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ORIGINAL ARTICLE

HDAC3 represses the expression of NKG2D ligands ULBPs in epithelial tumour cells: potential implications for the immunosurveillance of cancer

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The expression of the NKG2D ligands on cancer cells leads to their recognition and elimination by host immune responses mediated by natural killer and T cells. UL16-binding proteins (ULBPs) are NKG2D ligands, which are scarcely expressed in epithelial tumours, favouring their evasion from the immune system. Herein, we investigated the epigenetic mechanisms underlying the repression of ULBPs in epithelial cancer cells. We show that ULBP1–3 expression is increased in tumour cells after exposure to the inhibitor of histone deacetylases (HDACs) trichostatin A (TSA), which enhances the natural killer cell-mediated cytotoxicity of HeLa cells. Our experiments showed that the transcription factor Sp3 is crucial in the activation of the *ULBP1* promoter by TSA. Furthermore, by small interfering RNA-mediated knockdown and overexpression of HDAC1–3, we showed that HDAC3 is a repressor of ULBPs expression in epithelial cancer cells. Remarkably, TSA treatment caused the complete release of HDAC3 from the *ULBP1–3* promoters. HDAC3 is recruited to the *ULBP1* promoter through its interaction with Sp3 and TSA treatment interfered with this association. Together, we describe a new mechanism by which cancer cells may evade the immune response through the epigenetic modulation of the ULBPs expression and provide a model in which HDAC inhibitors may favour the elimination of transformed cells by increasing the immunogenicity of epithelial tumours.
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Introduction

NKG2D is an activating receptor expressed by natural killer (NK) and T cells (Bauer *et al*., 1999; Lopez-Larrea *et al*., 2008). In humans, NKG2D is a receptor for major histocompatibility complex (MHC) class I-related chain A and B molecules (MICA/B) and for the UL16-binding proteins (ULBPs)/RAET1 family (Cosman *et al*., 2001; González *et al*., 2008). NKG2D ligands (NKG2DLs) are not expressed by most benign cells, but are upregulated in cancer cells, which elicits a potent anti-tumour immune response *in vitro* and *in vivo* (Smyth *et al*., 2005; Sutherland *et al*., 2006; Nausch and Cerwenka, 2008). However, when the tumour is not rejected, the immune system sculpts or edits its phenotype, eliminating the most immunogenic cancer cells and, thereby, favouring the development of less immunogenic tumours (Dunn *et al*., 2004a). Importantly, cancer cells frequently develop several means to evade the immune system through the modulation of the expression of the NKG2D/NKG2DLs system (Waldhauer and Steinle, 2008). For instance, shedding of tumour-derived soluble NKG2DLs downregulates the NKG2D expression and functions (Groh *et al*., 2002; Kaiser *et al*., 2007). Nevertheless, enhancing the expression of these ligands may become a powerful strategy for immunotherapy against cancer.

UL16-binding proteins are NKG2D ligands frequently expressed in leukaemias, but they are scarcely detected in epithelial tumours (Pende *et al*., 2002; Salih *et al*., 2003). However, the molecular pathways regulating the ULBPs expression in cancer cells are poorly understood. As ULBPs stimulate anti-tumour immunity, further understanding of their regulation in epithelial cancer cells becomes particularly relevant. In this regard, we have shown that the ubiquitous transcription factors Sp1 and Sp3 are the main factors responsible for the basal expression of ULBP1 (López-Soto *et al*., 2006). Despite the fact that Sp1 and Sp3 are widely expressed, it is known that these factors may regulate gene expression through their interaction with chromatin-remodelling factors, such as histone deacetylases (HDACs), which have been reported to be associated with transcriptional repression (Doetzlhofer *et al*., 1999; Sun *et al*., 2002). Remarkably, it has been described that MICA/B are induced in cancer cells by treatment with HDACs inhibitors (HDACi), suggesting that chromatin structure may play a key role in the repression of NKG2DLs transcription (Armeanu *et al*., 2005; Skov *et al*., 2005; Diermayr *et al*., 2008).

Histone deacetylases are enzymes that catalyse the removal of acetyl groups from conserved lysine residues in the amino-terminal tails of histones. To date, 18

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HDACs have been cloned in mammalians and grouped into four classes based on their sequence homology to yeast HDACs. Class I HDACs (HDAC1, 2, 3 and 8) are widely expressed in many tissues, whereas class II HDACs (HDAC4–7, 9 and 10) display a restricted pattern of distribution (Verdin *et al*., 2003). Sirtuins are NAD^+-dependent HDACs and constitute the third class of HDACs (SIRT1–7) (Saunders and Verdin, 2007). Finally, class IV is only composed of HDAC11, which exhibits a certain similarity with the catalytic domains of both class I and II HDACs. HDACs are recruited to specific promoters to inhibit transcription through histone deacetylation. Likewise, they may also regulate gene expression by deacetylation of non-histone proteins such as transcription factors (Luo *et al*., 2000; Chen *et al*., 2001). Recent studies indicate that transcriptional repression mediated by HDACs plays a physiological role through the inhibition of differentiation and the maintenance of cell proliferation and survival. Consequently, the deregulation of HDACs expression detected in several human cancers has been associated with tumourigenesis (Glozak and Seto, 2007). However, opposite of genetic alterations, epigenetic changes are reversible and, therefore, suitable for pharmacological intervention. To this purpose, several HDAC inhibitors are currently undergoing clinical trials for the treatment of cancer. Several studies have demonstrated that the therapeutic effects of these drugs are linked to the induction of apoptosis and growth arrest in cancer cells (Xu *et al*., 2007), but also to the modulation of the anti-tumour immune response (Bolden *et al*., 2006; Minucci and Pelicci, 2006).

Here, we report that chromatin-remodelling processes are involved in the regulation of the ULBPs expression in epithelial cancer cells. Furthermore, our results demonstrate that HDAC3 is a repressor of these NKG2D ligands, suggesting that the use of specific inhibitors of HDAC3 may become a powerful strategy to enhance the immunogenicity of tumours through the activation of ULBPs expression in cancer cells.

Results

Inhibition of HDACs by trichostatin A (TSA) treatment induces ULBPs expression and immunogenicity in epithelial cancer cells

It has been widely reported that ULBPs are scarcely expressed on epithelial tumours. In order to investigate whether DNA hypermethylation may be a repressive mechanism of ULBP1 expression in cancer cells, we carried out bisulphite genomic sequencing of the CpG island encompassing the first exon and the minimal promoter of *ULBP1* gene in HeLa and U-937 cells. These studies showed a high degree of hypomethylation of this region (Supplementary Figures 1a and b). Similar results were obtained in the analyses of other epithelial cell lines, which scarcely expressed ULBP1, such as Caco-2 and HepG2 (data not shown). In agreement with these results, treatment of HeLa and HCT-116 cells with 5-aza-2′-deoxycytidine (5-Aza-CdR), an inhibitor of DNA methyltransferases, only caused a slight induction of the ULBP2 protein expression, but not of ULBP1 and ULBP3 in both cell lines (Supplementary Figure 1c). These experiments suggest that DNA methylation is not likely to be the main responsible mechanism for the silencing of ULBPs expression in the cancer cell lines analysed.

We next explored whether other epigenetic mechanisms, such as chromatin-remodelling processes, may be involved in the regulation of ULBP1 expression in epithelial cancer cells. First, we treated HeLa cells with different doses of TSA, an inhibitor of class I and class II HDACs, and ULBP1 mRNA and protein levels were analysed by real-time PCR and flow cytometry. These experiments showed that ULBP1 expression, which was slightly detected in untreated cells, was effectively induced on TSA exposure in a dose-dependent manner (Figures 1a and b).

These results prompted us to analyse whether HDACs inhibition was also involved in the regulation of other members of the ULBP family, such as ULBP2 and ULBP3. For this purpose, *ULBP* promoter constructs were transfected into several epithelial cell lines and, in turn, were treated with TSA or dimethylsulphoxide (DMSO). As shown in Figure 1c, exposure to the HDACi drastically increased *ULBP* promoters activities in all cell lines studied. In agreement with the luciferase assays, TSA treatment strongly increased *ULBP1–3* mRNA (Figure 1d) and surface protein levels (Figure 1e). In contrast, no significant changes in MHC-I expression were observed. Importantly, TSA treatment of HeLa cells led to an increase of the acetylation levels of histones H3 and H4 bound *in vivo* to the *ULBP* promoters, a process widely associated with transcriptional activation (Kouzarides, 2007) (Supplementary Figure 2). Remarkably, exposure to HDACi did not increase ULBPs expression in the primary mononuclear blood cells from healthy donors, suggesting that HDAC-mediated repression of these molecules is specific of tumours (data not shown).

