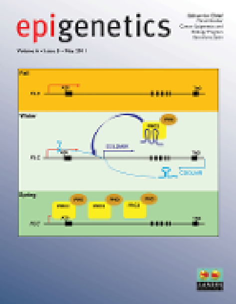

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Class I and III HDACs and loss of active chromatin features contribute to epigenetic silencing of *CDX1* and *EPHB* tumor suppressor genes in colorectal cancer

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Key words: colorectal cancer, transcriptional repression, HDACs, DNA methylation, β-catenin, *CDX1*, EphB receptors

Abbreviations: Aza, 5-Aza-2'-deoxycytidine; ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; DNMTs, DNA methyltransferases; *EPHB2-4*, *EPHB2*, *EPHB3* and *EPHB4*; ESCs, embryonic stem cells; HATs, histone acetyl transferases; HDACs, histone deacetylases; H3K4me3, histone H3 lysine 4 trimethylation; H3K9/14ac, histone H3 lysine 9/14 acetylation; H3K9me1, histone H3 lysine 9 monomethylation; H3K9me2, histone H3 lysine 9 dimethylation; H3K9me3, histone H3 lysine 9 trimethylation; H3K27me1, histone H3 lysine 27 monomethylation; H3K27me3, histone H3 lysine 27 trimethylation; H4K20me1, histone H4 lysine 20 monomethylation; IgG, immunoglobulin G; MBD, methylated DNA-binding domain; MSI, microsatellite-instability; NaBut, sodium butyrate; NIA, nicotinamide; PRC, polycomb repressive complex; qRT-PCR, quantitative reverse transcriptase PCR; RNAPII, RNA polymerase II; SPT, splitomicin; TSA, trichostatin A; TSS, transcriptional start site; VPA, valproic acid

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Aberrant Wnt/β-catenin signaling is a driving force during initiation and progression of colorectal cancer. Yet, the Wnt/β-catenin targets *CDX1*, *EPHB2*, *EPHB3* and *EPHB4* (*EPHB2-4*) act as tumor suppressors in intestinal epithelial cells and frequently appear to be transcriptionally silenced in carcinomas. The molecular mechanisms which underlie the apparent loss of expression of a subset of Wnt/β-catenin targets in a background of persistent pathway activity are largely unknown. To gain insight into this, we quantified expression of *CDX1* and *EPHB2-4* in human tissue specimens of case-matched colorectal normal mucosa, adenoma and invasive carcinoma. In particular *EPHB2-4* display biphasic, albeit not strictly coincident, expression profiles with elevated levels in adenomas and decreased transcription in approximately 30% of the corresponding carcinomas. Consistent with their divergent and variable expression we observed considerable heterogeneity among the epigenetic landscapes at *CDX1* and *EPHB2-4* in a model of colorectal carcinoma cell lines. Unlike the inactive *CDX1* locus, *EPHB2-4* maintain DNA hypomethylation of their promoter regions in the silent state. A strong reduction of active histone modifications consistently parallels reduced expression of *CDX1* and *EPHB3* and to some extent of *EPHB2*. Accordingly, treatment with inhibitors for DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) restored *CDX1* and *EPHB2-4* expression depending upon epigenetic features at their promoters but also upon cellular background. Overall our findings show that downregulation of *CDX1* and EphB receptor genes occurs independently and that different branches of epigenetic control systems including class I and III HDACs contribute to epigenetic silencing of Wnt/β-catenin targets during colorectal tumorigenesis.

Introduction

Aberrant activation of the Wnt/β-catenin pathway is an early event in most cases of colorectal cancer (CRC) tumorigenesis which is consistent with its role in control of stem and progenitor cell proliferation.[1] Initially, mutations in Wnt/β-catenin pathway components lead to cellular hyperproliferation and the formation of benign polyps which exhibit spatially restricted growth. Major effectors of cell positioning and compartmentalization within the intestinal crypts are the Wnt/β-catenin target genes *EPHB2-4*.[2] EphB receptor tyrosine kinases interact with membrane-bound ligands of the ephrin family and thereby modulate adhesive and repulsive cell properties, supporting an epithelial phenotype in many tissues.[2] At early stages of tumorigenesis, i.e., at

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Table 1. Expression changes* of *CDX1*, *EPHB2*, *EPHB3* and *EPHB4* in case-matched human tissue specimens of colorectal normal mucosa, adenoma and invasive carcinoma

Gene	Adenoma/Normal mucosa			Carcinoma/Normal mucosa			Carcinoma/Adenoma		
	down	=	up	down	=	up	down	=	up
CDX1	1/17 (6%)	14/17 (82%)	2/17 (12%)	3/18 (17%)	12/18 (67%)	3/18 (17%)	3/17 (18%)	11/17 (65%)	3/17 (18%)
EPHB2	1/15 (7%)	6/15 (40%)	8/15 (53%)	3/16 (19%)	6/16 (38%)	7/16 (44%)	5/15 (33%)	9/15 (60%)	1/15 (7%)
EPHB3	0/20 (0%)	3/20 (15%)	17/20 (85%)	1/21 (5%)	3/21 (14%)	17/21 (81%)	7/20 (35%)	10/20 (50%)	3/20 (15%)
EPHB4	1/17 (6%)	5/17 (29%)	11/17 (65%)	2/18 (11%)	4/18 (22%)	12/18 (67%)	5/17 (29%)	6/17 (35%)	6/17 (35%)

*Based on a threshold value of two-fold.

the transition of normal to dysplastic epithelial cells, functional EphB-ephrinB signaling effectively confines dysplastic epithelial cells to ephrinB-negative domains.[3] At the adenoma-carcinoma transition, dysplastic cells then breach this barrier and acquire tumor cell invasiveness.[4-6] Inactivation of the EphB-ephrin system can occur by mutational crippling or transcriptional silencing of EphB receptor genes.[2] In fact, *EPHB2* and *EPHB3* are transcriptionally inactive in a sizeable fraction of colorectal cancers.[4-9] Similar findings exist for *EPHB4*,[10] and *CDX1*, another Wnt/β-catenin target important for cellular differentiation and with potential tumor-suppressor functions.[11-14] However, the molecular mechanisms responsible for downregulation of *CDX1* and *EPHB2-4* in tumorigenesis despite continuous Wnt/β-catenin pathway activity are a matter of debate.[7,15-17]

One way to achieve transcriptional inactivation of tumor-relevant genes involves alterations of epigenetic features at regulatory regions.[18,19] Hypermethylation of promoter DNA is often found in transformed cells and can silence tumor suppressor genes. Loss of active and gain of repressive histone marks are additional characteristics of tumor cells.[18,19] Consistent with changes in DNA- and histone modification patterns, genes which encode the corresponding DNA- or histone-modifying proteins, are often deregulated in tumor cells. This includes HDACs, components of Polycomb repressive complexes (PRC) and de novo DNMTs.[20-23] Notably, epigenetic regulators appear to act in a coordinated fashion based on complex molecular interactions.[19,24,25] Thus, to fully understand epigenetic deregulation of tumor-relevant genes, a comprehensive knowledge of the associated changes in DNA methylation and histone modifications as well as the underlying enzymatic machinery is required. Currently, however, epigenetic landscapes characterizing different expression states of *CDX1* and *EPHB2-4* in CRC cells are only ill-defined. Silencing by DNA hypermethylation has consistently been described only for *CDX1*.[10,11,13,15,26,27] Patterns of histone modifications associated with different expression states have been determined neither for *CDX1* nor for the EphB receptor genes.

