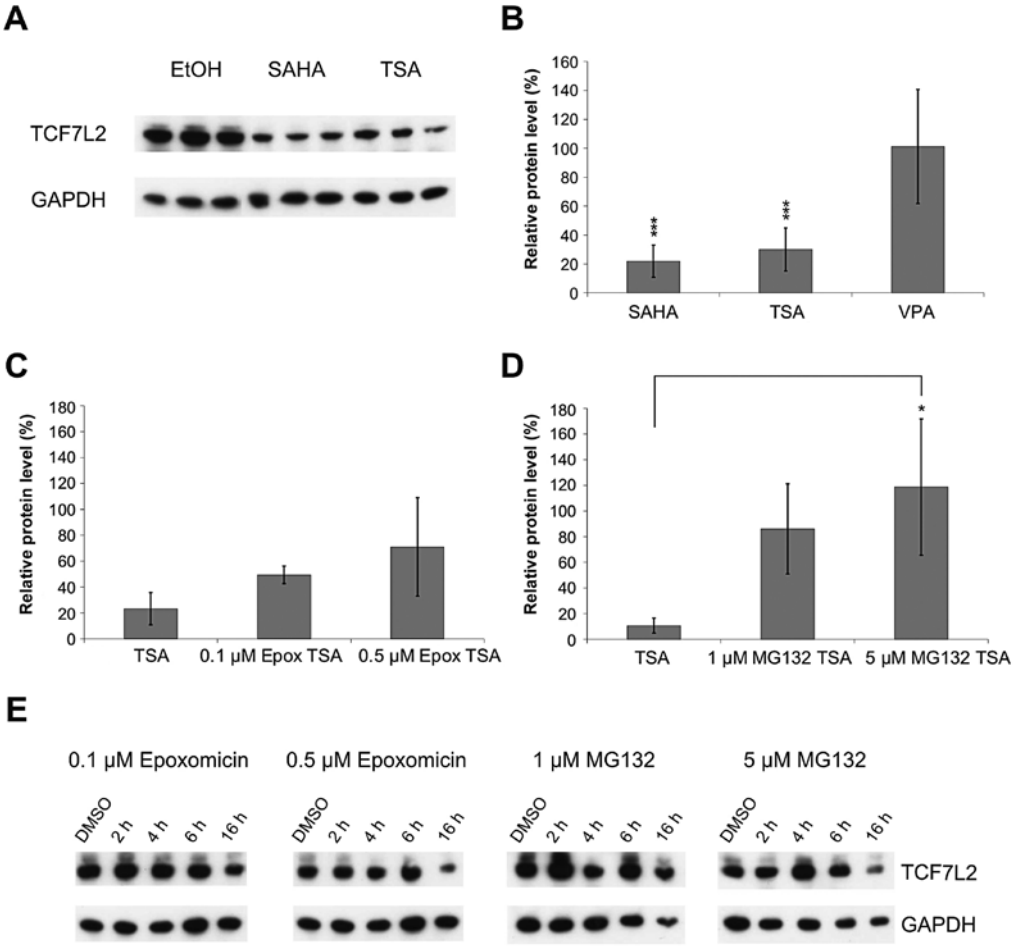

Figure 6. Analysis of TCF7L2 protein level revealing proteasome-dependent degradation after SAHA or TSA treatment. Diagrams show the densitometric analysis of western blots probed with TCF7L2 and GAPDH antibodies prepared from total lysates of HDI- and vehicle-treated HCT116 cells. (A) Representative images of the TCF7L2 and GAPDH western blot after 20 h SAHA and TSA treatment. (B) Densitometric analysis of TCF7L2 after 20 h treatment with 5 μM SAHA and 1 μM TSA in HCT116 cells. (C) Densitometric analysis of TCF7L2 protein after 16 h TSA and 6 h epoxomicin or (D) after 16 h TSA and 4 h MG132 treatment in HCT116. (E) Western blot analysis of TCF7L2 and GAPDH protein after treatment of HCT116 with different concentrations of the proteasome inhibitors, epoxomicin and MG132 alone shows no substantial regulation after 2 to 6 h treatment. Significant results are indicated by $^{*}p<0.05$; $^{**}p<0.01$ or $^{***}p<0.001$.