To further analyse the functional relevance of the TSA-mediated induction of ULBPs expression, we carried out cellular cytotoxicity assays by co-culturing HeLa cells, untreated or treated with TSA, and NKL, an NK cell line, which highly expresses NKG2D receptor. As shown in Figure 2, TSA treatment markedly increased the NK-mediated killing of target cells. Furthermore, blocking of ULBP2 surface protein with a monoclonal antibody dramatically inhibited the lysis of tumour cells, indicating that the enhanced cytotoxicity of HeLa cells on TSA treatment is dependent, at least in part, on the ULBP2/NKG2D binding. Overall, these results suggest that repression of ULBPs expression by HDACs may be a general feature of epithelial tumours, and induction of ULBPs by TSA enhances the recognition and NK-mediated elimination of cancer cells.

Identification of TSA-responsive elements in the ULBP1 promoter

As we have earlier characterized the transcriptional regulation of *ULBP1* in HeLa cells (López-Soto *et al*.,

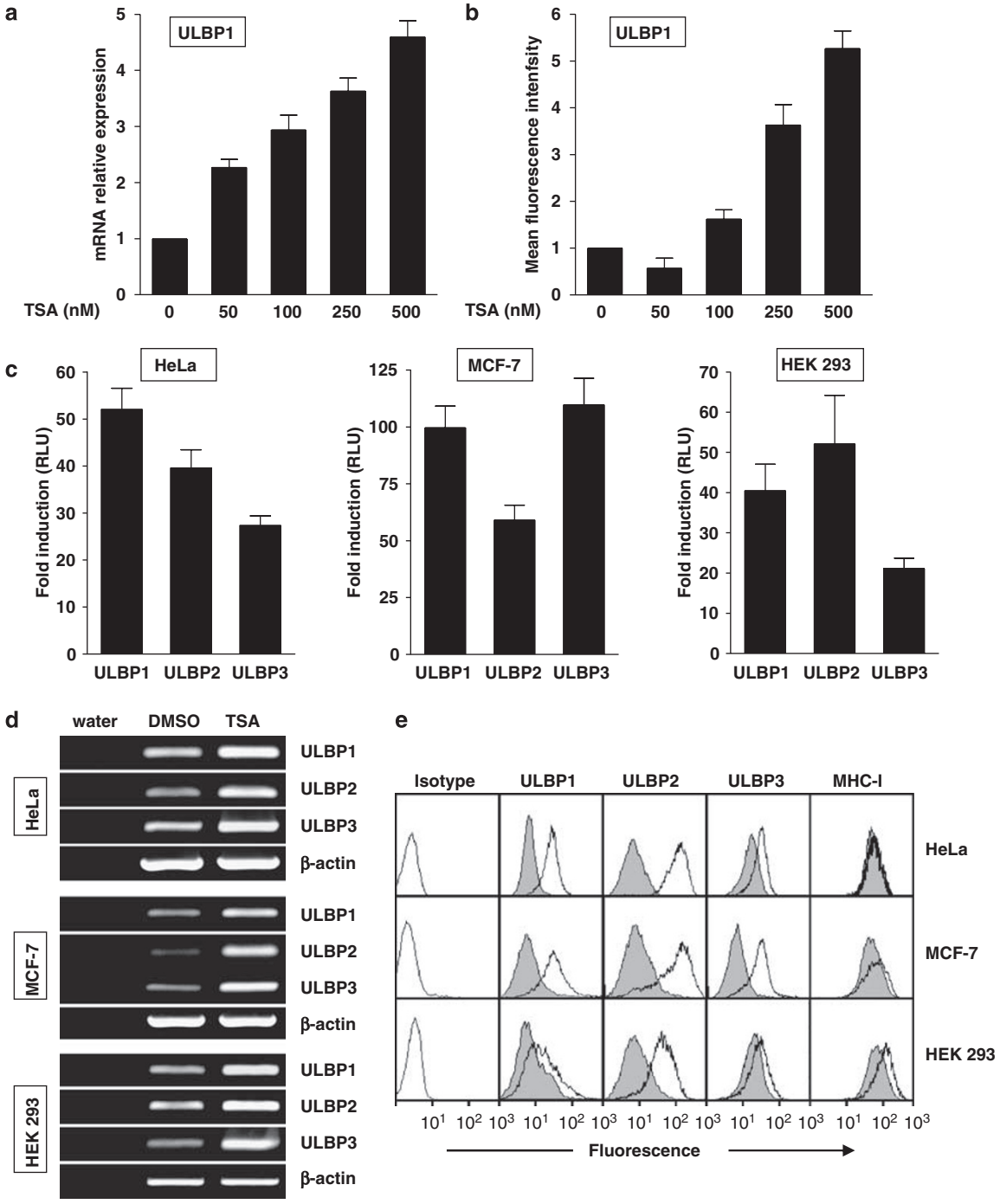

Figure 1 Treatment with trichostatin A (TSA) induces the expression of UL16-binding proteins (ULBPs)1–3 in epithelial cancer cell lines. (**a**) HeLa cells were exposed to different doses of TSA (0, 50, 100, 250 and 500 nM) for 4 h and total RNA was extracted. Real-time PCR was carried out using specific oligonucleotides for ULBP1 and β-actin. Values are means ± s.e.m. of three independent experiments normalized to β-actin. (**b**) Flow cytometry analyses were carried out to monitor ULBP1 protein expression on the surface of HeLa cells treated for 24 h with the same doses of TSA as those used in (**a**). Results are represented as the average of the mean fluorescence intensity (MFI) ratio of three independent experiments ± s.e.m. (**c**) Inhibition of histone deacetylases results in an induction of the *ULBP1–3* promoter activities in epithelial cells. HeLa, MCF-7 and HEK 293 cell lines were transiently transfected with promoter reporter constructs for *ULBP1–3* together with pRSV-β-gal vector and then treated with 100 nM TSA. After 18 h, reporter assays were carried out and luciferase activity was normalized with the β-galactosidase activity. Results are expressed as the average fold induction of relative luciferase activity (RLU) normalized to the vehicle-treated control of three independent experiments made in triplicate ± s.e.m. (**d**) Exposure to TSA increases the transcription of *ULBPs* in epithelial cell lines. *ULBP1–3* transcripts were analysed in HeLa, MCF-7 and HEK 293 cell lines treated with dimethylsulphoxide (DMSO) or 250 nM TSA for 4 h. (**e**) Increased protein expression of ULBP1–3 after TSA treatment. Flow cytometry analyses were carried out in HeLa, MCF-7 and HEK 293 cell lines treated with 250 nM TSA for 24 h, and ULBP1–3 and major histocompatibility complex class I (MHC-I) surface protein levels were determined. All the results shown are representative of three independent experiments.

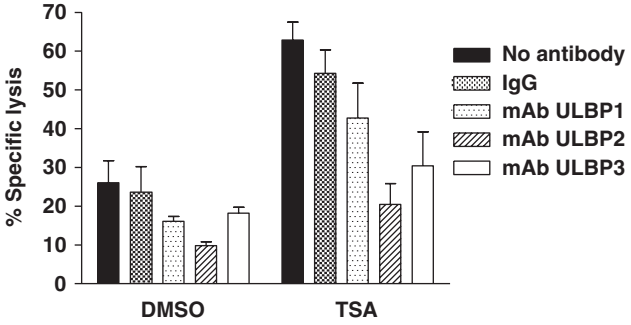

Figure 2 Induction of UL16-binding proteins (ULBPs)1–3 by treatment with trichostatin A (TSA) increases the susceptibility of HeLa cells to natural killer cell-mediated citotoxicity. HeLa cells treated and untreated with 250 nM TSA for 24 h were labelled with PKH67, incubated with monoclonal anti-ULBP antibodies or control IgG (all at 10 μg/ml) and co-cultured with NKL effector cells at a 1:10 ratio for 3 h. Results shown are representative of two experiments carried out in triplicates. DMSO, dimethylsulphoxide.