The goal of this study was to gain insight into the expression dynamics of a particular subset of Wnt/β-catenin target genes in CRC tumorigenesis and to investigate how epigenetic mechanisms contribute to their downregulation in carcinomas. To this end, we performed comparative quantitative expression analyses for *CDX1* and *EPHB2-4* in human tissue specimens of case-matched colorectal normal mucosa, adenoma and carcinoma. Furthermore, we employed a model of CRC cell lines to systematically analyze DNA methylation and histone modification patterns in relation to the expression status of *CDX1* and the EphB receptor genes. Our findings suggest biphasic, albeit not strictly coordinated, expression patterns with a surge in activity in adenomas followed by downregulation in the corresponding invasive carcinomas. In the CRC cell line model, reduced expression of *CDX1* and the EphB receptor genes is accompanied by massive changes of epigenetic features. Considerable heterogeneity among epigenetic landscapes and differential responses towards inhibition of DNMTs and HDACs reveal that the mechanisms of repression differ among *CDX1* and EphB receptors in a gene-specific manner and depending upon cellular background although class I and III HDACs generally appear to play critical roles in the silencing process.

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Results

Expression changes of *CDX1* and EphB receptor genes in case-matched colorectal tissue specimens. To acquire expression profiles for *CDX1* and the EphB receptor genes *EPHB2*, *EPHB3* and *EPHB4* during CRC tumorigenesis, we measured their expression in microdissected cells of a previously characterized group of human case-matched tissue specimens of colorectal normal mucosa, adenoma and invasive carcinoma using qRT-PCR.[28] A more than two-fold change between case-matched samples was defined as increase or decrease. For *CDX1*, transcript levels vary between normal mucosa, adenoma and carcinoma only in few cases (**Table 1 and Sup. Table 1**). In contrast, expression of *EPHB2-4* was increased in the majority of both adenomas and carcinomas when compared to normal mucosa (**Table 1 and Sup. Table 1**). This was most pronounced for *EPHB3* where 85% of the adenomas and 81% of the carcinomas, respectively, showed elevated expression compared to normal mucosa. Although EphB receptor gene expression was largely maintained from adenomas to carcinomas, there was a noticeable downregulation of *EPHB2* and *EPHB3* in 33% and 35% of cases, respectively (**Table 1 and Sup. Table 1**). Expression of *EPHB4* increased, decreased or remained unchanged with equal frequencies in carcinomas. Importantly, by comparing changes in *CDX1* and EphB receptor expression and the occurrence of nuclear β-catenin in the tissue specimens, we find that their expression can be decreased in carcinomas despite the presence of nuclear β-catenin-positive tumor cells (**Sup. Table 2 and Sup. Fig. S1**; for example see cases 54, 67, 68). Thus, it appears that certain Wnt/β-catenin target genes can be subject to secondary downregulation in carcinomas which is not simply the result of diminished Wnt pathway

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activity. Furthermore, comparison of expression changes in the case-matched tissue specimens provided no evidence for a coordinated regulation of *CDX1* and EphB receptor genes during CRC tumorigenesis (**Sup. Table 1**).

Within carcinomas, there was a trend towards reduced *EPHB2* levels in CRCs with lymph node metastasis and poorer differentiation (data not shown), which is in agreement with previous findings and confirms its clinical relevance.[3,4,7,16] An association of *CDX1* or EphB receptor gene expression with Microsatellite-Instability (MSI) status or T category, respectively, was not determined because the majority of the investigated carcinomas was MSI negative and T3.[28]

Differential expression of *CDX1* and EphB receptor genes in human colorectal cancer cell lines. To further probe the divergent expression observed in carcinoma samples we investigated mRNA amounts and protein levels of *CDX1* and *EPHB2-4* in the CRC cell lines LS174T, SW480, HCT116 and HT29, and the non-CRC cell lines HEK293 und U2OS by qRT-PCR and western blotting. The CRC cell lines carry activating pathway mutations, and show high expression of the Wnt/β-catenin target *AXIN2* (**Sup. Fig. S2**), except for HCT116 in which the *AXIN2* promoter is silenced by DNA hypermethylation.[29] Importantly, as seen with the different cases of invasive carcinoma tissue specimens (**Table 1 and Sup. Table 1**), the distinct CRC cell lines differ with respect to expression of *CDX1* and *EPHB2-4* (**Fig. 1 and Table 2**). The highest expression levels of all four genes are found in LS174T, whereas in the other CRC and non-CRC cell lines these genes are expressed at much lower levels or not at all. Taken together, this identifies a set of CRC cell lines which reflect heterogenous expression of *CDX1* and *EPHB2-4* in human tissue specimens of CRC and which can serve as a model for mechanistic investigations.

Differential methylation of *CDX1* and the EphB receptor genes. To analyze whether differential expression of *CDX1* and *EPHB2-4* is due to epigenetic modifications, we first tested whether differences in the DNA methylation status correlate with alterations in their expression. CpG islands were identified at the promoter regions and the first introns of the genes under investigation (**Fig. 2A**), and DNA methylation patterns in these regions were examined by affinity precipitation using recombinant methylated DNA-binding domain (MBD) protein. PCR amplification of precipitated material revealed differential methylation at the distal promoter, the transcriptional start site (TSS) and the first intron of *CDX1* which are unmethylated in LS174T, but are highly methylated in SW480, HCT116, HT29 and HEK293 (**Fig. 2B**). In contrast, the *EPHB2* and *EPHB4* loci appear to be unmethylated in the CRC cell lines at all positions investigated (**Fig. 2B**). Similarly, with the sole exception of an intronic site, *EPHB3* is neither methylated in the region upstream of the TSS, nor in the region spanning the TSS in any of the CRC cell lines tested (**Fig. 2B and Sup. Fig. S3**). Preliminary results from bisulfite sequencing of genomic DNA also indicate that the *EPHB3* promoter is not methylated (data not shown). We additionally confirmed these results using the methylation-sensitive enzyme HpaII and its methylation-insensitive isoschizomere MspI. Genomic DNA was digested by HpaII and MspI and used as template for PCR. Untreated genomic DNA was used as amplification control. The results of the analyses also indicate that the *EPHB3* intronic site is methylated in SW480, HCT116, HT29 and HEK293 but not in LS174T, whereas the TSS appears to be unmethylated in all CRC cell lines (**Sup. Fig. S3**). Taken together, despite similar patterns of transcriptional activity, *CDX1* and *EPHB2-4* exhibit differences with respect to DNA methylation.

The observed differences in methylation of promoter DNA correlate with differential sensitivity of *CDX1* and *EPHB2-4* expression towards the DNMT inhibitor Aza (**Fig. 2C**). Consistent with the absence of DNA methylation at the promoter regions of *EPHB2-4*, their expression was not or only marginally affected by treatment with Aza regardless of cellular background (**Fig. 2C**). In contrast, Aza-treatment resulted in a concentration-dependent increase in *CDX1* expression of up to 30-fold in SW480 and HCT116. Yet, no increase in *CDX1* expression was observed in HT29 despite similar levels of DNA methylation. As expected from the lack of DNA methylation at *CDX1* in LS174T cells, Aza-treatment did not result in expression changes in these cells (**Fig. 2C**). Thus, as seen in previous studies, aberrant DNA methylation is able to silence *CDX1* gene expression,[13,27] but additional factors which seemingly vary depending upon cellular background, can play a role. Furthermore, repressive mechanisms other than DNA methylation are engaged in EphB receptor downregulation and *CDX1* and *EPHB2-4* are silenced by distinct mechanisms.