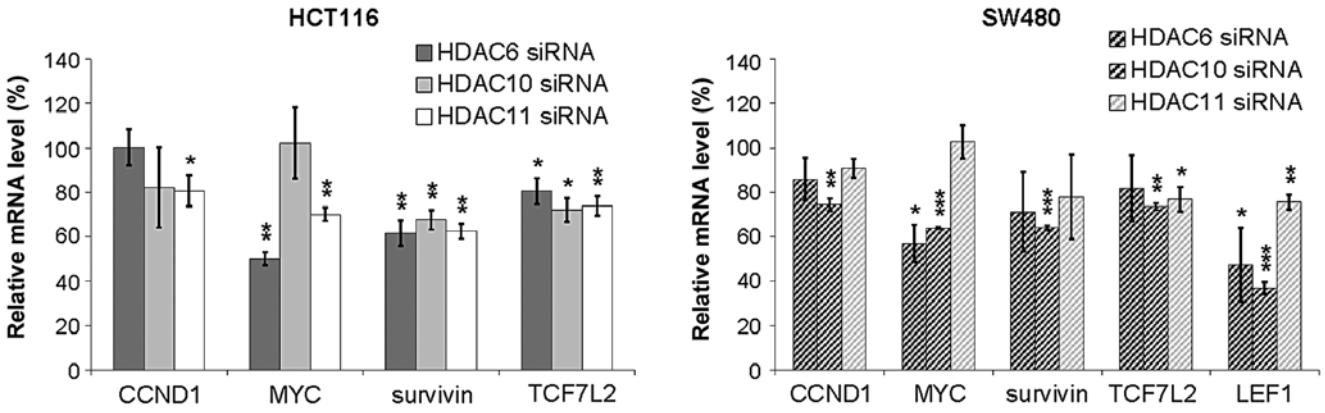

Figure 7. Effect of *HDAC* knockdown on Wnt target gene expression. Wnt target gene expression was determined 48 h after *HDAC* siRNA transfection in HCT116 and SW480 cells. Results are means of three independent experiments. The relative expression levels of the genes *CCND1*, *MYC*, *survivin*, *TCF7L2* and *LEF1* are shown in comparison to the levels in cells transfected with non-targeting siRNA (100% relative expression). Significant results are indicated by $^{*}p<0.05$; $^{**}p<0.01$ or $^{***}p<0.001$.

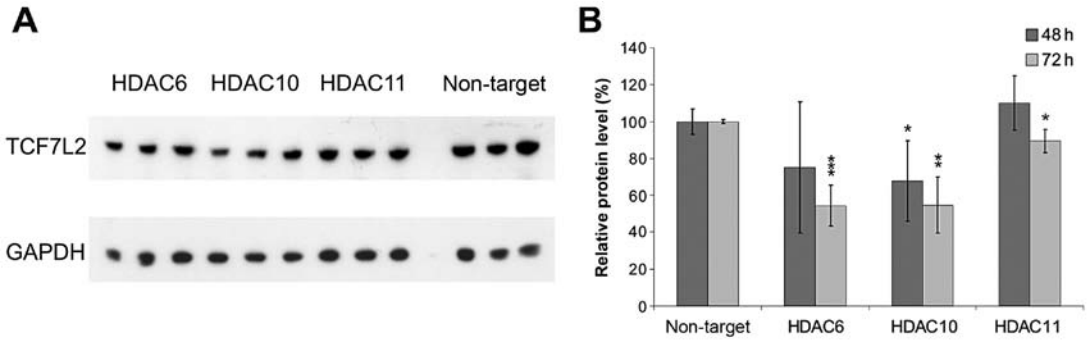

Figure 8. Analysis of TCF7L2 protein level upon *HDAC* knockdown. (A) Representative images of the TCF7L2 and GAPDH western blot analysis 72 h after siRNA transfection. (B) Densitometric analysis of the TCF7L2 western blots prepared from total lysates of *HDAC* and non-targeting siRNA transfected HCT116 cells. The TCF7L2 protein was significantly reduced 72 h after HDAC knockdown in HCT116. Significant results are indicated by $^{*}p<0.05$; $^{**}p<0.01$ or $^{***}p<0.001$.

the endogenous Wnt target genes *CCDN1*, *MYC*, *survivin*, *LEF1* and *TCF7L2* 48 h after HDAC siRNA transfection.

In HCT116 cells, HDAC 6 or 10 knockdown reduced the expression of several Wnt target genes, whereas HDAC11 knockdown had an effect on all analyzed genes (Fig. 7). In SW480 cells, the transfection with HDAC10 siRNA led to a significant decrease in all analyzed Wnt target genes, while the siRNAs for HDAC 6 or 11 reduced only the expression of two of the five analyzed genes (MYC and LEF1 or LEF1 and TCF7L2, respectively) (Fig. 7).

Western blot analysis was performed to determine the effects of HDAC knockdown on the TCF7L2 protein level. The knockdown of HDACs 6 or 10 led to a reduction of the TCF7L2 protein level to approximately 50% of the initial protein level after 72 h ($p<0.001$ and $p<0.01$) (Fig. 8); while HDAC11 knockdown only slightly decreased the TCF7L2 protein level. Thus, HDACs 6 and 10 may be involved in the SAHA and TSA induced proteasomal degradation of TCF7L2 leading to the reduced expression of Wnt target genes.

Discussion

Through the regulation of a vast number of different target genes, active Wnt signaling is implicated in different aspects of tumorigenesis, including cell proliferation, apoptosis, angiogenesis and metastasis.