2006), we decided to gain insight into the mechanisms responsible for the TSA-mediated induction of *ULBP1* transcription in this cell line. To determine the specific region of the *ULBP1* promoter involved in the response to the HDACi, we used reporter plasmids containing progressive deletions of the 5′-flanking sequence of the *ULBP1* gene. The activity of the *ULBP1* minimal promoter (−137/+28) was increased ~50-fold on TSA treatment, a similar result to that observed with the full-length promoter (−862/+28). Likewise, a marked induction of the promoter activity (20-fold) was still detected with the (−127/+28) construct. Furthermore, the region spanning from −137 to −108 was essential in the TSA response, as its deletion practically abolished the *ULBP1* induction by HDACi (Figure 3a). A search for potential binding sites for transcription factors in this region revealed the presence of two CRE-like sequences (CRE(1) and CRE(2)), one GC box (GC(3)) and an overlapping AP-2/GC(4) sequence, which we earlier described as a negative regulatory element of the *ULBP1* promoter activity in HeLa cells (López-Soto *et al*., 2006). The functional relevance of these binding sites in the TSA responsiveness was studied by mutation of their sequences in the *ULBP1* minimal promoter (−137/+28). Interestingly, all the mutations carried out decreased the TSA-mediated activation to a greater or lesser extent, which could be explained by the overlapping of these transcription factor binding sites. However, the most relevant result was obtained with the mutation of the GC(4)/AP-2 binding element, which inhibited the TSA-induced promoter activity by 65% (Figure 3b), suggesting that this sequence is the main element involved in the TSA-mediated induction of the *ULBP1* promoter.

Sp3 is the major transcription factor responsible for the induction of ULBP1 promoter activity by TSA

To determine the nuclear proteins interacting with the TSA-responsive element and the minimal promoter of *ULBP1*, we next carried out supershift assays and chromatin immunoprecipitation (ChIP) analyses using specific antibodies. Consistent with our earlier data (López-Soto *et al*., 2006), these experiments showed that the transcription factors Sp1, Sp3 and AP-2-α were bound to this region of the *ULBP1* promoter (Figures 4a and b). Interestingly, this binding was not significantly changed by exposure to HDACi either *in vitro* or *in vivo*, suggesting that altering the binding properties of these transcription factors is not the main mechanism underlying the ULBP1 induction by TSA.

To further assess the role of Sp1 and Sp3 in the ULBP1 response to TSA, a small interfering RNA approach was used (Figure 4c). Although no significant effect was observed with the silencing of Sp1, depletion of Sp3 dramatically reduced (by ~75%) the increase of the *ULBP1* promoter activity by TSA (Figure 4d). Considering the marked activation of ULBP1 expression observed after TSA treatment in HEK 293 (Figures 1c–e), a cell line which does not express AP-2-α (López-Soto *et al*., 2006), we have ruled out the possibility that this transcription factor may be involved in the ULBP1 response to HDACi. Therefore, these results suggest that Sp3 plays a key role in the induction of ULBP1 by TSA in HeLa cells.

HDAC3 represses the ULBP promoters activities

As inhibition of HDACs by TSA treatment induced ULBPs expression, we next tried to elucidate which HDACs were involved in the repression of ULBPs. First, we transfected expression vectors for HDAC1–3 in combination with the *ULBP* promoter constructs in HeLa cells. HDAC1 overexpression slightly reduced the promoter activity, whereas HDAC2 increased the transcriptional activity of *ULBP1*. By contrast, HDAC3 coexpression markedly repressed the *ULBP1* promoter activity (Figure 5a). Interestingly, among the remaining HDACs that are inhibited by TSA (HDAC4 through HDAC11), only HDAC7, which has been described as associated with HDAC3 activity *in vivo* (Fischle *et al*., 2001), led to a marked inhibition of the *ULBP1* promoter activity (Supplementary Figure 3). As shown in Figures 5b and c, HDAC1 and HDAC3 overexpression had no significant effect on *ULBP2* and *ULBP3*. By contrast, co-transfection of the HDAC2 plasmid induced these promoter activities. To test whether the lack of response of ULBP2 and ULBP3 to HDAC3 was specific to HeLa cells, we overexpressed HDAC3 in HCT-116, a cell line which has been reported that this HDAC represses the *p21* promoter activity (Wilson *et al*., 2006). Our experiments showed that HDAC3 expression inhibited the ULBP1-3 promoters in this cell line. Furthermore, the degree of repression observed was higher than that observed with the *p21* promoter (Figure 5d). Interestingly, transfection of a deletion mutant of HDAC3 (1–373), which lacks deacetylase activity (Yang *et al*., 2002), scarcely suppressed the *ULBP* promoter activities, suggesting that the deacetylase activity of HDAC3 was necessary for the repression of *ULBP* promoters in these cells (Supplementary Figures 4a, b and c). Moreover, transfection of a GFP-HDAC3 expression plasmid into

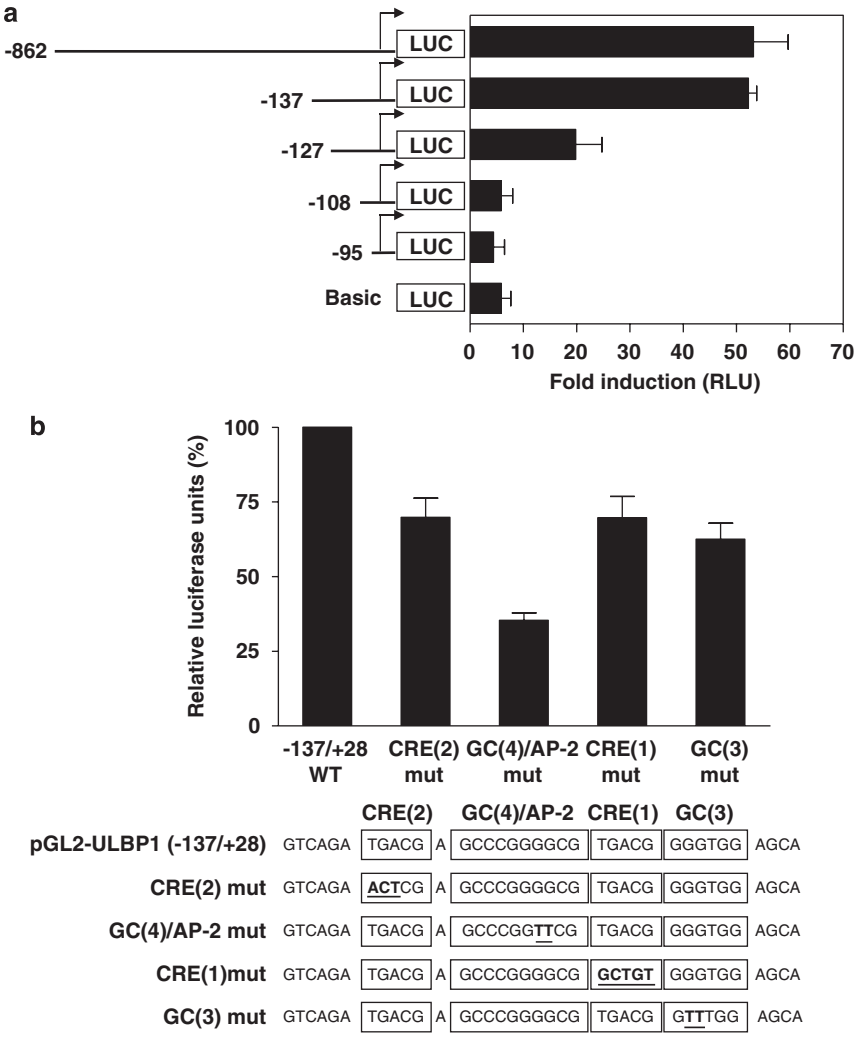

Figure 3 Analysis of trichostatin A (TSA)-responsive elements in the UL16-binding protein (*ULBP*)1 promoter. (**a**) Reporter plasmids containing progressive deletions of the 5′-end of *ULBP1* were transiently transfected into HeLa cells together with the pRSV-β-gal vector, and reporter activities were measured at 18 h after TSA (100 nM) or dimethylsulphoxide (DMSO) treatment. Luciferase activity was normalized with the β-galactosidase activity. Results are expressed as the average fold induction of luciferase activity relative to the vehicle-treated control of three independent experiments made in triplicate ± s.e.m. (**b**) A schematic presentation of the potential binding sequences for transcription factors present in the minimal promoter of *ULBP1* is shown. Reporter plasmids containing different mutations of the *ULBP1* minimal promoter were transiently transfected into HeLa cells as described in panel **a**. Mutated sequences have been underlined in the scheme. Results are expressed as the average fold induction of luciferase activity relative to the vehicle-treated control of three independent experiments made in triplicate ± s.e.m. The luciferase activity obtained with the wild-type construct was set as 100%.

HCT-116 cells resulted in a marked reduction of the ULBPs protein levels on the surface of GFP-positive cells. (Figure 5e).