Decreased promoter occupancy by RNA polymerase II and loss of active histone modifications accompany reduced expression of *CDX1, EPHB2* and *EPHB3*. Next, we performed chromatin immunoprecipitations (ChIP) to investigate if post-translational histone modifications could provide mechanistic explanations for reduced transcription of *CDX1* and *EPHB2-4*. Active histone marks [trimethylated histone H3 lysine 4 (H3K4me3), acetylated histone H3 lysines 9 and 14 (H3K9/14ac), monomethylated histone H3 lysine 27 (H3K27me1), monomethylated histone H3 lysine 9 (H3K9me1), monomethylated histone H4 lysine 20 (H4K20me1)],[30] as well as repressive histone marks [trimethylated histone H3 lysine 27 (H3K27me30, di- and trimethylated histone H3 lysine 9 (H3K9me2; H3K9me3)],[30] were analyzed. In addition, promoter occupancy by RNA polymerase II (RNAPII) was monitored. The results of these analyses (**Fig. 3 and Sup. Figs. S4–S6**) are summarized in **Table 2**. In strongly expressing LS174T cells, *CDX1* and the EphB receptor genes are associated with RNAPII and active histone modifications. Loss of *CDX1* expression correlates with the disappearance of RNAPII and active histone marks. Similarly, at *EPHB3* RNAPII levels and active histone marks decrease concomitant with reduced transcription. At *EPHB2* and *EPHB4,* we observe a similar regression of some active marks (H3K27me1, H3K9me1 and H4K20me1) in SW480, HCT116 and HT29 cells. However, promoter occupancy by RNAPII and levels of H3K4me3 and H3K9/K14ac are maintained despite a decline in expression. As an exception, H3K4me3 and H3K9/K14ac are reduced at *EPHB2* in HT29 cells. Unexpectedly, repressive histone marks are not associated with any of the investigated genes in SW480

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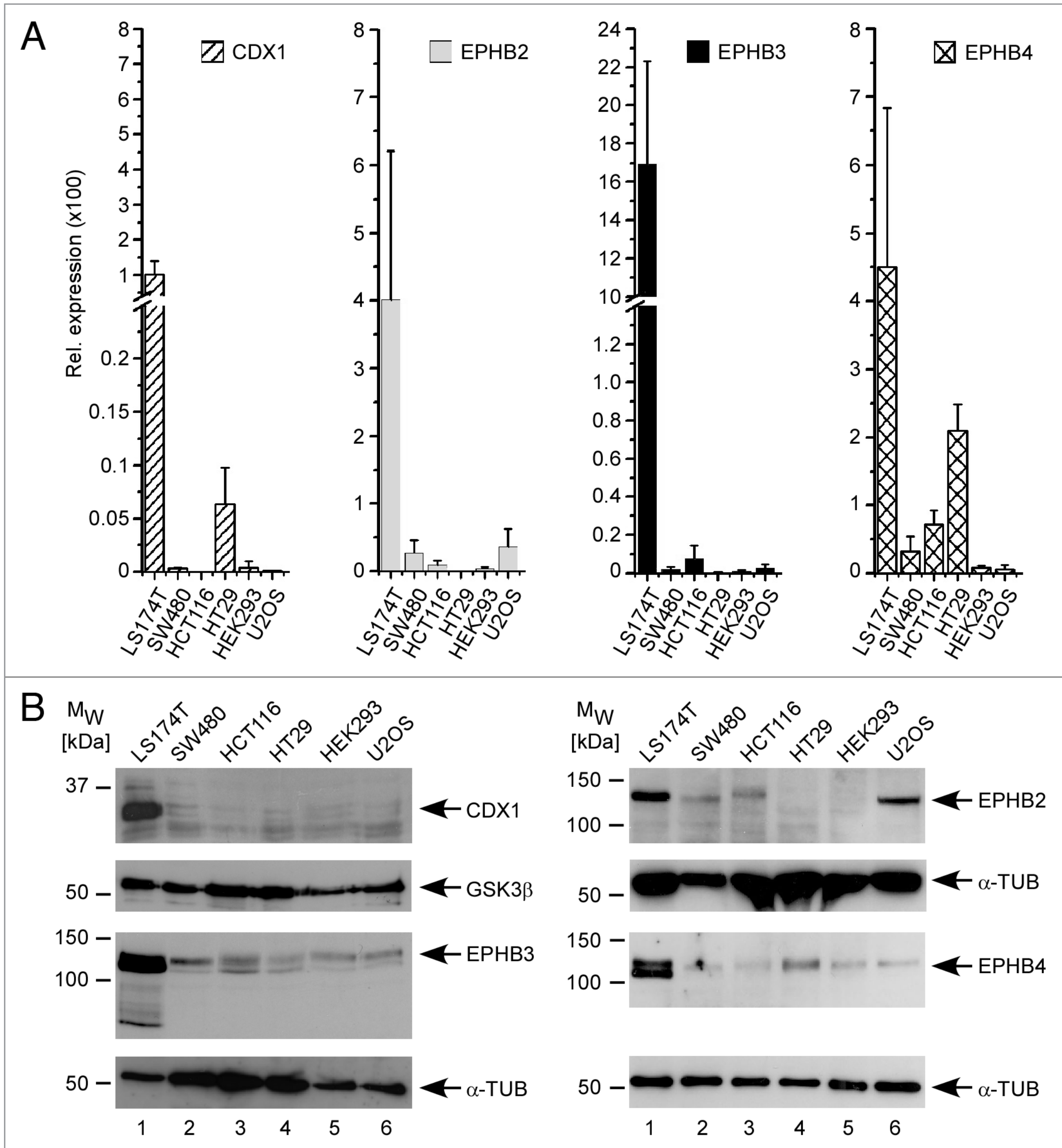

Figure 1. Differential expression of the Wnt/β-catenin target genes *CDX1*, *EPHB2*, *EPHB3* and *EPHB4* in the human CRC cell lines LS174T, SW480, HCT116, HT29 and the non-CRC cell lines HEK293 and U2OS. (A) mRNA expression levels of *CDX1* and *EPHB2-4* were quantified via qRT-PCR and normalized to α-*TUBULIN*. Bars represent relative expression levels (rel. expression) multiplied by 100 for clarity. Average values and standard deviations from at least three independent experiments are shown. (B) Protein expression analyses of CDX1, EPHB2, EPHB3 and EPHB4 using western Blot. Nuclear extracts (for detection of CDX1) or whole cell lysates (for detection of EPHB2-4) were used for western blotting and immunodetection. GSK3β and α-TUBULIN (α-TUB) immunodetection was used to monitor equal loading of nuclear extracts and whole cell lysates, respectively. M_W = molecular weight.

and HCT116 cells. Only HT29 cells show a modest enrichment of H3K27me3 and H3K9me3 at several positions of the *CDX1* locus, at the TSS and intronic regions of *EPHB3* and *EPHB4*, as well as at a promoter-distal and an intronic site of *EPHB2* (**Sup. Fig. S6**). Nonetheless, the CRC cell lines express components of the Polycomb repressive complexes 1 and 2 including the H3K27 methylase EZH2 as well as several methyltransferases which target H3K9 (**Sup. Fig. S7**). Also, bulk histone preparations from the CRC cell lines contain H3K9me2, H3K9me3 and H3K27me3 (**Sup. Fig. S7**). Therefore, absence of repressive histone marks appears to be a specific feature of the inactive *CDX1* and *EHPB2-4* loci rather than the result of a general deficiency in repression systems based on H3K9 and H3K27 methylation in certain CRC cell lines. Taken together, these findings reveal further variability in the epigenetic landscapes of the genes under investigation and suggest that a decrease of active chromatin characteristics underlies the differential expression of certain Wnt/β-catenin target genes in CRC cell lines.

