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TSA downregulates *Wilms tumor gene 1* (*Wt1*) expression at multiple levels

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ABSTRACT

The Wilms tumor gene *WT1* encodes a zinc-finger transcription factor that is inactivated in a subset of pediatric kidney cancers. During embryogenesis, *WT1* is expressed in a time- and tissue-specific manner in various organs including gonads and kidney but also in the hematopoietic system. Although widely regarded as a tumor suppressor gene, wild-type *WT1* is overexpressed in a variety of hematologic malignancies, most notably in acute lymphoblastic leukemia as well as myelodysplastic syndromes. Reduction of *WT1* expression levels leads to decrease of proliferation and apoptosis of leukemic cells, suggesting that in certain contexts *WT1* might act as an oncogene. We show here that histone deacetylase inhibitors like Trichostatin A (TSA) can promptly and dramatically downregulate *Wt1* expression levels in different cell lines. This effect was mostly due to the cessation of transcription and was mediated by sequences located in intron 3 of *Wt1*. In addition, TSA also caused enhanced degradation of the Wt1 protein by the proteasome. This was at least in part due to induction of the ubiquitin-conjugating enzyme UBCH8. Thus, downregulation of *Wt1* expression might contribute to the beneficial effects of histone deacetylase inhibitors that are currently used in clinical trials as cancer therapeutics.

INTRODUCTION

The Wilms tumor gene *WT1* was originally identified as a tumor suppressor gene lost in 10–15% of Wilms tumors (1,2) and is a member of the GC- rich TATA-less and CCAAT-less class of RNA pol II genes (3). The *WT1* transcript encompasses 3.5 kb and encodes a four zinc-finger containing protein with an essential role in the development of several organs, most notably the kidney (4–6). More than 20 different *WT1* gene products with molecular masses of 52–65 kDa are generated by a combination of alternative RNA splicing, the usage of different start codons and RNA editing (7).

Expression of the wild-type *WT1* gene has been found in most cases of acute myelocytic leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelocytic leukemia (CML) and myelodysplastic syndrome (MDS) at higher levels than those in normal bone marrow or peripheral blood (8–10). WT1 is used as a prognostic factor and marker for minimal residual disease in cases of acute leukemia (9,11). Furthermore, various types of solid tumors, including lung, breast, thyroid, esophageal and colorectal cancers express wild-type *WT1* at higher levels compared to those in corresponding normal tissues (12).

In several studies, the role of Wt1 in cell proliferation, differentiation and leukemogenesis has been analyzed. In the chronic myeloid leukemia cell line K562 as well as in primary leukemic cells from human patients, *WT1* antisense oligomers inhibited growth via reduction of WT1 protein levels (13). In the same cell line, ribozyme-mediated downregulation of *WT1* led to inhibition of cell proliferation and apoptosis (14). Similarly, siRNA-mediated reduction of *WT1* mRNA levels in various leukemic cell lines including those from AML and CML patients inhibited proliferation and induced apoptosis (15). Taken together, all these studies indicate that Wt1 may be necessary for leukemic or solid tumor growth survival and that under certain circumstances *Wt1* could act as an oncogene (12). This is corroborated by the recent observation in mice that the chimeric oncoprotein AML1-ETO exerts its leukemogenic function in cooperation with *Wt1* expression (16).

Conversely, removal of WT1 may have an anticancer effect. That this is indeed the case was recently demonstrated by vaccination of patients with AML, MDS as well as lung or breast cancer with a WT1 peptide. WT1 vaccination led to an increase in WT1-specific cytotoxic T lymphocytes and subsequent cancer regression without

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damage to other normal tissues (17). Thus, in particular cancers WT1 could be a therapeutic target and downregulation of WT1 might be a promising anticancer strategy.

For transcription to begin in eukaryotes, concerted actions of multiple protein factors are required. The major hurdle in activating transcription *in vivo* is the highly compacted nature of chromatin, which prevents access of the transcription machinery to the DNA template. Posttranslational modifications of histones such as acetylation, phosphorylation, methylation, ubiquitination, ADP-ribosylation, sumoylation and biotinylation are assumed to be important factors that control chromatin accessibility and subsequent gene transcription. It is this association between histone modification and the activity state of the chromatin for which the expression 'histone code' has been coined (18).

The best understood modification is acetylation of core histones, which is carried out by histone acetyl transferases (HATs); the steady state levels of acetylation are maintained by the opposing activities of HATs and histone deacetylases (HDACs) (19). So far at least 18 HDACs have been identified in humans and have been grouped into four different classes (20). Class I members (HDACs 1–3 and 8) are most closely related to the *Saccharomyces cerevisiae* transcriptional regulator RPD3. Class II HDACs (4–7, 9 and 10) display similarity to yeast HDA1; class III comprises the NAD^+-dependent sirtuin deacetylases SIRT 1–7 and class IV consists of proteins related to the recently identified human HDAC11, which shares features of both class I and II. HDACs associate with a number of oncoproteins and tumor suppressors and in case of their aberrant activation or inactivation the concomitant HDAC activity can lead to undue changes in gene expression and in turn to diseases, e.g. cancer (21). This observation has stimulated the identification and characterization of HDAC inhibitors as means to counteract disease-associated aberrant gene expression.