To further analyse the putative role of HDAC1–3 in the transcriptional regulation of ULBPs, we next silenced the expression of these HDACs in HeLa cells by using a small hairpin RNA approach (Supplementary Figure 5). Knockdown of HDAC1 did not exert any effect on the *ULBP* luciferase activities (Figures 6a–c). In contrast, silencing of HDAC2 strongly inhibited the *ULBP* transcriptional activities. Finally, in agreement with our earlier results of HDACs overexpression, depletion of HDAC3 strongly enhanced the *ULBP* promoter activities. Moreover, knockdown of HDAC3 in HeLa cells markedly increased the ULBPs surface levels in comparison with cells transfected with a control shRNA vector (Figure 6d). The enhancement of ULBP1 protein expression on HDAC3 depletion was also observed by western blot analysis, whereas ULBP2 and ULBP3 antibodies were unable to detect these proteins by this technique (Supplementary Figure 6). Taken together, these experiments suggested that, although HDAC2 may play a positive role in regulating the *ULBP* promoters, HDAC3 is a repressor of the ULBPs expression in epithelial cancer cells.

Treatment with TSA decreased the binding of HDAC3 to the ULBP promoters

To investigate whether the treatment with TSA could modify the binding of HDAC1–3 to the *ULBP*

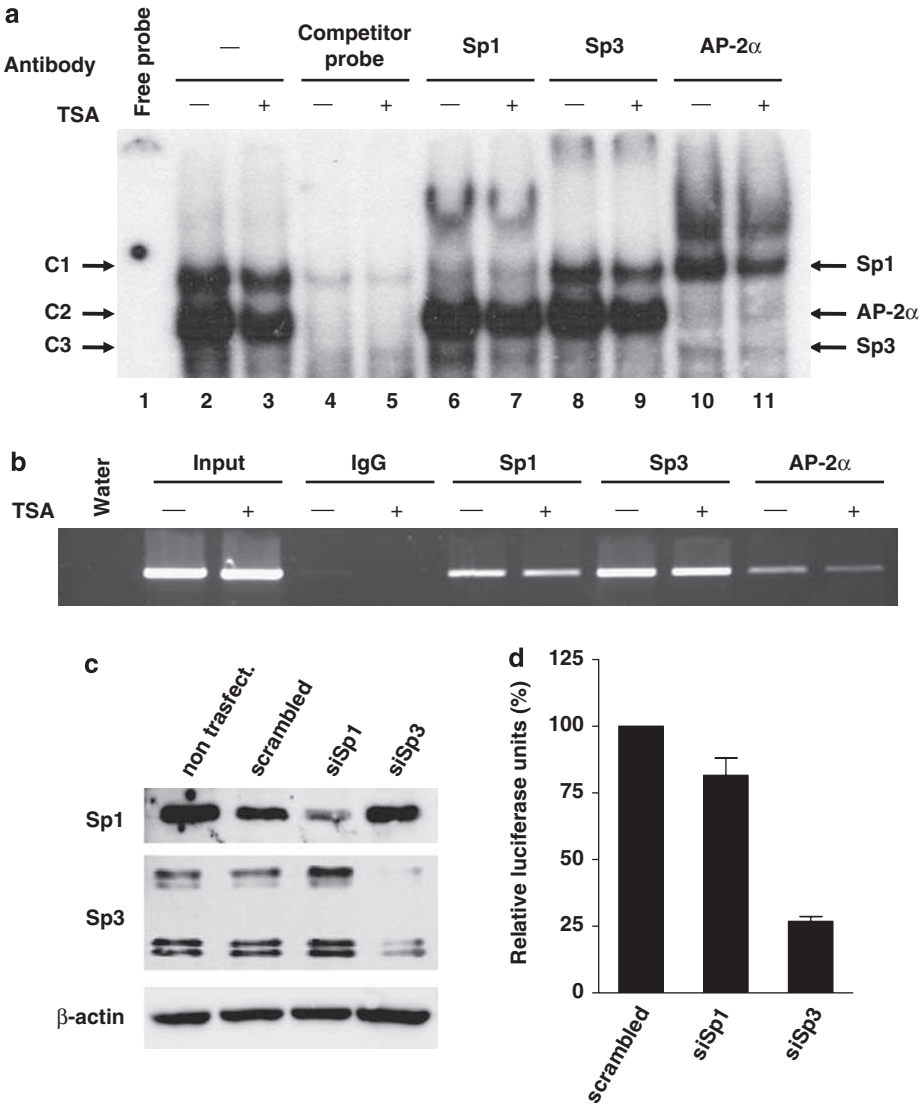

Figure 4 Sp3 is the major factor responsible for trichostatin A (TSA)-mediated activation of the UL16-binding protein (*ULBP*)1 promoter. (**a**) TSA treatment does not alter the binding of Sp1, Sp3 and AP-2-α to the ULBP1 promoter *in vitro*. A supershift assay was carried out to analyse the binding pattern of Sp1, Sp3 and AP-2-α to the main TSA-responsive sequence of the ULBP1 promoter (−131/−99). Antibodies for Sp1, Sp3 and AP-2-α were preincubated with HeLa nuclear extracts obtained after dimethylsulphoxide (DMSO) (−) or TSA (+) treatment before the addition of the probe. Lane 1 shows the free probe and lanes 2–3 show the binding between the probe and the nuclear extracts. In lanes 3–4, an unlabelled ULBP1 100-fold molar excess probe was included. (**b**) *In vivo* binding of Sp1, Sp3 and AP-2-α to the *ULBP1* minimal promoter was examined by chromatin immunoprecipitation analysis. Cross-linked chromatin was immunoprecipitated from sonicated lysates of HeLa cells treated with DMSO (−) or TSA (+) using normal rabbit IgG (lanes 4–5) or antibodies against Sp1 (lanes 6–7), Sp3 (lanes 8–9) and AP-2-α (lanes 10–11) transcription factors. After reversing the cross-linking, DNA was precipitated and PCR was carried out using primers to amplify the *ULBP1* promoter. The PCR products were then run on 2% agarose gels and visualized by ethidium bromide staining. Lane 1 contains a negative control for PCR and lanes 2–3 contain input DNAs used as controls. These data are representative of two independent experiments with similar results. (**c**) Western blotting of the expression of Sp1 and Sp3 in HeLa cells transiently transfected with small interfering RNAs (siRNAs) specific for Sp1 and Sp3 or scrambled. β-actin expression was analysed as a loading control. A representative blot is shown. (**d**) Reporter gene analyses of *ULBP1* minimal promoter in HeLa cells co-transfected with Sp1, Sp3 or scrambled siRNAs. Luciferase activities were measured at 24 h after TSA (100 nM) or DMSO treatment. Results are expressed as the average fold induction of luciferase activity relative to the vehicle-treated control of three independent experiments made in triplicate ± s.e.m. The luciferase activity obtained with the co-transfection of the scrambled siRNA was set as 100%.

promoters *in vivo*, we carried out ChIP analysis in HeLa cells. These experiments showed that these HDACs were bound to the *ULBP* promoters in basal conditions. Exposure to TSA did not significantly alter the occupancy by HDAC2, whereas HDAC1 recruitment slightly decreased in both *ULBP1* and *ULBP2* promoters. Remarkably, TSA treatment led to the complete release of HDAC3 from the *ULBP1–3* promoters (Figure 7a).