Involvement of class I and class III HDACs in the repression of *CDX1, EPHB2* and *EPHB3*. Functional relevance of reduced histone acetylation levels for *CDX1* and EphB expression was tested by use of three different HDAC inhibitors: TSA, acting on class I and class II HDACs; SPT and NIA, acting predominantly on the NAD^+-dependent sirtuins (class III HDACs).[31,32]

Table 2. Summary of expression data and epigenetic features for *CDX1* and EphB receptor genes in different CRC cell lines

Locus	Cell line	Expression	RNA-Pol II	H3K4me3[a] H3K9/14ac	H3K27me3 H3K9me3	^{5}meCpG
CDX1	LS174T	+++	+++	+++	-	-
	SW480	-	-	-	-	+
	HCT116	-	-	-	-	+
	HT29	+	-	-	+/-	+
EPHB2	LS174T	+++	n.a.[b]	+++	-	-
	SW480	-	n.a.	+++	-	-
	HCT116	-	n.a.	+++	-	-
	HT29	-	n.a.	+	+/-	-
EPHB3	LS174T	+++	+++	+++	-	-
	SW480	-	+	+	-	+[c]
	HCT116	-	+	+	-	+[c]
	HT29	-	+	+	+/-	+[c]
EPHB4	LS174T	+++	+++	+++	-	-
	SW480	-	+++	+++	-	-
	HCT116	+	+++	+++	-	-
	HT29	++	+++	+++	+/-	-

[a]Additional active histone modifications (H3K27me1, H3K9me1 and H4K20me1) behave in a similar fashion (**Sup. Fig. S5**). [b]n.a., not analyzed due to lack of primer pairs suitable for qPCR. [c]Elevated DNA methylation in first intron only.

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CRC cell lines were treated with increasing concentrations of each of these inhibitors. Additionally, treatment with TSA was combined with either SPT or NIA. Subsequent expression analyses using qRT-PCR revealed stable expression levels in LS174T cells (**Fig. 4A**). Similarly, HDAC inhibition had no effect on *CDX1* and *EPHB4* expression (**Fig. 4A** and data not shown). However, TSA-, NIA- and SPT-treatment led to reactivation of *EPHB2* specifically in HT29 cells. Combined treatment, especially of TSA and NIA (last lane in **Fig. 4A**), almost doubled the effect. The results for *EPHB3* were even more striking. In all of the CRC cell lines with low *EPHB3* gene expression, HDAC inhibition by TSA or NIA restored *EPHB3* expression (**Fig. 4A**).

To narrow down the HDACs involved in the regulation of *EPHB2* and *EPHB3*, we used VPA and NaBut, specifically acting on class I and class IIa HDACs, as well as MS-275, acting on class I HDACs only. Expression levels of *CDX1* and the EphB receptor genes were compared to those in TSA-treated cells. In case of *EPHB2* and *EPHB3*, the class I specific inhibitor MS-275 restored expression to levels similar to or even exceeding those seen with TSA and the other reagents (**Fig. 4B**). MS-275 and NaBut also affected *CDX1* expression in SW480 and HT29 (**Fig. 4B**) revealing a contribution of class I HDACs to the repression of *CDX1* as well. No change in expression of *EPHB4* was observed (data not shown). Taken together, these results suggest that class I HDACs in addition to class III HDACs are important to subdue expression of *CDX1*, *EPHB2* and *EPHB3* in certain CRC cells.

To obtain evidence that *CDX1*, *EPHB2* and *EPHB3* are immediate targets of HDACs we established temporal profiles for changes in their expression upon treatment of CRC cell lines with MS-275 and NIA over time courses from 3 to 24 h. In parallel, we analyzed the behavior of the *CDKN1A/p21^{CIP1}* gene which is an accepted and well characterized direct target of class I HDACs.[33-35] Furthermore, we performed ChIP analyses to monitor changes in H3K9/14 acetylation at these loci. Increases in *CDX1*, *EPHB2* and *EPHB3* transcript levels were seen in SW480 cells as early as three hours after addition of either 3 μM or 9 μM MS-275, respectively (**Fig. 5A** and left parts). A more delayed response of *CDX1*, *EPHB2* and *EPHB3* was observed in HT29 cells (**Fig. 5B** and left parts). In HCT116 cells, *CDX1* and *EPHB2* levels remained unchanged upon MS-275 treatment as before (see **Fig. 4**), while upregulation of *EPHB3* resembled that in HT29 cells (data not shown). NIA elicited responses of *EPHB3* in SW480 and HT29 cells and of *EPHB2* in HT29 cells with temporal profiles similar to MS-275 (**Sup. Fig. S8**). Of note, the observed time courses of MS-275-induced reactivation of *CDX1*, *EPHB2* and *EPHB3* are in complete accordance with the behavior of *CDKN1A/p21^{CIP1}* both in SW480 and HT29 cells (**Fig. 5** and left parts). Moreover, ChIP analyses detected significantly elevated levels of H3K9/14ac at the *CDX1*, *EPHB2* and *EPHB3* and *CDKN1A/p21^{CIP1}* loci in the presence of MS-275 (**Fig. 5** and right parts). Taken together, these observations strongly suggest that HDACs are directly involved in the downregulation of *CDX1*, *EPHB2* and *EPHB3* promoter activity.

Upregulation of HDACs has been linked to colorectal tumorigenesis.[22,36,37] Therefore, we investigated whether differences in expression of the class I enzymes *HDAC1*, *HDAC2*, *HDAC3* and *HDAC8* and the class III members *SIRT1* and *SIRT2* might underlie the formation of distinct epigenetic landscapes at the *CDX1*, *EPHB2*, *EPHB3* and *EPHB4* loci. Expression analyses were performed by RT-PCR (**Fig. 6**). However, we did not