Trichostatin A (TSA), a *Streptomyces* product was originally identified as a fungicidic antibiotic. It inhibits all class I and II HDACs and has potent antiproliferative properties in cancer cells (22,23). Differential display analysis revealed that expression of only 2–5% of genes in TSA-treated cells is significantly altered (24). The basis for this gene selectivity is not yet understood, but it suggests that only a highly restricted set of genes is sensitive to changes in histone acetylation (25,26). Another HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA) inhibits the growth of prostate cancer cells in culture as well as in a xenograft model (27). Moreover, TSA also inhibits hypoxia-induced angiogenesis in the Lewis lung carcinoma model (28). Thus, histone deacetylases are promising drug targets and clinical trials using first generation HDAC inhibitors as anticancer reagents are currently under way (29).

Here, we report the effect of HDAC inhibitors like TSA on *Wt1* expression. TSA led to a drastic downregulation of *Wt1* mRNA levels in different human and mouse cell lines. This effect was mostly due to a reduction of *Wt1* transcription. In addition, TSA induced degradation of Wt1 protein via the proteasome. This effect was at least in part mediated by the ubiquitin-conjugating enzyme UBCH8.

MATERIALS AND METHODS

Cell lines and drug treatment

Mouse mesonephric M15 as well as Leydig like TM3 cells and human embryonic kidney 293 cells were maintained in DMEM, human chronic myelogenous leukemia K562 cells were kept in RPMI medium supplemented with 10% FCS (Gibco, Paisley, Pennsylvania, USA) at 37°C with 5% CO_2. For drug studies, exponentially growing cells were trypsinized, seeded at 40% confluency, grown for 24 h and then treated with TSA (Sigma, St. Louis, Missouri, USA), valproic acid (VPA, Sigma), SAHA (Merck, Whitehouse Station, New Jersey, USA) or MS-275 (Alexis Biochemicals, San Diego, California, USA) for 24 h. In actinomycin D (Sigma) experiments, cells were pretreated with 4 μM actinomycin D for 1 h prior to TSA addition.

In order to assess the requirement for ongoing protein synthesis, M15 cells were treated with TSA (500 nM) and cycloheximide (50 μg/ml) as described above for 20 h. Similarly, M15 cells were treated with TSA (500 nM) and/or lactacystine (10 μM) (Sigma) for 6 and 20 h. To determine the half-life of Wt1 protein in the presence of TSA (500 nM), VPA (1.5 mM) or SAHA (5 μM), M15 cells were treated with inhibitors alone or in combination with cycloheximide (50 μg/ml). Cells were harvested at indicated time points and used for further analysis.

RNA isolation and quantitative RT–PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. RNA samples were treated with DNase RQ1 (Promega, Madison, Wisconsin, USA) to remove any genomic DNA. cDNA was synthesized from 2 μg total RNA using the SuperScript II RT kit (Invitrogen).

Real-time PCR reaction was carried out employing the QuantiTect SYBR green real-time PCR kit (Qiagen, Hilden, Germany) on a Biorad iCycler in a 96-well format. PCR conditions were: 95°C for 15 min, followed by 40 cycles of three-step PCR including melting for 30 s at 95°C, annealing for 30 s at 60°C and elongation for 30 s at 72°C. Primers used were: *Wt1F AGTTCCCCAACCA TTCCTTC*, *Wt1R TTCAAGCTGGGAGGTCATTT*, *WT 1F CAGTTCCCCAACCACTCATT, WT1R AAGCTGG GATGTCATTTGGT, UBCH8F GATGCCAATGTCC TGGTGT*, *UBCH8R GCAAATCTGTCCGTTCTCGT*, *β-ActinF TGTTACCAACTGGGACGACA*, *β-ActinR GGGGTGTTGAAGGTCTCAAA*, *GAPDHF AACAGCG ACACCCACTCCTC, GAPDHR GGAGGGGAGATTCA GTGTGGT*. Expression levels were determined in one plate for all samples simultaneously and normalized to the corresponding amounts of *β-Actin* or *GAPDH* cDNA measured within the same plate. Relative expression levels where calculated using the $2^{-\Delta\Delta CT}$ method (30).