Additionally, we carried out ChIP assays to assess whether HDAC3 regulates the chromatin structure at the *ULBP* promoter regions. The experiments showed that depletion of HDAC3 expression in HeLa cells resulted in a marked enhancement of histones H3 and

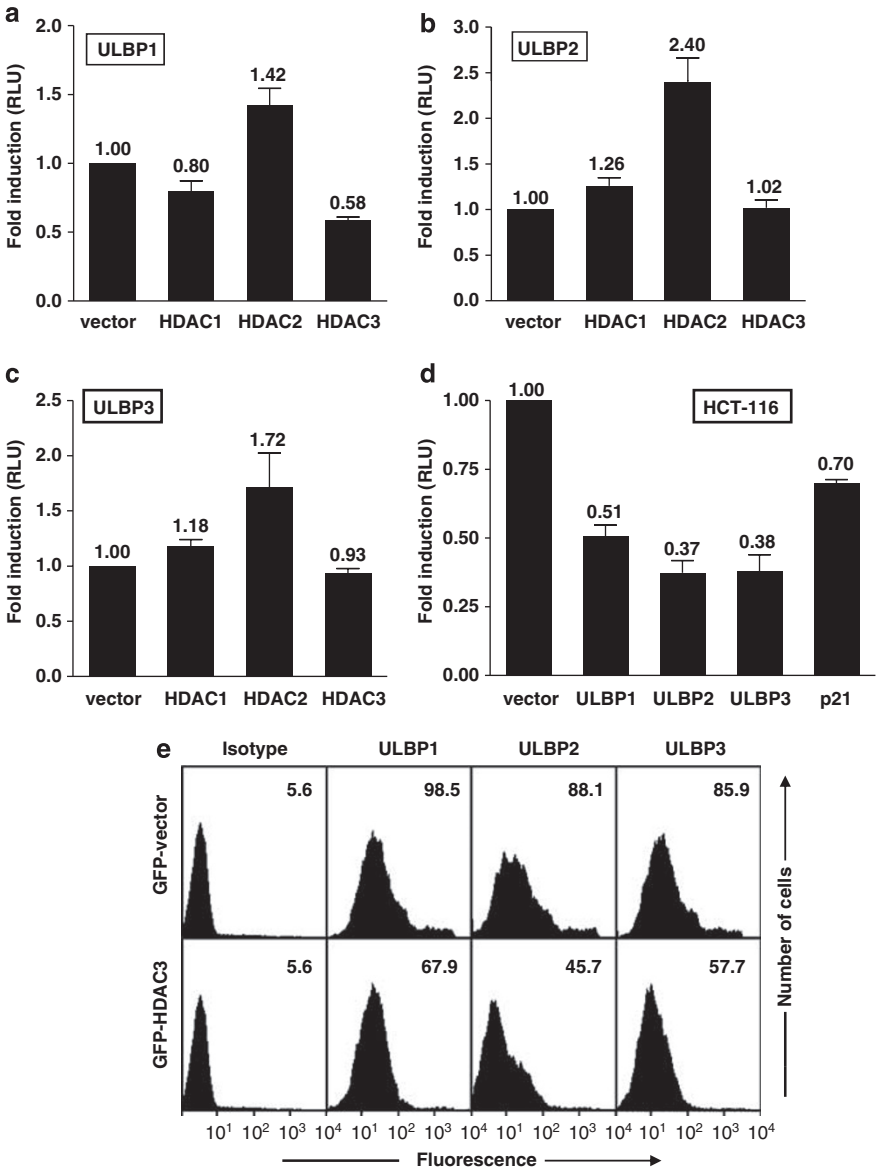

Figure 5 Opposite roles for histone deacetylase (HDAC)2 and HDAC3 in the regulation of UL16-binding protein (*ULBP*) promoters. (**a**–**c**) Backbone vector and plasmids that express HDAC1, HDAC2 and HDAC3 were co-transfected with the *ULBP1–3* promoter reporter constructs and the pRSV-β-gal vector into HeLa cells. Luciferase activities were measured at 72 h after transfection and normalized with the β-galactosidase activity. Data are representative of three independent experiments made in triplicate ± s.e.m. (**d**) HDAC3 represses *ULBP* and *p21* promoter activities in HCT-116 cells. Reporter constructs for *ULBP1–3* and *p21* were co-transfected with the HDAC3 expression vector in combination with the pRSV-β gal vector and luciferase activities were evaluated as indicated in panel **a**. (**e**) Repression of ULBP proteins expression by HDAC3 in HCT-116 cells. pEGFP or GFP-HDAC3 vectors were transfected into HCT-116 cells, and ULBPs protein levels were evaluated on the surface of GFP-positive cells by flow cytometry 72 h later. Data are representative of two independent experiments.

H4 acetylated associated to the *ULBP1–3* promoters (Figure 7b). Consistent with the finding that HDAC3 repressed the ULBPs expression, these data strongly suggest that induction of *ULBPs* transcription by TSA was mediated by a marked decrease in HDAC3 recruitment and local hyperacetylation of the *ULBP1–3* promoters *in vivo*.

HDAC3 is recruited to the ULBP1 promoter by Sp3 in HeLa cells

Considering Sp3 and HDAC3 were the main mediators involved in the ULBP1 response to TSA, co-immunoprecipitation experiments were carried out to investigate whether Sp3 interacts with HDAC3 *in vivo*. To this end, HeLa cells were transfected with plasmids that expressed either wild-type FLAG-HDAC3 (1–428) or C-terminal deleted FLAG-HDAC3 (1–373), and whole-cell extracts were immunoprecipitated using anti-FLAG agarose beads. Indeed, endogenous Sp3 co-immunoprecipitated with both forms of HDAC3 in our cellular model. Furthermore, treatment with TSA almost completely abolished the association between Sp3 and HDAC3 in both cases (Figure 8a). Remarkably, the dissociation of HDAC3 from Sp3 elicited by TSA treatment was not

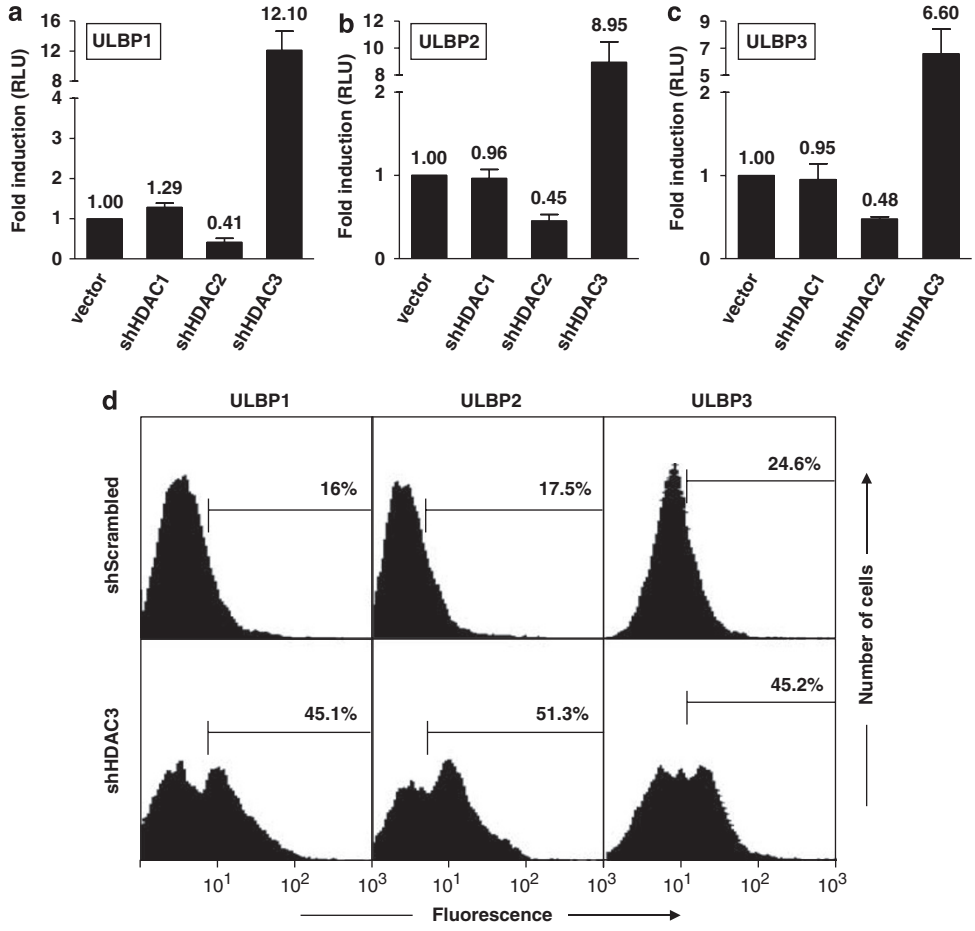

Figure 6 Histone deacetylase (HDAC)3 represses UL16-binding proteins (ULBPs) expression. (**a**–**c**) The pBS/U6 vector or plasmids that express small hairpin RNAs (shRNAs) against HDAC1, HDAC2 or HDAC3 were co-transfected with the *ULBP1–3* reporter constructs into HeLa cells, and the pRSV-β-gal vector and luciferase activities were measured at 72 h after transfection and normalized against the β-galactosidase activity. All results are expressed as the average fold induction of luciferase activity relative to control vector transfection of three independent experiments made in triplicate ± s.e.m. (**d**) Depletion of HDAC3 increases ULBPs protein expression. HeLa cells were co-transfected with plasmids encoding scrambled or HDAC3 shRNAs (5 μg) in combination with pEGFP vector (1 μg), and ULBP1–3 protein levels were monitored by flow cytometry 72 h later. Numbers denote the fragment of gated cells expressing ULBPs above 5% of cells transfected with shScrambled in each case. Data are representative of three independent experiments.

mediated by changes in the expression of this HDAC (Figure 8b). Consistently, small interfering RNA-mediated decrease of Sp3 expression in HeLa cells markedly reduced the *in vivo* recruitment of HDAC3 to the *ULBP1* promoter (Figure 8c). Overall, these results suggest that binding of HDAC3 to the *ULBP1* promoter could be mediated by its association with Sp3 and this interaction could be modulated by TSA treatment.