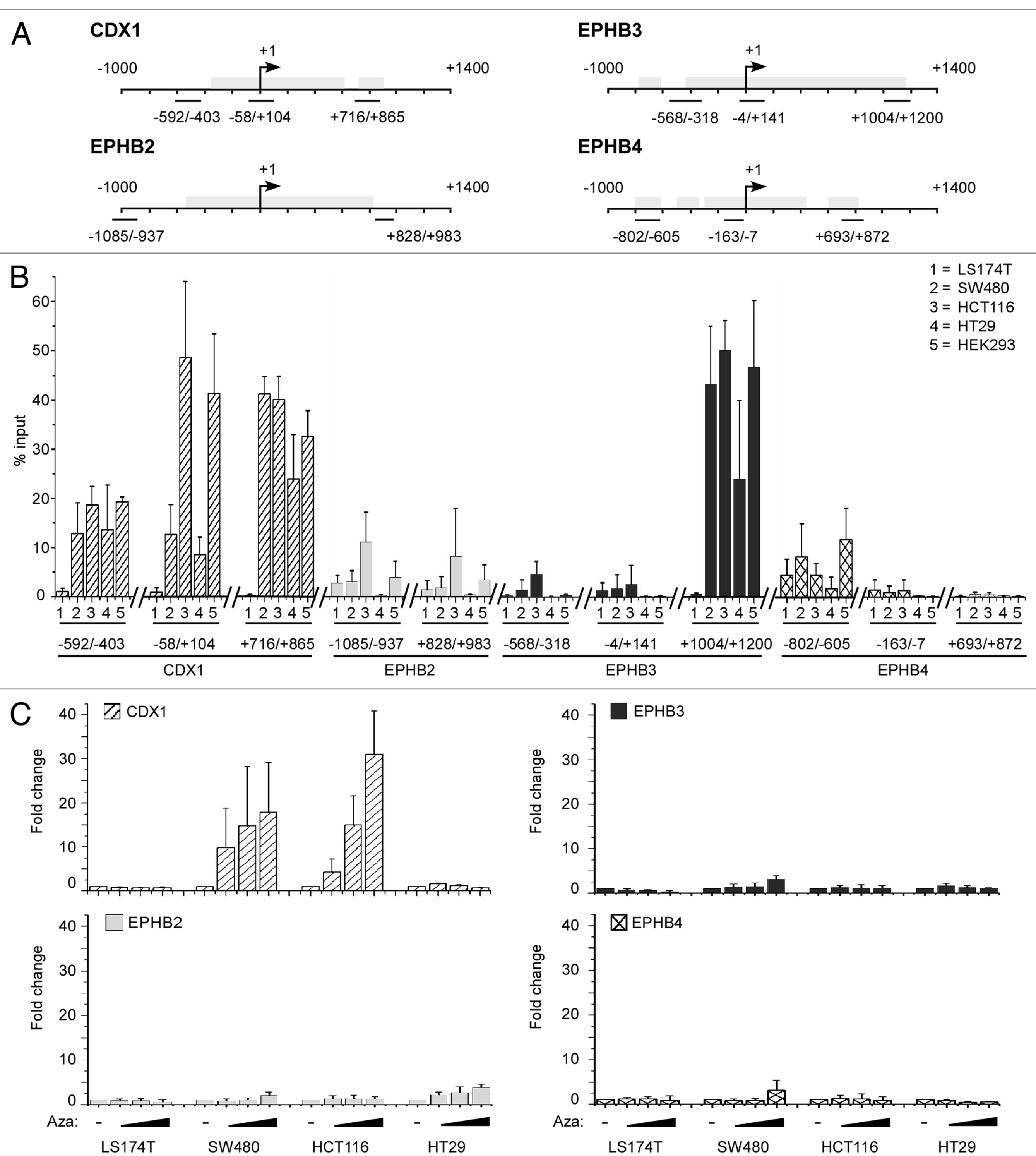

Figure 2. Analysis of DNA methylation of *CDX1*, *EPHB2*, *EPHB3* and *EPHB4*. (A) A schematic depiction of the *CDX1, EPHB2, EPHB3* and *EPHB4* loci showing the TSS (arrow, +1), the CpG island (shaded gray) and the positions of PCR amplicons used for the analyses. Numbers refer to nucleotide positions relative to the TSS. (B) Analyses of DNA methylation by affinity precipitation using a recombinant MBD protein, binding CpG-methylated DNA fragments. Precipitated DNA fragments were analyzed via quantitative PCR (qPCR) with the indicated primer pairs. Bars represent the relative amount of DNA recovered at each site and in each cell line compared to the corresponding input control (% input). Average values and standard deviations from at least three independent experiments are shown. (C) The CRC cell lines were treated with 2 μM, 10 μM or 50 μM Aza for 72 h. Total RNA was isolated from untreated and Aza-treated cells and cDNA was synthesized. qRT-PCR was performed using specific primer pairs for *CDX1* and *EPHB2-4*. Expression levels were normalized to *GAPDH* expression. Bars represent the fold change of expression in Aza-treated cells compared to the untreated control. Average values and standard deviations from three independent experiments are shown.

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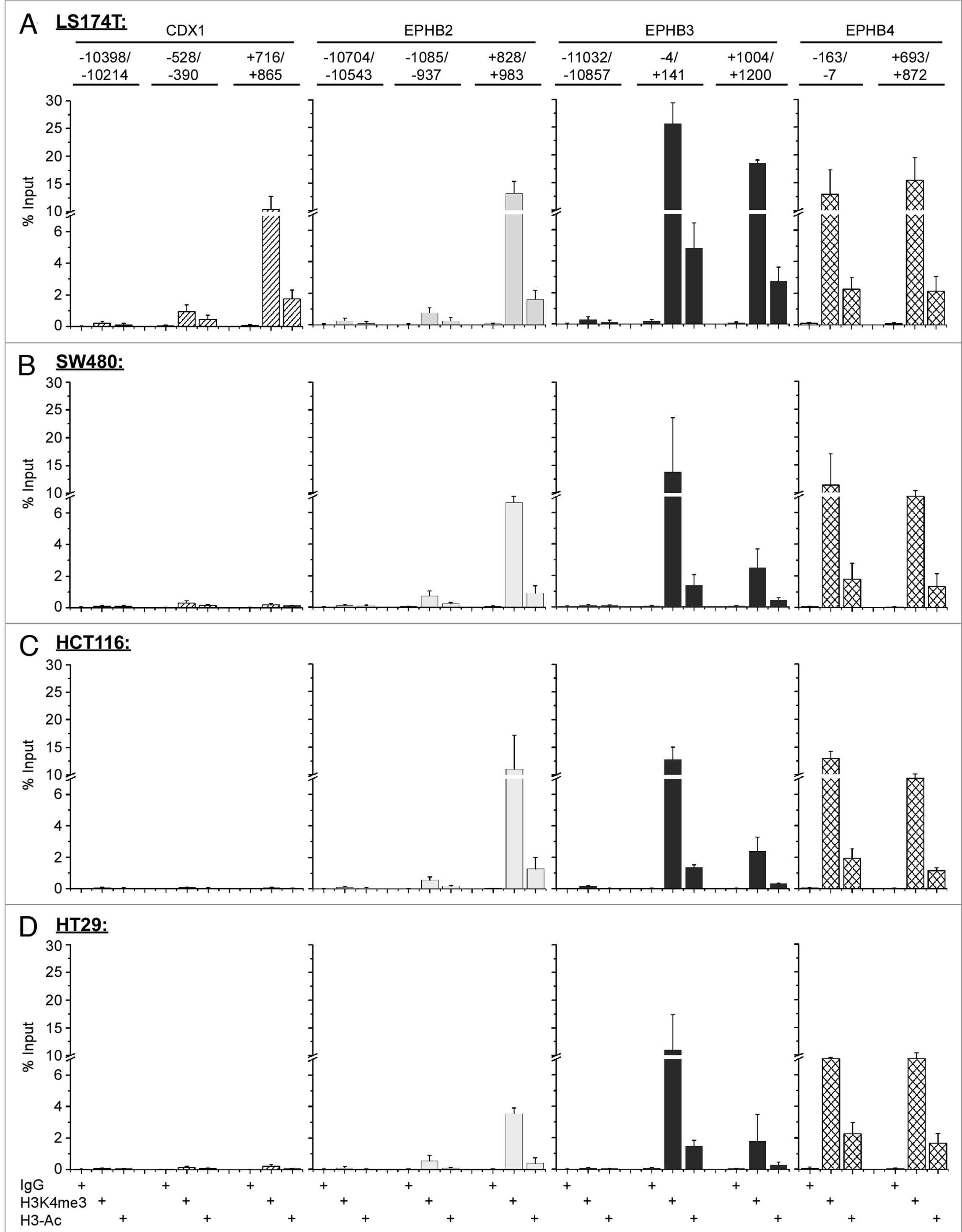

Figure 3. For figure legend, see page 617.

Figure 3 (See opposite page). Reduced levels of active histone modifications coincide with decreased transcriptional activity of *CDX1* and *EPHB3*. Occurrence of the active histone marks H3K4me3 and H3K9/K14ac was investigated in the cell lines shown using ChIP. Precipitated DNA was analyzed via qPCR using the indicated primer pairs. Numbers refer to nucleotide positions relative to the TSS. Bars represent the relative amount of DNA recovered at each site and in each cell line compared to the corresponding input control (% input). All data were normalized to histone H3 density. Results from ChIP experiments with isotype control antibodies (IgG) are shown for all primer pairs in all cell lines. Average values and standard deviations from three independent experiments are shown.