Chromatin immunoprecipitation (ChIP) assay

A total of 2×10^6 M15 cells (treated with 500 nM TSA or ethanol for 24 h) were cross-linked with 1% formaldehyde for 10 min at 37°C. Cells were resuspended in 250 µl SDA lysis buffer (1% SDS, 50 mM Tris, pH 8.1, 10 mM EDTA, supplemented with protease inhibitors) followed by sonication to an average DNA length of 200–1000 bp. The sample was cleared by centrifugation and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1 and 167 mM NaCl, supplemented with protease inhibitors). Aliquots of the diluted samples were kept as input controls. Anti-acetylated histone H4 (AcH4) antibody (Upstate, Charlottesville, Virginia, USA) was added to each tube, which were rotated at 4°C overnight. A total of 30 µl of protein A beads was then added to each of the tubes, which were further rotated for 2 h at 4°C. The beads were washed once with 1 ml of the following buffers: (i) low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1 and 150 mM NaCl); (ii) high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1 and 500 mM NaCl); (iii) LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA and 10 mM Tris, pH 8.1) and (iv) TE buffer (10 mM Tris, pH 8.1, 1 mM EDTA). The beads were eluted with 250 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) and incubated overnight at 65°C to reverse cross-links. The eluates were treated with proteinase K and extracted with phenol/chloroform followed by ethanol precipitation. The DNA was dissolved in TE buffer and analyzed by PCR. The following PCR primers were used; promoter: *CCTCCTGGCTCCTCCTCTT* (sense) and *CGCTGCCT TGAACTCCTTAC* (antisense); intron (688–847): *AGA TTGGGTGGGGGAATG* (sense) and *CCAAGGATGGG AGAGAAAGA* (antisense); intron (2015–2212): *CACTT GCATCTTTGGTGCTT* (sense) and *TTGCTCCCATTT TCTCTGCT* (antisense); intron (5478–5614): *TAGCT TCGGAGTCCATTTCC* (sense) and *GTGTCTGCGTCC CTCACC* (antisense); intron (8658–8758): *TGTAATGAC CCTGTCACGAA* (sense) and *GTTACACGGGCTGGAC AACT* (antisense). PCR products were amplified for 32 cycles.

Immunoblot analysis

Cells were harvested, washed with PBS and lyzed in ice-cold protein lysis buffer (50 mM Tris pH 7.6, 400 mM NaCl, 0.5% NP-40, 1 mM PMSF and 1 × protease inhibitor cocktail (Roche, Mannheim, Germany)). Lysates were clarified by centrifugation (15000*g* for 15 min at 4°C) and the supernatant was analyzed immediately or stored at −80°C. Equivalent amounts of protein (25–40 µg) were resolved by 10–12% SDS–PAGE and transferred onto a PVDF membrane (Hybond P, Amersham Biosciences, Piscataway, New Jersey, USA). Membranes were incubated with anti-Wt1 (1:1000, Santa Cruz, California, USA) anti-β-Actin (1:5000, Sigma), anti-acetylated histone H4 (1:5000, Upstate) anti-UBE2L6 (1:1000, Abgent, San Diego, California, USA), anti-HA (1:1000, Cell Signaling, Danvers, Massachusetts, USA) or anti-V5 (1:10000, Invitrogen) primary antibodies overnight at 4°C. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia, Uppsala, Sweden) followed by exposure to autoradiography film (Amersham Pharmacia). To detect acetylated histone H4, cells were lyzed in protein loading buffer (0.313 M Tris pH 6.8, 10% SDS, 40% glycerol, 0.05% bromophenol blue and 2% β-ME) and sonicated three times for 20 s each and separated on 15% SDS–PAGE. Quantification of immunoreactive bands on films was performed using the Bio-Rad Quantity One Software program. Values for Wt1 were divided by those for β-Actin and are depicted as percent relative to the control set as 100%.

In vivo ubiquitination assay was performed as described (31). Ubiquitinated Wt1 was detected by western blotting using an anti-HA antibody.

Nuclear run-off assay

Nuclear run-off assay was performed as described (32). Radiolabeled RNA was hybridized with a Hybond-N nylon membrane (Amersham) containing immobilized fragments of *Gapdh* (1 µg of a 558 bp fragment) and *Wt1* (2 µg of a EcoRI-ApaI fragment). Hybridization was performed overnight at 65°C with 1×10^6 c.p.m. labeled RNA per sample using 2 ml of the Rapid-hyb buffer (Amersham) according to the manufacturer's recommendations. To quantitate the signals, radioactive spots were excised from the membranes and measured in a scintillation counter.

Constructs, transfection and reporter gene assay

For the *Wt1* promoter construct, a 3318 bp *Wt1* upstream fragment was amplified by PCR with primers *mWt1F2KpnI aCGTGGTACCGCCAGTGTCTCTTTTC TTCCA* and *P1XhoI ACGTCTCGAGCGCTG CCTTGA ACTCCTTACC* containing a KpnI and an XhoI restriction site, respectively, using a *PAC* clone harboring the entire *Wt1* genomic locus (33). PCR was performed as mentioned above except elongation was carried out for 3 min at 72°C using Triple-Master enzyme mix (Eppendorf, Hamburg, Germany). PCR products were gel purified and ligated into the pGEMT-Easy vector (Promega). The promoter fragment was removed from the vector by XhoI/KpnI digestion and cloned into pGL3-Basic. Cloning of *Wt1* intron 1 and intron 3 into the pGEMT-Easy vector has been described (33). After excision with NotI and subsequent fill-in reaction by Klenow enzyme, introns 1 and 3 were cloned into a SmaI-linearized and dephosphorylated pGL3-Promoter vector to create pGL3Int1 and pGL3Int3, respectively.