ULBP1 expression is reduced, whereas HDAC3 is overexpressed in colon cancer

As it has been reported that HDAC3 expression is deregulated in colon cancer (Wilson *et al*., 2006), we decided to examine whether ULBPs expression was also inhibited by HDACs in this malignancy. Thus, treatment of several colorectal adenocarcinoma cell lines (HT-29 M6, SW480 and HCT-116) with TSA markedly enhanced the ULBP1 and ULBP2 surface levels, whereas ULBP3 and MHC-I expression was poorly increased (Figure 9a), indicating that chromatin-remodelling processes are actively involved in regulating the ULBPs expression in colon cancer cells.

We next analysed the protein expression of ULBP1–3 by immunohistochemistry in 26 snap-frozen specimens of colon carcinomas and normal tissues obtained from patients who had undergone surgical resection. Only expression of ULBP1, but not of ULBP2 or ULBP3, was detected in tissue samples using the antibodies currently available. Thus, ULBP1 staining was observed in the apical part of the colonocytes obtained from normal colon samples. However, ULBP1 expression gradually decreased in the colorectal carcinomas of higher tumour grade (Figure 9b and Table 1). By contrast, weak expression of HDAC3 was observed in the nucleus of normal colonocytes, whereas a high number of the colorectal carcinomas analysed (65.3%) displayed a strong nuclear staining of HDAC3 (Figure 9b and Table 1). Interestingly, in adenomas and more differentiated carcinomas, where the tumour

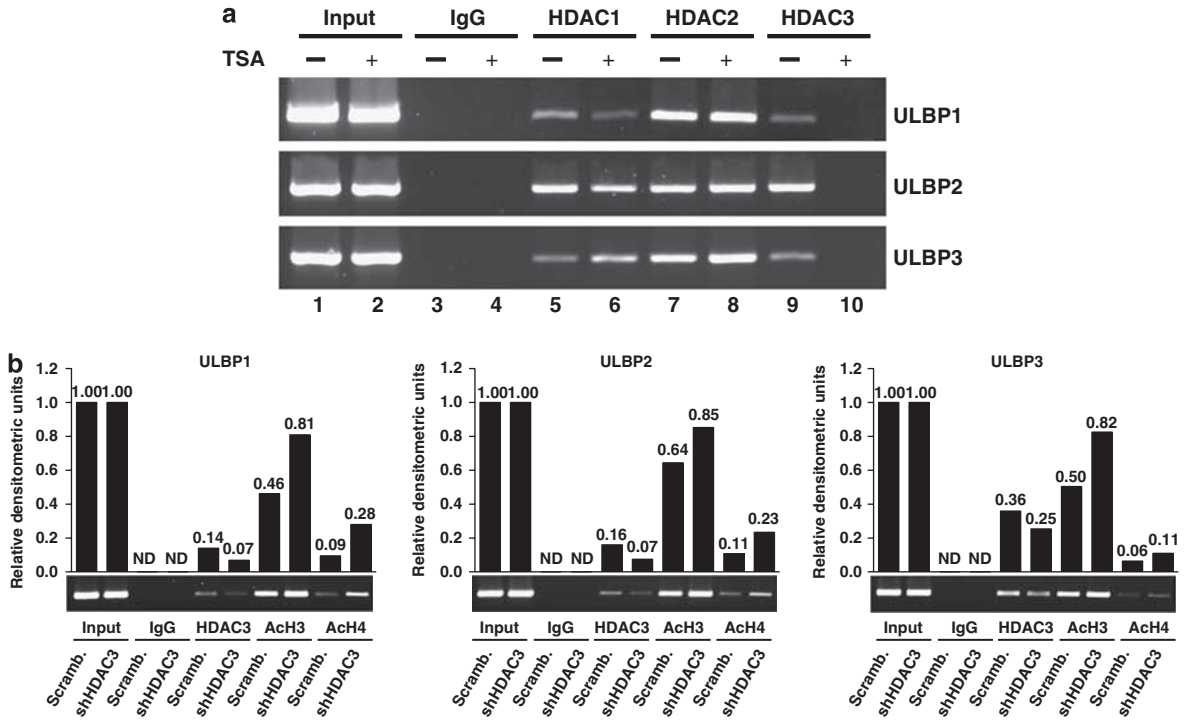

Figure 7 Loss of histone deacetylase (HDAC)3 interaction with the UL16-binding protein (*ULBP*)1–3 promoters after trichostatin A (TSA) treatment. (**a**) *In vivo* binding of HDAC1, HDAC2 and HDAC3 to the *ULBP1* (top panel), *ULBP2* (middle panel) and *ULBP3* (bottom panel) promoters was examined by chromatin immunoprecipitation analysis. Cross-linked chromatin was immunoprecipitated from sonicated lysates of HeLa cells treated with dimethylsulphoxide (DMSO) (−) or 250 nM TSA (+) using normal rabbit IgG (lanes 3–4) or antibodies against human HDAC1 (lanes 5–6), HDAC2 (lanes 7–8) and HDAC3 (lanes 9–10) proteins. After reversing the cross-linking, DNA was precipitated and PCR was carried out using primers to amplify the *ULBP1*, *ULBP2* and *ULBP3* promoter regions. Lanes 1–2 contain input DNAs used as controls. These data are representative of two independent experiments with similar results. (**b**) Depletion of HDAC3 expression increases H3 and H4 histones acetylation at the *ULBP1–3* promoters. Cross-linked chromatin was immunoprecipitated from sonicated lysates of HeLa cells transfected with scrambled or HDAC3 small hairpin RNAs for 72 h, and chromatin was immunopreciptated using normal rabbit IgG (lanes 3–4), with specific antibodies against HDAC3 (lanes 5–6) or with antibodies against acetylated H3 (AcH3) (lanes 7–8) and H4 (AcH4) (lanes 9–10) histones. After reversing the cross-linking, DNA was precipitated and PCR was carried out using primers to amplify the *ULBP1* (left panel), *ULBP2* (middle panel) and *ULBP3* (right panel) promoter regions. Lanes 1–2 contain input DNAs used as controls. Signal intensities obtained from ethidium bromide-stained gels were quantified using the ImageJ software (NIH) and normalized to input DNA signals. These data are representative of two independent experiments with similar results. ND, non-detected.

cells keep a polarized form, a cytoplasmic expression of HDAC3 was also observed. Thus, HDAC3 is over-expressed in colon cancer samples, whereas ULBP1 expression is low or absent in these tumours.

Discussion

The expression of NKG2D ligands in transformed cells leads to their recognition and elimination by the immune system. However, a loss of immunogenicity is a common hallmark of cancer cells that evade the tumour-controlling features of the host defense system (Dunn *et al*., 2004b). Several reports have demonstrated that non-mutational epigenetic events, such as DNA methylation and chromatin remodelling, are involved in tumour-immune evasion processes (Maio *et al*., 2003). In this sense, we investigated whether the epigenetic mechanisms may account for the repression of the ULBP family of NKG2D ligands in epithelial cancer cells. Our results suggest that DNA methylation does not play a major role in the regulation of ULBPs expression. By contrast, treatment with a chromatin-remodelling agent, such as TSA, resulted in a marked increase of ULBPs transcription and protein levels in several epithelial cancer cells, whereas no effect was detected in the mononuclear blood cells from healthy donors, suggesting that epigenetic repression of ULBPs is specific to tumour cells, which may undergo evasion strategies to increase immune resistance.

We show that exposure to TSA stimulates the NK-mediated lysis of HeLa cells through the induction of ULBPs, mainly ULBP2, expression, whereas this treatment did not elicit a significant effect on the MHC-I surface levels. These data clearly support the potential therapeutic relevance of inducing ULBPs expression in tumour cells through the use of HDACi in order to improve the host immune response against cancer.