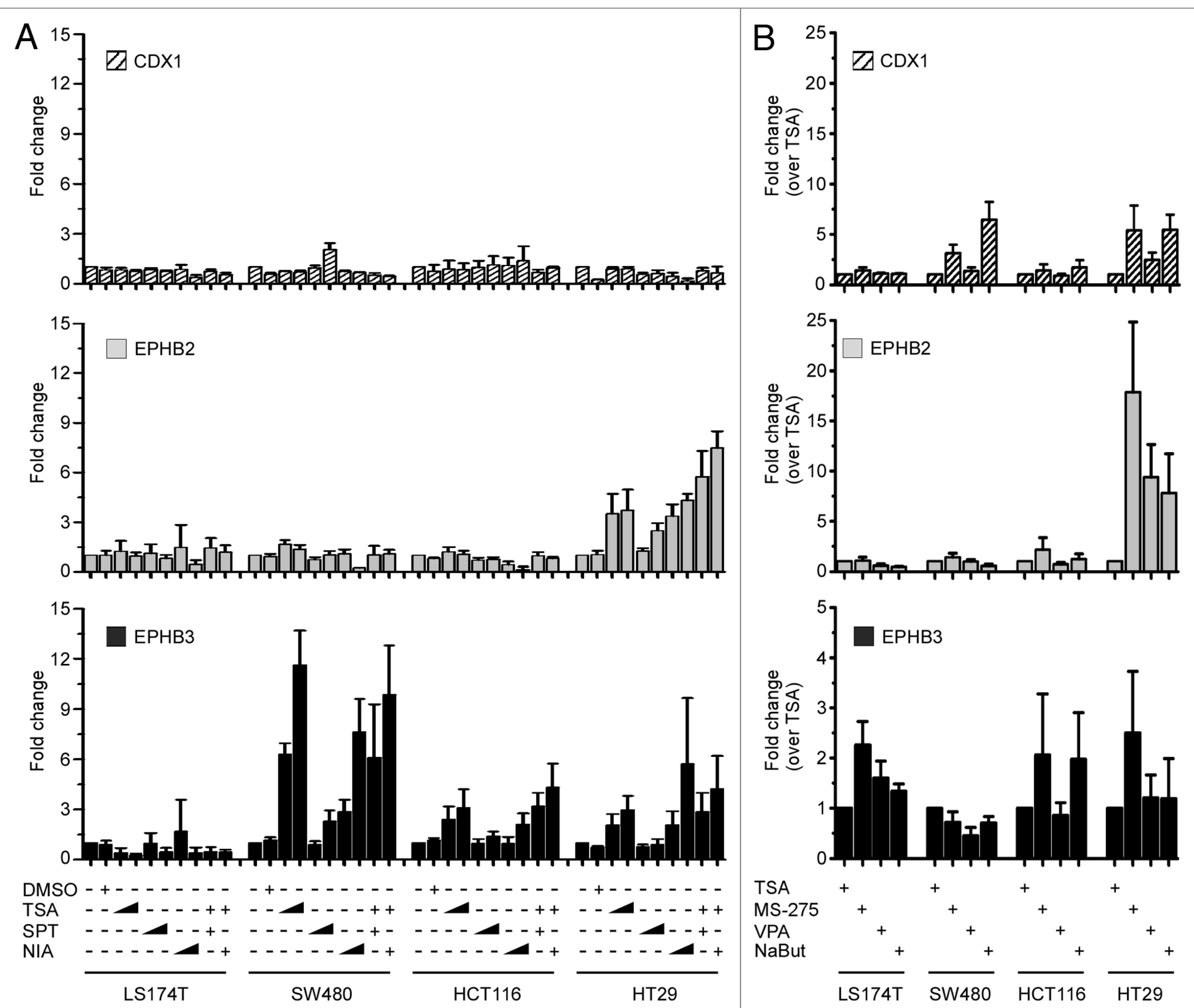

Figure 4. Class I and III HDACs regulate *CDX1* and EphB receptor gene expression. (A) The human CRC cell lines shown were treated with SPT (300 μM or 600 μM) or NIA (15 mM or 30 mM) for 20 h or with TSA (75 nM or 150 nM) for 3.5 h. For combined treatment, 75 nM TSA and 300 μM SPT or 15 mM NIA, respectively, were used. Total RNA was isolated from untreated and HDAC inhibitor-treated cells and cDNA was synthesized. qRT-PCR was performed with specific primer pairs for *CDX1*, *EPHB2* and *EPHB3*. Expression levels were normalized to *GAPDH* expression. Bars represent the fold change of expression of HDAC inhibitor-treated cells compared to the untreated control. Solvent had no effect (DMSO control). Average values and standard deviations from three independent experiments are shown. (B) The indicated human CRC cell lines were treated with either 150 nM TSA for 3.5 h or with 3 μM MS-275, 2 mM VPA or 2 mM NaBut for 24 h. Control cells received the solvent DMSO only. Upon RNA isolation and cDNA synthesis, qRT-PCR was performed with primer pairs specific for *CDX1*, *EPHB2* and *EPHB3*. Expression levels were normalized to *GAPDH* expression. Bars represent the fold change of expression over the TSA-induced effect which was set to one. Average values and standard deviations from three independent experiments are shown.

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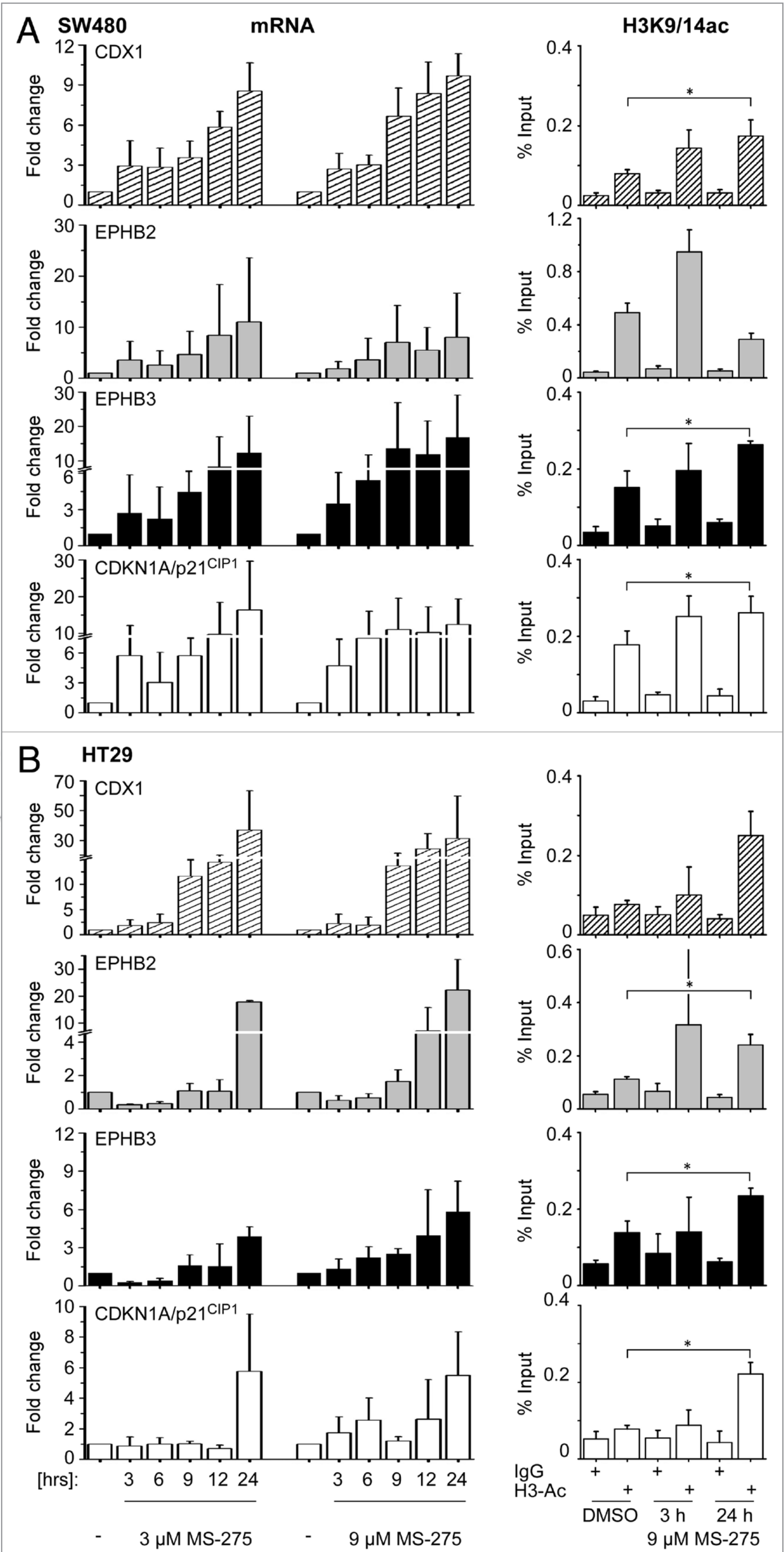
Figure 5. Increased transcriptional activity of *CDX1, EPHB2, EPHB3* and *CDKN1A/p21*^{CIP1} after treatment with MS-275 coincides with elevated levels of histone acetylation. (A and B) Left parts: The human CRC cell lines SW480 (A) and HT29 (B) were treated with 3 μM MS-275 or 9 μM MS-275, respectively, for the indicated times. Control cells received DMSO as solvent control. Upon RNA isolation and cDNA synthesis, qRT-PCR was performed with specific primer pairs for *CDX1*, *EPHB2*, *EPHB3* and *CDKN1A/p21*^{CIP1}. Expression levels were normalized to *GAPDH* expression. Bars represent the fold change of expression of HDAC inhibitor-treated cells compared to the solvent control (DMSO). Average values and standard deviations from three independent experiments are shown. Right parts: Occurrence of the active histone mark H3K9/K14ac after treatment with 9 μM MS-275 for 3 h or 24 h, respectively, was investigated using ChIP. Precipitated DNA was analyzed via qPCR using the following primer pairs: *CDX1* (-174, -30), *EPHB2* (+828/+983), *EPHB3* (+1,004/+1,200) and *CDKN1A/p21*^{CIP1} (-375, -230). Numbers refer to nucleotide positions relative to the TSS. Bars represent the relative amount of DNA recovered at each site compared to the corresponding input control (% input). All data were normalized to histone H3 density. Results from ChIP experiments with isotype control antibodies (rab IgG) are shown. Average values and standard deviations from three independent experiments are given. p-values were calculated using the paired, two-tailed Student's t test. *$p < 0.05$.