For short-term stable transfections, 2×10^6 M15 cells were transfected with 10 µg of reporter plasmid or the respective empty vector (pGL3-Basic or pGL3-Promoter) as control, along with 1 µg of pBABE-puro using SuperFect (Qiagen) according to the manufacturer's recommendations. Selection was started after 24 h using 1 µg puromycin/ml. At day 5 after transfection, 1×10^5 cells of the selected mass populations were seeded into

24-well culture dishes and grown overnight followed by treatment with the indicated concentrations of TSA for further 24 h. Subsequently, reporter gene activity was assessed using the Luciferase Assay System (Promega). Values were normalized to protein content in each sample and divided by those obtained for the control vector containing samples. All transfection results are presented as the average from at least three independent experiments.

For *in vivo* ubiquitination assay, 293 cells were transfected with 5 μg *Wt1* expression construct together with an equal amount of a *His-ubiquitin* expression plasmid. 20 h after transfection cells were treated with lactacystine for additional 20 h.

For *UBCH8* overexpression studies, 293 cells were transfected in 6 cm plates with 0.5, 1 and 2 μg of a plasmid encoding UBCH8 harboring a V5 epitope tag in absence or presence of 1 μg *Wt1* expression construct. The pcDNA3.1 plasmid transfected cells were used as a control. Cells were harvested 24 h posttransfection and Wt1 levels were determined by western blot.

siRNA transfection

For *UBCH8* knockdown experiments 293 cells were seeded at 1×10^5 cells per well in a 6-well plate in 2 ml medium 1 day prior to transfection. siRNA (ON-TARGET plus SMARTpool duplex, Dharmacon, Lafayette, Colorado, USA) directed against *UBCH8* was diluted to 100 μl in serum-free media to achieve a final concentration of 50 nM, and 10 μl siFect Transfection Reagent (Qiagen) was added. Samples were vortexed, incubated at room temperature for 10 min and then added drop-wise to the cells. Twenty-four hours post-transfection media was replaced and cells were grown additionally for 36 h, followed by incubation in presence of 500 nM TSA for 20 h. Transfection without siRNA and TSA was used as a control.

RESULTS

Differential effects of HDAC inhibitors on *Wt1* expression

While we were analyzing epigenetic changes at the murine *Wt1* locus in *Wt1* expressing and *Wt1* nonexpressing cells, we observed a dramatic downregulation of *Wt1* mRNA levels following treatment of M15 cells with TSA. This effect was dose- and time-dependent (Figure 1A). TSA had no effect on *β-Actin* expression suggesting that the effect on *Wt1* expression was specific and not due to cell death. We then wanted to quantify the effect and used real-time PCR. Within 24 h TSA at 100 nM concentration caused *Wt1* downregulation to about 40% and at 500 nM to $< 5\%$ of the original levels (Figure 1A, left). With 500 nM TSA maximal reduction was reached after 12 h (Figure 1A, right).

We further analyzed whether this effect was specific for M15 cells, which express high levels of endogenous *Wt1* (Figure 1E) or could be observed in other cell types. We therefore treated the two human cell lines 293 and K562 as well as murine TM3 cells with TSA and could observe significant reduction of *Wt1* mRNA levels in all cell lines tested (Figure 1B and data not shown). However, TSA concentrations required as well as the magnitude of the effect varied between the different cell lines. In order to assess a possible effect of TSA on cell cycle progression, we have analyzed cell cycle distribution of TSA-treated cells by flow cytometric analysis. While M15 cells accumulated in G2 phase, K562 cells showed a G1 arrest (Supplementary Figure 1A). Thus, the effect of TSA on cell cycle did not correlate with its effect on *Wt1* expression. Of note, analysis of cell viability indicated a toxic effect of TSA only at a concentration of 1 μM (Supplementary Figure 1B) and confirmed that the effects on *Wt1* expression were not due to cell death.

To test whether TSA was unique among HDAC inhibitors in that it downregulated *Wt1*, we included SAHA, MS-275 and VPA in our experiments. M15 cells were treated for 24 h with various concentrations of the three respective substances and *Wt1* mRNA levels were measured by quantitative real-time PCR. In SAHA-treated cells, *Wt1* mRNA levels were strongly reduced with increasing drug concentrations (Figure 1C, left), albeit much higher doses than with TSA were required. In contrast, VPA had no significant effect on *Wt1* expression (Figure 1C, right). Acetylation of histone H4 was used as a control for VPA activity. When we used the HDAC inhibitor MS-275, significant reduction in *Wt1* expression levels could be observed (Figure 1D, left); this effect was even more dramatic in K562 cells (Figure 1D, right). These data suggest that the loss of *Wt1* mRNA amount is independent of the levels of *Wt1* expression (Figure 1E); moreover, it is a phenomenon shared by many but not all HDAC inhibitors. TSA and SAHA inhibit both class I and class II HDACs, whereas VPA and MS-275 have been reported to be class I-selective (34,35). Thus, the differential effect of TSA/SAHA/MS-275 and VPA on *Wt1* gene regulation cannot be attributed to a specific class of HDACs.