Studies performed with other NKG2DLs showed that TSA treatment decreased the HDAC1 association at their promoters (Kato *et al*., 2007). However, our experiments did not show a relevant role for HDAC1 in the regulation of *ULBP* promoters. Recently, it has been described that inactivation of HDAC2 by truncating

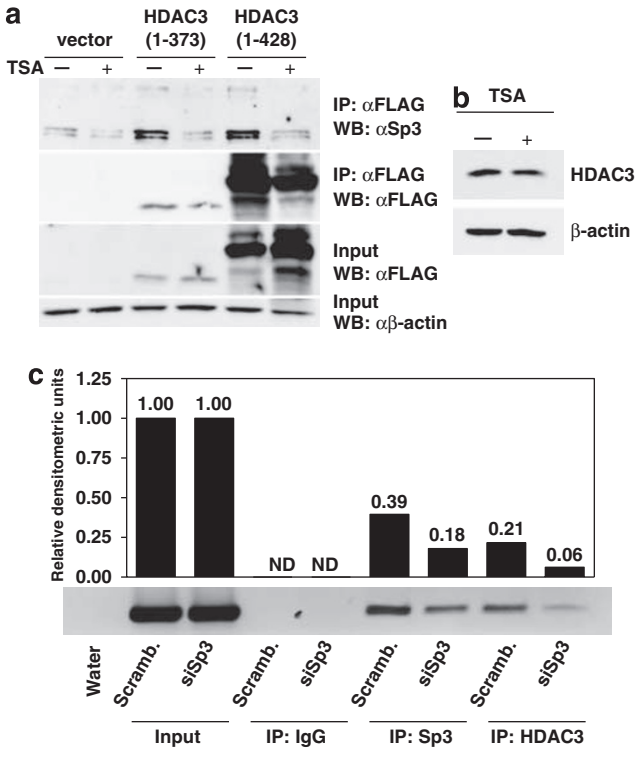

Figure 8 Analysis of histone deacetylase (HDAC)3 and Sp3 interaction. (**a**) HeLa cells were transfected with plasmids that express full-length FLAG-HDAC3 (1–428), FLAG-HDAC3 deletion (1–373) or the backbone vector. Lysates were prepared from transfected HeLa cells treated with dimethylsulphoxide (DMSO) (−) or 250 nM trichostatin A (TSA) (+) for 24 h and were immunoprecipitated (IP) with anti-FLAG-M2 agarose-conjugated beads followed by western blot (WB) analysis with anti-Sp3 (top panel). Protein expression efficiencies were assessed by western blot with anti-FLAG antibody (middle panel). β-actin expression was analysed as a loading control (bottom panel). Blots are representative of two independent experiments (**b**) HDAC3 protein levels were analysed by western blotting in HeLa cells treated with DMSO or 250 nM TSA for 24 h. (**c**) Chromatin immunoprecipitation assays showing reduced occupancy of HDAC3 to the UL16-binding protein (*ULBP*)1 promoter in cells expressing small interfering RNAs (siRNAs) targeting Sp3. Chromatin of HeLa cells transfected with scrambled and Sp3 siRNAs for 48 h was immunoprecipitated with a control IgG (lanes 4–5) or using specific antibodies for Sp3 (lanes 6–7), HDAC3 (lanes 8–9), and purified DNA was amplified by PCR using primers designed to amplify the *ULBP1* proximal promoter. Lane 1 contains a negative control for PCR and lanes 2–3 contain input DNAs used as controls. Signal intensities obtained from ethidium bromide-stained gels were quantified using the ImageJ software (NIH) and normalized to input DNA signals. Data are representative of two independent experiments. ND, non-detected.

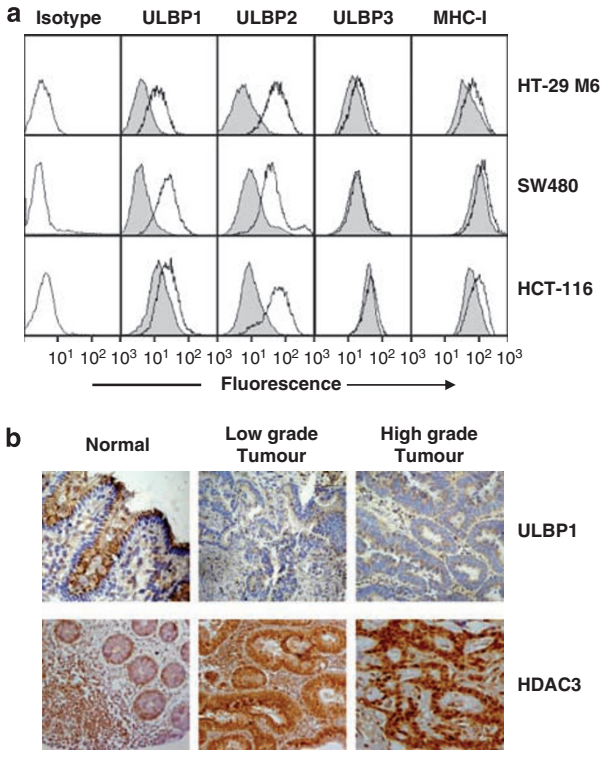

Figure 9 UL16-binding protein (ULBP)1 expression decreases with colon cancer tumour grade. (**a**) ULBP1–3 protein expression increases after trichostatin A (TSA) treatment in colon cancer cell lines. Flow cytometry analyses were carried out in HT-29 M6, SW480, and HCT-116 colon adenocarcinoma cells treated with dimethylsulphoxide (DMSO) or 250 nM TSA for 24 h, and ULBP1–3 and major histocompatibility complex class I (MHC-I) surface protein levels were determined. The results shown are representative of three independent experiments. (**b**) Analysis of ULBP1 and histone deacetylase (HDAC)3 expression in human colon cancer. Immunohistochemistry analysis of the protein expression of ULBP1 and HDAC3 were carried out in normal colon tissues (left panel), low-grade (middle panel) and high-grade (right panel) colon adenocarcinoma samples.

mutation upregulates several tumour-promoting genes in cancer cells (Ropero *et al*., 2008). Consistent with this finding, our results also suggest that HDAC2 may favour the immunogenicity of cancer cells by enhancing the transcriptional activity of *ULBPs*. Remarkably, our data showed that HDAC3 is a repressor of the ULBPs expression. Thus, knockdown of HDAC3 in HeLa cells markedly increased the ULBP1–3 promoter activities and protein levels, whereas overexpression of this HDAC reduced the *ULBP1* transcriptional activity. HDAC3 was also able to repress the ULBP2 and ULBP3 promoters and protein expression in HCT-116 colon cancer cells through its deacetylase activity, whereas no repression was achieved in HeLa cells. This result could be explained if higher amounts of endogenous HDAC3 were bound to these promoters, thereby leading to an HDAC3-saturated repressed state. Furthermore, we showed that TSA promotes the complete dissociation of HDAC3 and, thereby increases the acetylation status of H3 and H4 histones in the vicinity of *ULBP* promoters. Consistent with our findings, it has been recently reported that liver-specific deletion of *Hdac3* in a genetically modified mouse model resulted in an increased expression of two members of the ULBP/RAET1 family (Knutson *et al*., 2008). Likewise, silencing of HDAC3 expression in colon cancer resulted in an enhancement of apoptosis (Wilson *et al*., 2006). According to the pro-survival role of HDAC3 in cancer cells (Karagianni and Wong, 2007), our discovery that HDAC3 is a repressor of the ULBPs expression may represent an additional mean by which tumour cells evade the immune-surveillance. In agree-

Table 1 UL16-binding protein 1 (ULBP1) and histone deacetylase 3 (HDAC3) expression in colorectal adenocarcinomas

Histopathological grade	*Number of tumours*	*ULBP1 expression*	*HDAC3 expression*
Well-differentiated	10	8 (80%)	5 (50%)
Moderately differentiated	7	3 (42%)	5 (71%)
Poorly differentiated	9	3 (33%)	7 (77%)

ment, our results showed that HDAC3 is upregulated in colon cancer samples, whereas ULBP1 expression is reduced in colorectal tumours *in vivo*.