detect differential expression of any of theses enzymes which could explain the differential occurrence of H3K9/K14ac. Therefore, despite the functional importance of HDACs, these findings rule out that solely altered expression levels of these enzymes are responsible for decreased histone H3 acetylation at regulatory regions of *CDX1* and *EPHB2-4*.

Discussion

Previous observations of deregulation of *CDX1* and EphB receptor genes based on separate analyses in different collections of cell lines and patient-derived samples raised the possibility that certain Wnt/β-catenin targets might be coordinately repressed in CRC tumorigenesis and progression.[4,11,13] A rationale for synchronized silencing could be highly redundant functions of the genes affected or cumulative effects.[4,38] Indeed, we show here that transcript levels of EphB receptor genes, especially *EPHB3*, markedly increase in adenomas and are subsequently downregulated at the transition from adenoma to carcinoma in up to 35% of cases, supporting the view that their repression confers

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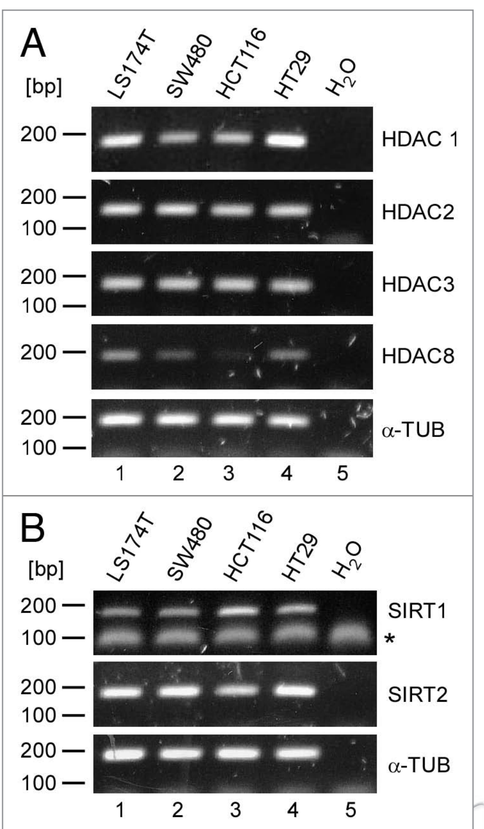

Figure 6. Class I and III HDACs are not differentially expressed in the CRC cell lines. (A) mRNA expression of class I HDAC members was analyzed using semiquantitative RT-PCR with specific primer pairs for *HDAC1*, *HDAC2*, *HDAC3* and *HDAC8*. (B) mRNA expression of the class III HDACs (sirtuins) *SIRT1* and *SIRT2* was analyzed as described above. Amplification of *α-TUBULIN* (α-TUB) was used as control. Negative control reactions (H_2O) received no cDNA. *primer artifact.

growth advantages and invasiveness to tumor cells.[4,5,10] However, our parallel qRT-PCR analyses in case-matched tissue specimens of normal mucosa, adenoma and carcinoma revealed divergent expression profiles of *CDX1* and *EPHB2-4* and thus clearly refute the hypothesis of coordinate misregulation. On the other hand, variable downregulation in carcinomas fits our findings that distinct mechanisms operate in the inactivation of *CDX1* and *EPHB2-4*. It is also consistent with their non-overlapping expression in vivo.[16,38] Overall this suggests that the various EphB receptors affect different aspects of tumor cell physiology, such as the ability of CRC tumor cells to metastasize which seems to be linked more strongly to *EPHB2*.[6,8,9,15]

The highly variable *CDX1* and *EPHB2-4* expression in CRC tissue specimens and their strongly reduced expression in carcinomas and in carcinoma cell lines with an active Wnt/β-catenin pathway are remarkable. A general defect in pathway activity is unlikely to underlie this downregulation. This would be difficult to reconcile with the observed divergent behavior of *CDX1* and *EPHB2-4*. Furthermore, active Wnt/β-catenin signaling is essential at all stages of tumor formation.[1] On the other hand, epigenetic mechanisms would be ideally suited to impinge on the control of individual Wnt/β-catenin target genes. Indeed, hypermethylation of promoter DNA and repressive histone marks can render genes refractory to Wnt induction during embryogenesis.[39] Interestingly, one of the models proposed to explain epigenetic silencing of tumor relevant genes is based on the observation that transformed cells increasingly acquire features of embryonic stem cells (ESCs).[18,19] Thus, genes under control of polycomb repressive complexes in ESCs are predisposed to epigenetic silencing in tumor cells.[40-42] BMI1 and EZH2, components of PRCs, are upregulated in cancer cells and this may contribute to the reconstitution of gene expression patterns similar to ESCs.[18,19] Known physical interactions between DNMTs and PRCs in cancer cells could account for the additional recruitment of DNMTs to PRC targets and explain aberrant promoter hypermethylation of tumor-relevant genes.[24] This model is consistent with chromatin features at the inactive *CDX1* locus in HT29 cells. The dual action of DNMTs and PRCs could also explain why Aza treatment in HT29 cells failed to restore *CDX1* expression. However, H3K27me3, the mark laid down by the EZH2 lysine methylase, is absent from *CDX1* in SW480 and HCT116 cells while promoter hypermethylation nonetheless occurs. Clearly, variable mechanisms of repression can operate at a single locus and embryonic modes of gene regulation need not necessarily be recapitulated in CRC tumorigenesis.