TSA downregulates *Wt1* at the transcriptional level

Downregulation of *Wt1* mRNA upon TSA treatment prompted us to investigate the responsible mechanism. One possibility could be that TSA may affect the stability of *Wt1* mRNA. Alternatively, TSA could directly influence *Wt1* transcription. To investigate the first possibility, M15 cells were treated with the transcriptional inhibitor actinomycin D 1 h before the addition of TSA. Subsequently, steady-state *Wt1* mRNA was measured by real-time PCR. Most of the repressive effect of TSA on *Wt1* mRNA levels could be blocked by pretreatment with actinomycin D (Figure 2A). This suggested that TSA does not significantly alter *Wt1* mRNA stability. In order to examine whether TSA would act at the level of transcription, we performed a nuclear run-off assay using untreated and TSA-treated M15 cell nuclei. Already, after 3 h, TSA treatment profoundly suppressed synthesis of new *Wt1* transcripts (Figure 2B and C). Longer TSA treatments (12 and 24 h) resulted in further loss in *Wt1* mRNA synthesis. These results suggest that in response to TSA treatment, *Wt1* is downregulated at the transcriptional level and that TSA has no significant effect on *Wt1* mRNA stability.

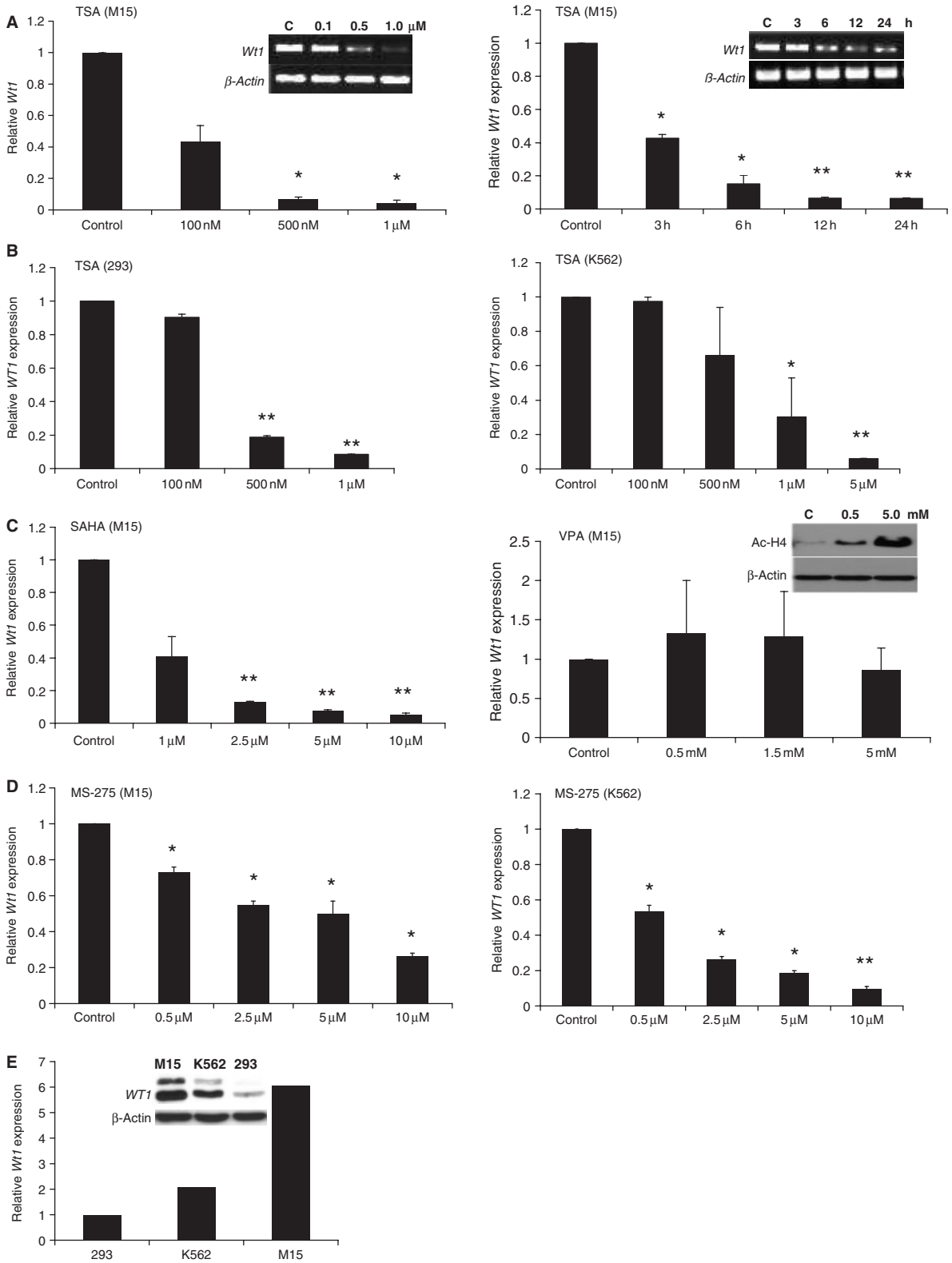

Figure 1. HDAC inhibitors affect *Wt1* expression. (**A**) Quantitative real-time PCR of *Wt1* expression in M15 cells that had been treated for 24 h with TSA at different concentrations (*left*) or with 500 nM TSA for different time points (*right*). (**B**) Analysis as in (A) employing the human cell lines 293 (*left*) and K562 (*right*). (**C**) Analysis as in (A) using the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA, *left*) and valproic acid (VPA, *right*) and M15 cells. (**D**) Analysis as in (A) using the HDAC inhibitor MS-275 in M15 (*left*) and K562 cells (*right*). (**E**) Relative *Wt1* expression in different cell lines was measured by quantitative real-time PCR. HDAC inhibitor treatment was done for 24 h when not specified otherwise. Insets in (A) show gel pictures of the respective RT–PCR products; the inset in (C) shows a western blot performed with an anti-acetylated histone H4 antibody to confirm VPA activity. The inset in (E) shows WT1 protein levels. Bars represent ±SD from three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.005$; paired Student's *t*-test).

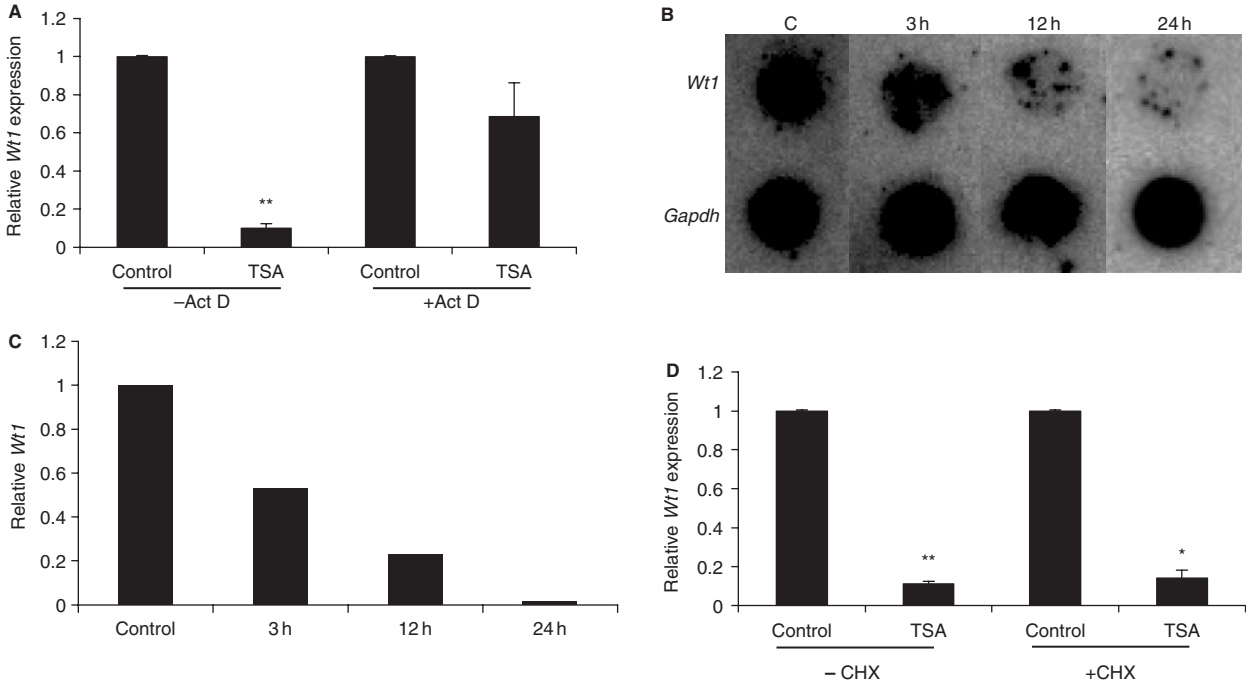

Figure 2. TSA downregulates *Wt1* expression at the level of transcription. (**A**) Effect of TSA on *Wt1* mRNA stability. M15 cells were treated with TSA or vehicle only in absence or presence of actinomycin D for 20 h. Subsequently, quantitative real-time PCR of *Wt1* mRNA expression was performed. (**B**) TSA represses *Wt1* transcription. M15 cells were treated with TSA for 3, 12 and 24 h and nuclear run-off analysis using probes against *Wt1* and *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) was performed. (**C**) Quantification of the autoradiographs shown in (B). *Wt1* signals were normalized against *Gapdh* and untreated control sample was set as 1. (**D**) Effect of protein synthesis inhibition on TSA-dependent downregulation of the *Wt1* mRNA level. M15 cells were exposed to vehicle or TSA (500 nM) in absence or presence of cycloheximide (CHX, 50 μg/ml) for 20 h. Subsequently, quantitative real-time PCR of *Wt1* mRNA expression was performed. $^{*}P < 0.05$; $^{**}P < 0.005$; paired Student's *t*-test.