In this study, we observed the rapid induction of apoptotic colorectal carcinoma cell death and transcriptional down-regulation of Wnt target genes upon treatment with the hydroxamic acid HDIs SAHA and TSA. While SAHA and TSA rapidly induced apoptosis and cell death in colorectal carcinoma cells, we noted only slight effects upon VPA treatment. Likewise, *APC* mutant cells were relatively insensitive to VPA-induced apoptosis (20). However, VPA has been shown to be a potent inducer of apoptosis in various cancer models, including colorectal carcinoma cell lines (21), when cell viability was determined after 72-h treatment. In contrast, we found that after 24 and 48 h of treatment HCT116 and SW480 cells were much more sensitive to hydroxamic acid HDIs than to the fatty acid VPA.

The transcriptional regulation of Wnt target genes also displayed a differential mode of regulation upon HDI treatment. While TSA and SAHA downregulated Wnt target genes, VPA had no effects on the expression of most genes. In several different cellular contexts, the downregulation of single Wnt targets such as *CCND1* or *survivin* upon HDI treatment has been described (22,23). *MMP7* expression was reduced by TSA and butyrate in chondrosarcoma cells (24). In addition, we found the two Wnt transcription factors *TCF7L2* and *LEF1*

to be downregulated by hydroxamic acids. However, as all HDIs induced histone hyperacetylation, the impact of HDIs on the Wnt pathway must be explained by a mechanism independent of histone hyperacetylation.

Recently, a growing number of non-histone targets of HDACs has been described, one of them being p53. Acetylation of p53 increases its binding affinity to target promoters and also its transactivation during DNA damage response (5). β-catenin also was shown to be a target of acetylation (25) which increases its affinity for TCF/LEF factors (26). However, we did not find a change in the β-catenin acetylation level upon TSA treatment.

Another non-histone target of HDACs is HSP90. HDI treatment disrupts HSP90 client protein interactions with subsequent proteasomal degradation of client proteins, e.g., Bcr-Abl (27). We analyzed if TCF7L2 might be regulated in a similar mode as HSP90 client proteins. Interestingly, TSA and SAHA induced depletion of TCF7L2 which could be attenuated by co-treatment with proteasome inhibitors. Given that TCF7L2 is the main transcription factor mediating Wnt target gene expression in colorectal carcinoma cells (28), TCF7L2 depletion might be the primary event in the observed Wnt target gene downregulation.

To elucidate the role of HDACs 6, 10 and 11, which are inhibited by hydroxamic acids but not by VPA (29), in Wnt signaling, we studied Wnt target gene expression and TCF7L2 protein level by siRNA experiments. Single knockdowns of HDACs 6, 10 and 11 reduced the expression of at least some of the analyzed Wnt target genes. In addition, knockdown of HDACs 6 or 10 induced a strong reduction in TCF7L2 protein level, while knockdown of HDAC11 induced a minor reduction. These results suggest that inhibition of HDACs 6 and 10 leads to the attenuation of Wnt signaling through the depletion of its main transcription factor TCF7L2. Interestingly, it was previously published that inhibition of HDACs 6 and 10 induces hyperacetylation of HSP90 leading to release and degradation of client proteins like VEGFR (30-32). Moreover, HSP90 inhibitors were shown to downregulate Wnt target gene expression (33). Taken together our data could indicate that TCF7L2 might be an HSP90 client protein, which has not been described yet.

To sum up the mode of HDI action on Wnt signaling, we suggest the following line of events: HDIs of the hydroxamate class inhibit the predominantly cytosolic HDACs 6 and 10 which results in hyperacetylation of a yet unidentified interaction partner of TCF7L2; thereby the release of TCF7L2 from its interaction partner is induced and TCF7L2 is subjected to proteasomal degradation. Concomitantly, Wnt target gene expression is downregulated and colorectal carcinoma cell death is induced. In numerous studies, the attenuation of Wnt signaling results in cancer cell death, e.g., TCF7L2 antisense RNA inhibits proliferation and *in vivo* tumor formation of liver cancer cell lines (34). The re-introduction of wild-type *APC* induces apoptosis in colorectal carcinoma cells (35). Likewise, a Wnt1 antibody or small molecule inhibitors of Wnt signaling stimulate apoptosis in different types of human cancer cells (36,37). Other reports show that active Wnt signaling inhibits apoptosis in cancer cells (38). Thus, TCF7L2 depletion and attenuation of Wnt signaling renders cancer cells sensitive to apoptotic stimuli.

In conclusion, we describe the depletion of TCF7L2 by HDIs of the hydroxamate class, through the inhibition of HDAC6 and HDAC10, leading to an attenuation of Wnt signaling. TCF7L2 degradation might be the primary event in the observed Wnt target gene downregulation and apoptotic colorectal carcinoma cell death. These findings provide a molecular rationale for the use of HDIs against colorectal carcinomas with activated Wnt signaling.

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