In SW480 and HCT116 cells, reduced expression of *EPHB2-4* occurs in the absence of repressive histone marks and hypermethylation of promoter DNA. For *EPHB2* and *EPHB4* there are conflicting results as to the contribution of DNA methylation to their inactivation in tumor cells.[7,10,15,17] DNA methylation at *EPHB3* had not been systematically analyzed in the past.[17] We believe that the observed hypomethylation of all three EphB receptor genes in our cellular model reflects genuine properties of these loci since we could show hypermethylation of *CDX1* and *SFRP2* (not shown) under the same experimental conditions. However, our observations of pronounced heterogeneity and divergence concerning expression patterns and epigenetic features of *CDX1* and *EPHB2-4* in different tissue specimens and cellular backgrounds provide a potential explanation for the discordant literature data. Nonetheless, our findings support previous reports showing that diminished expression of *EPHB2-4* is based on mechanisms other than DNA methylation.[7,17]

While it remains unclear what brings about reduced expression of *EPHB4*, a striking finding of our study is that the *CDX1* and *EPHB3* loci show reduced levels of the active histone marks in non-expressing CRC cell lines. This holds true also for *EPHB2* in HT29 cells. Generally, a decrease in H3K4 trimethylation and H3 acetylation is paralleled by a strong decline in gene expression.[43,44] Histone methylation and acetylation are regulated by the precise interplay between antagonistic groups of lysine methylases/demethylases and HATs/HDACs, respectively.[18,19] Changes in HAT activity due to mutations are known to play a role in certain forms of leukemia, whereas in epithelial cancers, HDACs feature more prominently.[22,33,36,37,45] The results of our inhibitor studies clearly reveal the repressive influence of class I and class III HDACs on *CDX1*, *EPHB2* and *EPHB3*.

How could HDACs be targeted to *CDX1*, *EPHB2* and *EPHB3* in a context-dependent manner? Overexpression seemingly

suffices to grant epigenetic modifiers access to their targets.[19,23] However, our results did not reveal differences in expression levels of the HDACs investigated. HDACs can be recruited to promoters as part of co-repressor complexes through interactions with DNA-binding transcriptional repressors.[46,47] Recently, the NFκB family member c-Rel was described as a negative regulator of *EPHB2* in the CRC cell line SW620.[7] NFκB proteins are known to interact with HDACs[48] raising the possibility that the HDAC inhibitor-sensitive repression of *EPHB2* which we detect in HT29 cells, involves a c-Rel/HDAC complex. However, we do not think that this mechanism is generally involved in the regulation of EphB receptor genes because repression of *EPHB2* is refractory to HDAC inhibition in SW480 and HCT116 cells. Also, bioinformatic analyses of the *EPHB3* and *EPHB4* promoter sequences did not identify c-Rel binding sites (not shown). Yet, this does not exclude the possibility that other transcriptional repressors bind to these promoters and recruit epigenetic modifiers including HDACs.

In summary, expression profiling of *CDX1* and EphB receptor genes in CRC tissue specimens and a set of CRC cell lines as well as the comprehensive study of DNA methylation and histone modifications at these four Wnt/β-catenin target genes shows that mechanisms which lead to their transcriptional repression are highly divergent and, moreover, can vary for a given locus depending upon cellular background. The observed mechanistic heterogeneity underlying gene-specific and context-dependent deregulation of tumor suppressors may reflect the multi-faceted nature of genetic and epigenetic changes found in colorectal carcinomas. In view of therapeutic applications this may necessitate careful investigations of epigenetic features in individual tumor samples, yet, our data further support the notion that HDAC and DNMT inhibitors are promising agents for the treatment of solid cancers.

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Materials and Methods

Cell culture and inhibitor treatment. The human cell lines HT29 and U2OS were obtained from the American Type Culture Collection. SW480, HCT116 and HEK293 cell lines were obtained from the Max Planck Institute of Immunobiology, Freiburg. The cell line LS174T was a gift from T. Brabletz (Freiburg). All cell lines were authenticated by expression and sequence analyses of indicative genes. Cells were cultured in Dulbecco's modified Eagle's medium (PAN-Biotech) supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml penicillin streptomycin (Invitrogen), 10 mM HEPES buffer (PAN-Biotech) and 1% MEM non-essential amino acids (Invitrogen). For the inhibition of the DNMTs, 2 x 10^5 cells were seeded per well of 6-well plates. After 16 h the cells were treated with 2 μM, 10 μM or 50 μM 5-Aza-2'-deoxycytidine (Aza) (Sigma-Aldrich) which was replenished every 24 h. For HDAC inhibition, 1 x 10^6 cells were seeded per well of six-well plates. The next day, cells were treated with either 15 mM or 30 mM nicotinamide (NIA), 300 μM or 600 μM splitomicin (SPT), 2 mM valproic acid (VPA), 2 mM sodium butyrate (NaBut) (Sigma-Aldrich), or 3 μM MS-275 (Enzo), respectively. Treatment with 75 nM or 150 nM trichostatin A (TSA) (Sigma-Aldrich) was initiated 3.5 h before harvest. For time courses, cells were treated with either 3 μM or 9 μM MS-275, respectively, as indicated.

Tissues. Cells of normal colorectal mucosa (n = 21), low- (n = 14) and high-grade (n = 6) intraepithelial neoplasia and invasive colorectal carcinomas (n = 21) were obtained by microdissection from formalin-fixed and paraffin-embedded tissue specimens of 21 patients resected for sporadic colorectal cancer.[28,49] Since there was no statistical difference among adenomas showing low-grade versus high-grade intraepithelial neoplasia, both groups were combined and are presented as "adenoma." The use of tissues had been approved by the local ethics committee.

RNA isolation and reverse transcriptase PCR. Isolation of total RNA from cell lines, cDNA synthesis and reverse transcriptase PCR (RT-PCR) analysis were performed as before[39] using the NucleoSpin RNA II kit (Macherey and Nagel) and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. RNA from human tissue specimens in sufficient yield and quality was isolated and transcribed into cDNA as described in reference 28, using the MMLV reverse transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed in an iQ5 multicolor real-time PCR detection system (Bio-Rad) using SYBR green reaction mix (Peqlab), primers listed in **Supplemental Table 3**, and an equivalent of 50 ng RNA per sample in case of cell lines. The expression data were normalized to α-*TUBULIN* or *GAPDH* expression as indicated in figure legends. Expression data from human tissue specimens were normalized to β*2-MICROGLOBULIN*.[50]

Western blotting. Preparation of whole cell lysates and nuclear extracts, western blotting and signal detection was performed as before in reference 51. CDX1 was detected using rabbit polyclonal antibodies (1:1,000) kindly provided by R. Kemler, Freiburg. Additional antibodies used were: mouse monoclonal anti-EPHB2 (1:1,000) (Santa Cruz; sc-100298), mouse monoclonal anti-EPHB3 (1:1,000) (Abnova; H00002049-M01), mouse monoclonal anti-EPHB4 (1:1,000) (Invitrogen 35-2900), mouse monoclonal anti-α-TUBULIN (1:10,000) (Sigma-Aldrich; T9026) and mouse monoclonal anti-GSK3β (1:1,000) (BD Biosciences; 610201).

Analysis of DNA methylation. Genomic DNA from 5 x 10^6 cells was isolated using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol and sonicated on ice 10 times for 10 s each at amplitude setting 15 using a Branson sonifier model W-450D. Average DNA fragment size was 200 to 500 bp. Analysis of DNA methylation was done with 100 ng fragmented DNA and the MethylCollector™-kit (Active Motif) following the manufacturer's protocol. Quantitative PCR (qPCR) was performed using 4 μl of the precipitated DNA. 50 ng of fragmented genomic DNA not subjected to the MethylCollector™ served as reference material and were used for normalization. All primers used are listed in **Supplemental Table 4**.

Chromatin immunoprecipitation. ChIP assays were performed as previously described in reference 39. Antibodies used were rabbit anti-trimethyl histone H3K4 (Abcam; ab8580), rabbit anti-acetyl histone H3K9/14 (Upstate; 06-599), rabbit anti-histone H3 (Abcam; ab1791) and isotype control immunoglobulin

(IgGs) (Santa Cruz; sc-2027). DNA was analyzed by qPCR with primers listed in **Supplemental Table 4**. One microliter of precipitated DNA and 2% of the input material were used as templates. Results from experiments with modification-specific antibodies are presented as % of input DNA after normalization using data from ChIPs with pan-H3 antibodies to correct for variations in nucleosome density.

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Note

Supplemental materials can be found at:
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