We then asked, whether downregulation of *Wt1* expression by TSA required new protein synthesis. We therefore pretreated M15 cells with cycloheximide to inhibit new protein synthesis and then challenged the cells with TSA for 20 h. As shown in Figure 2D, such treatment could not prevent TSA-dependent *Wt1* mRNA downregulation. This result supports the notion that new protein synthesis is not needed for TSA-dependent downregulation of *Wt1* expression.

To identify the TSA responsive region in the *Wt1* gene, a 3.3 kb *Wt1* promoter fragment as well as the large introns 1 and 3 of the murine *Wt1* gene (Figure 3A) were cloned into the pGL3-Basic LUC and pGL3-Promoter LUC vectors, respectively. These reporter gene constructs, together with a puromycin resistance-conferring construct were transfected into M15 cells, which were subsequently selected by puromycin for 4 days. Subsequently, mass populations were split, treated with different concentrations of TSA for 20 h and finally luciferase activity was determined. Under these conditions, TSA had no effect on the *Wt1* promoter activity (Figure 3B). At the highest concentration of 10 nM, TSA treatment was found to increase the activity of intron 1. Whether this might reflect a promoter activity that has been described to reside in intron 1 of the *Wt1* gene (36) remains to be determined. In contrast, we could observe a TSA-induced and dose-dependent downregulation of *Wt1* intron 3 activity. This effect could already be observed at the lowest concentration used, namely 1 nM. The TSA concentrations used here were considerably lower than those required for the reduction of endogenous *Wt1* expression (Figure 1A). We assume that this might be due to differences in the genomic context as well as in copy number of the respective fragments. We conclude from these studies, that elements in intron 3 of *Wt1* are involved in the repression of *Wt1* expression by HDAC inhibitors.

To characterize such a regulatory element with better resolution, we examined epigenetic alterations within intron 3 of the *Wt1* gene in M15 cells. For this, we performed chromatin immunoprecipitation using an antibody against acetylated histone 4 (H4) and subsequent PCR analysis. When we used primers specific for a genomic region at the 5′ end of intron 3 (spanning position 688–847), we observed a decrease in acetylation of H4 upon TSA treatment (Figure 3C); this decrease was less pronounced when primers were placed more 3′ and absent in two more downstream regions of intron 3 as well as in a promoter fragment. We conclude from these experiments that TSA-mediated downregulation of *Wt1* expression is associated with specific changes in histone modification within intron 3 of the *Wt1* locus.

TSA promotes proteasomal degradation of Wt1 protein

To determine the effect of TSA on Wt1 protein levels, western blot analysis was performed. In TSA-treated M15 cells, a significant decrease of Wt1 protein was observed (Figure 4A). Densitometric analysis revealed