Reporter analyses showed the dependence of the GC/AP-2 binding site in the TSA-mediated induction of *ULBP1* promoter. Sp1, Sp3 and AP-2-α interact with this sequence and this association was not significantly altered by TSA. We have reported earlier that Sp1, and mainly Sp3, are the key transactivators of the ULBP1 basal expression (López-Soto *et al*., 2006). In addition, we show that Sp3 is also critical for the induction of ULBP1 by TSA. GC-boxes are Sp1/Sp3 binding sites, which play crucial roles in HDACi-responses (Sowa *et al*., 1999; Yokota *et al*., 2004). Likewise, it has been shown that Sp1, but not Sp3, is important for the induction of *MICA* promoter activity by HDACi (Andresen *et al*., 2007). Consistently, our results suggest that diverse molecular mechanisms may be involved in the HDACi-mediated regulation of the expression of different NKG2DLs. Several works have reported that Sp3 interacts with HDAC1 and HDAC2 (Sun *et al*., 2002; Ammanamanchi *et al*., 2003). However, to our knowledge, this is the first time that an association between Sp3 and HDAC3 has been shown. In agreement with the ChIP analyses, the decrease of the binding between HDAC3 and Sp3 on TSA treatment and the observation that the *in vivo* association of HDAC3 with the *ULBP1* promoter diminished in Sp3-depleted cells reinforces the hypothesis that HDAC3 is recruited to the *ULBP1* regulatory region by interaction with this transcription factor. It is also conceivable that TSA might modify the acetylation status of any of these interacting proteins or other members of the complex. In this regard, it has been reported that Sp3 activity is tightly regulated by acetylation and acetylated Sp3 plays a role as a transcriptional activator (Ammanamanchi *et al*., 2003; Wooten-Blanks *et al*., 2007).

On the basis of our results, a model for the epigenetic regulation of ULBP1 expression in epithelial tumour cells may be mediated by Sp3-recruited HDAC3, which in turn regulates the structure of chromatin in the vicinity of the *ULBP1* promoter. Therefore, TSA treatment might modulate this interaction, leading to the release of HDAC3. Likewise, this process may favour the access of other molecular partners, such as p300, which has been reported to acetylate Sp3 (Braun *et al*., 2001), as well as the recruitment of the general transcription machinery to the *ULBP1* promoter (Figure 10).

Overall, we showed that chromatin remodelling regulates the expression of ULBPs and treatment with TSA effectively enhances their protein levels in epithelial cancer cells. Our results are in agreement with the recent findings that other NKG2DLs are also induced by HDACi, which turn the use of chromatin-remodelling drugs into a powerful strategy to increase cancer cell susceptibility to NK-mediated cytotoxicity. In this sense, our finding that HDAC3 is a repressor of ULBPs expression in epithelial cancer cells suggests that the design of specific inhibitors that selectively target this HDAC may improve the therapeutical efficiency of these drugs against cancer through the enhancement of anti-tumour immune responses.

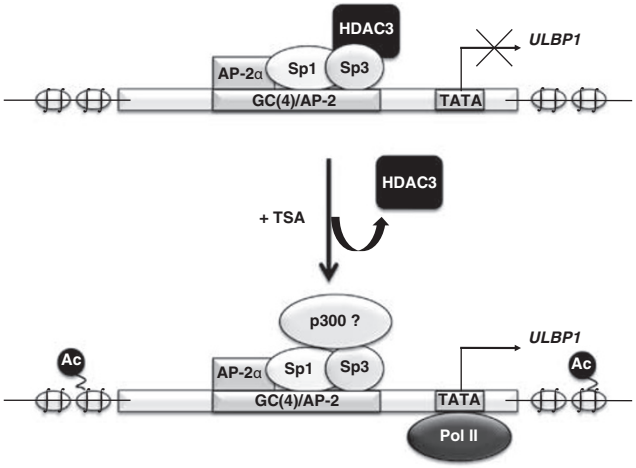

Figure 10 Hypothetical model of UL16-binding protein (*ULBP*)*1* activation by trichostatin A (TSA). Histone deacetylase (HDAC)3 is recruited to the *ULBP1* promoter by the transcription factor Sp3, leading to the repression of the *ULBP1* transcription. Inhibition of HDACs activity by TSA treatment leads to the release of HDAC3 to the *ULBP1* promoter and, thereby, to the enhancement of the acetylation status of histones in the vicinity of this region, resulting in the activation of the *ULBP1* expression. Likewise, the remodelling of the complexes interacting with the *ULBP1* promoter on TSA treatment might favour the recruitment of other proteins, such as the histone acetyl-transferase p300, increasing *ULBP1* transcription.

Materials and methods

Cell culture

HeLa, HEK 293, MCF-7, SW480, HCT-116, U-937 (from the American Type Culture Collection) and HT-29 M6 cells (provided by Dr García de Herreros, IMIM, Barcelona, Spain) were maintained as described elsewhere.

Cytotoxicity assays

HeLa cells were treated with 250 nM TSA (Sigma-Aldrich, St Louis, MO, USA) or dimethylsulphoxide (DMSO) for 24 h, and 10^5 cells were labelled using the PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich) as described before with

minor modifications (Kottilil *et al*., 2006). In blocking experiments, target cells were incubated for 45 min with monoclonal antibodies against ULBP1 (M295), ULBP2 (M311), ULBP3 (M551) (kindly provided by Amgen, Seattle, WA, USA) or control IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) added at a concentration of 10 μg/ml. Then, NKL cells activated overnight with interleukin-2 (IL-2, PeproTech, London, UK) were added at a 10:1 ratio (effector:target cells) and incubated with the target cells for 3 h at 37°C, 5% CO_2. Afterwards, cells were stained with 7AAD (Sigma-Aldrich) to test cell viability. The per cent NK-specific lysis was calculated using the formula: NK-specific lysis = ((per cent 7AAD staining of sample − per cent 7AAD staining of negative control)/(100 − per cent 7AAD staining of negative control)) × 100.

RNA isolation and reverse transcriptase–PCR
Total RNA was extracted using the NucleoSpin kit (Macherey-Nagel, Düren, Germany) and was reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Barcelona, Spain) and oligo(dT). PCR reactions were carried out using the primers provided in Supplementary Table 1. PCR conditions used for ULBPs amplification are detailed in Supplementary materials.

Plasmids, transfections and luciferase assays
Description of the reporter and expression plasmids used and transfection procedures are described as Supplementary materials.

EMSA and ChIP assays
EMSA assays were carried out as described earlier (López-Soto *et al*., 2006). Briefly, nuclear extracts from HeLa cells treated or untreated with TSA for 24 h were prepared as described elsewhere (Andrews and Faller, 1991). Binding reactions were carried out using a radiolabelled double-stranded oligonucleotide corresponding to positions −131/−99 of the *ULBP1* promoter (TGACGAGCCCGGGGCGT GACGGGGTGGAGCATC). In supershift assays, 2 μg of anti-Sp1, -Sp3 or -AP-2-α antibodies were incubated for 60 min at 4°C with the nuclear extracts before adding the probe. ChIP experiments were carried out using the ChIP assay kit from Upstate Biotechnology as described in Supplementary Materials.

Western blotting and co-immunoprecipitation
Western blot experiments were carried out as described earlier (López-Soto *et al*., 2006). Blots were incubated overnight at 4°C with specific primary antibodies against ULBP1 (R&D systems, Minneapolis, MN, USA), Sp1, Sp3, HDAC1, HDAC2 (all from Santa Cruz Biotechnology), HDAC3 (Abcam, Cambridge, UK) and β-actin (Sigma-Aldrich). Finally, blots were incubated with horseradish peroxidase-conjugated secondary antibodies and developed with a chemiluminescent detection kit (Millipore, Bedford, MA, USA). Co-immunoprecipitation experiments were carried out as described in Supplementary materials.

Flow cytometry
Cells treated with TSA for 24 h were incubated with primary monoclonal antibodies against ULBP1, ULBP2 and ULBP3 (Amgen) or with W6/32 anti-MHC-I for 30 min on ice. After washing, cells were stained with a phycoerythrin-labelled goat anti-mouse secondary antibody (Serotec, Oxford, UK) for 30 min in the dark, and were analysed on a BD Biosciences FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA). Staining intensities were defined by the mean fluorescence intensity (MFI) ratios standardized using the following formula: ((MFI treatment) − (MFI Isotype control)/(MFI vehicle) − (MFI Isotype control)).

Immunohistochemistry
Human colon samples embedded in optimum cutting temperature and frozen in liquid nitrogen were used. Sections (7-μm thick) were blocked with 1% bovine serum albumin and incubated with monoclonal antibodies against ULBP1 (4G7, provided by Dr T Spies, Fred Hutchinson Cancer Research Center, Seattle, WA, USA), ULBP2 and ULBP3 (Amgen). Sections were washed with PBS and incubated with an anti-mouse EnVision system-labelled polymer (Dako-Cytomation, Barcelona, Spain) for 30 min, washed in buffer solution, visualized with diaminobenzidine and counterstained with Mayer's hematoxylin. This study involving human individuals was approved by the ethical committee of the Hospital Universitario Central de Asturias.

Conflict of interest

Dr Seto's work has been funded by the NIH. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)