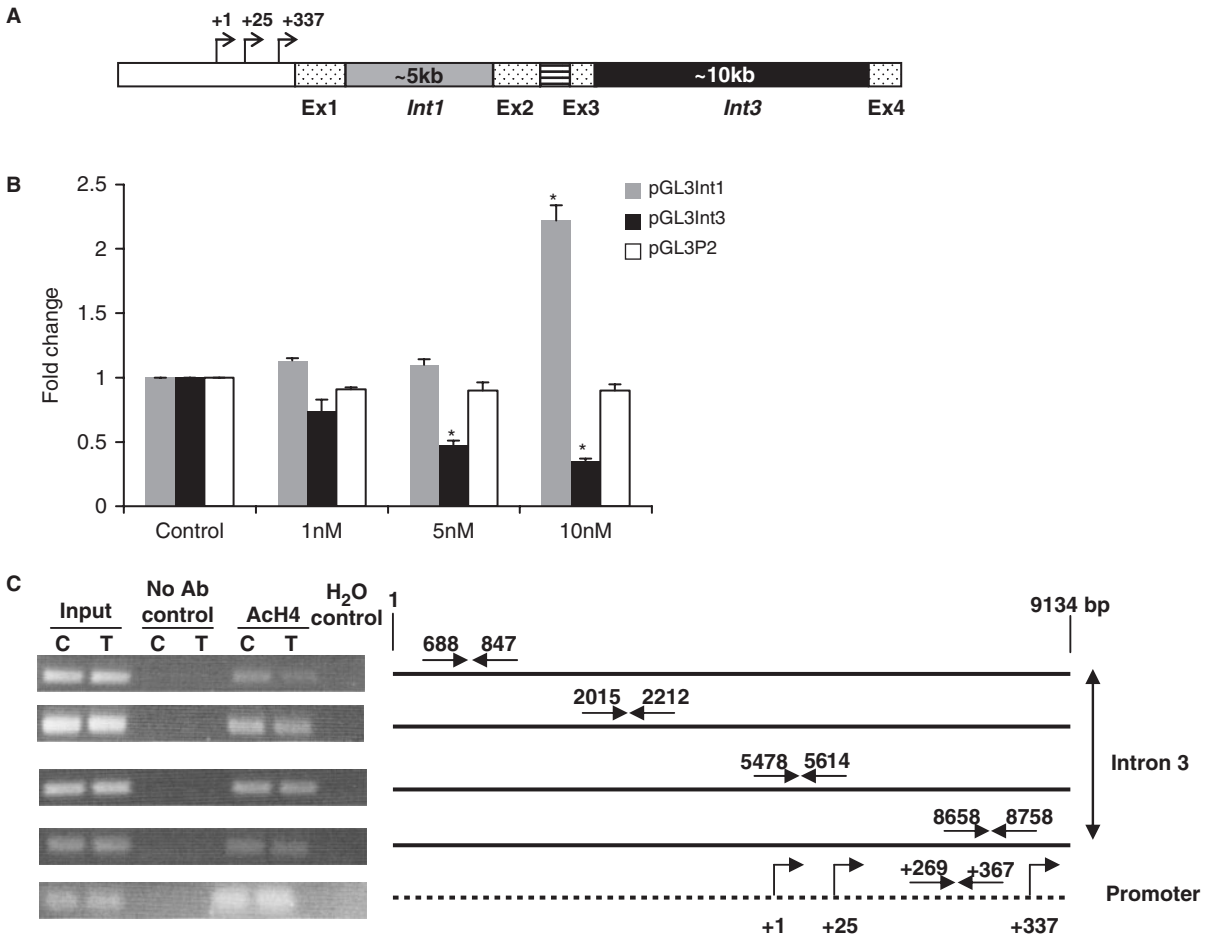

Figure 3. A TSA-responsive element is located in intron 3 of *Wt1*. (**A**) Schematic representation of part of the murine *Wt1* gene. The three transcriptional start sites are marked by arrows. Ex, exons; Int, introns. (**B**) M15 cell populations transfected with constructs harboring a *Wt1* 3.3 kb promoter fragment (pGL3P2), intron 1 (pGL3Int1) or intron 3 of *Wt1* (pGL3Int3), respectively, were treated with the indicated concentrations of TSA for 24 h. Luciferase activity was determined, normalized to the protein content in each sample and divided by the values obtained for cell populations containing the respective empty control vector. Values for the EtOH treated control samples were set as 1.0. Means and ± SD of three independent experiments are shown ($^*P < 0.05$; paired Student's *t*-test). (**C**) ChIP analysis of the *Wt1* locus in M15 cells (*left*). Schematic representation of the primers (indicated by arrows) in the *Wt1* promoter and intron 3 region selected for ChIP analysis (*right*). Cells were treated with 500 nM of TSA or ethanol for 24 h. Immunoprecipitation was performed with an anti-acetylated H4 (AcH4) antibody. C, control; T, TSA.

that upon 4 h of TSA treatment 30% protein was lost, while after 8 and 16 h 60 and 95% of Wt1 protein had been degraded, respectively (Supplementary Figure 2A). Surprisingly, simultaneous treatment of M15 cells with TSA and cycloheximide attenuated TSA mediated degradation of the Wt1 protein such that after 16 h almost 40% of Wt1 protein was still present. A comparable effect was obtained with SAHA, while VPA led to a more subtle Wt1 degradation, which, however, was also reversed by cycloheximide (Supplementary Figure 2B). Interestingly, a significant and cycloheximide-sensitive WT1 degradation was also detectable in human leukemic K562 cells (Figure 4B). This observation suggested the possibility of some kind of protease induction, responsible for Wt1 degradation.

Proteases responsible for the degradation of Wt1 have not been reported yet. In order to examine the involvement of candidate proteases in Wt1 degradation, we used various protease inhibitors. NH_4Cl is a weak base known to inhibit lysosomal hydrolases by reducing the acidification of the endosomal/lysosomal compartment. Pepstatin A is an inhibitor of acid proteases and leupeptin inhibits serine and cysteine proteases. Application of these protease inhibitors together with TSA did not prevent Wt1 protein degradation (data not shown). Similarly, addition of ALLN, ALLM and z-VAD-fmk, inhibitors of calpain I, II and caspases, respectively, had no significant effect. However, cotreatment with lactacystine, which is a highly specific inhibitor of the 20S proteasome subunit suppressed the TSA mediated degradation of the Wt1 protein in M15 cells (Figure 4C). After 20 h of treatment, Wt1 protein levels were 3-fold higher in TSA and lactacystine-treated cells than in the case of TSA alone (Figure 4D).

The involvement of the proteasome in Wt1 degradation suggests that Wt1 might be ubiquitinated. We therefore transfected cells with a *Wt1* expression vector together with a plasmid encoding a His-tagged ubiquitin molecule. Subsequent analysis by purification of the cell extract via nickel chelate chromatography and western blotting demonstrated that Wt1 is indeed ubiquitinated and that

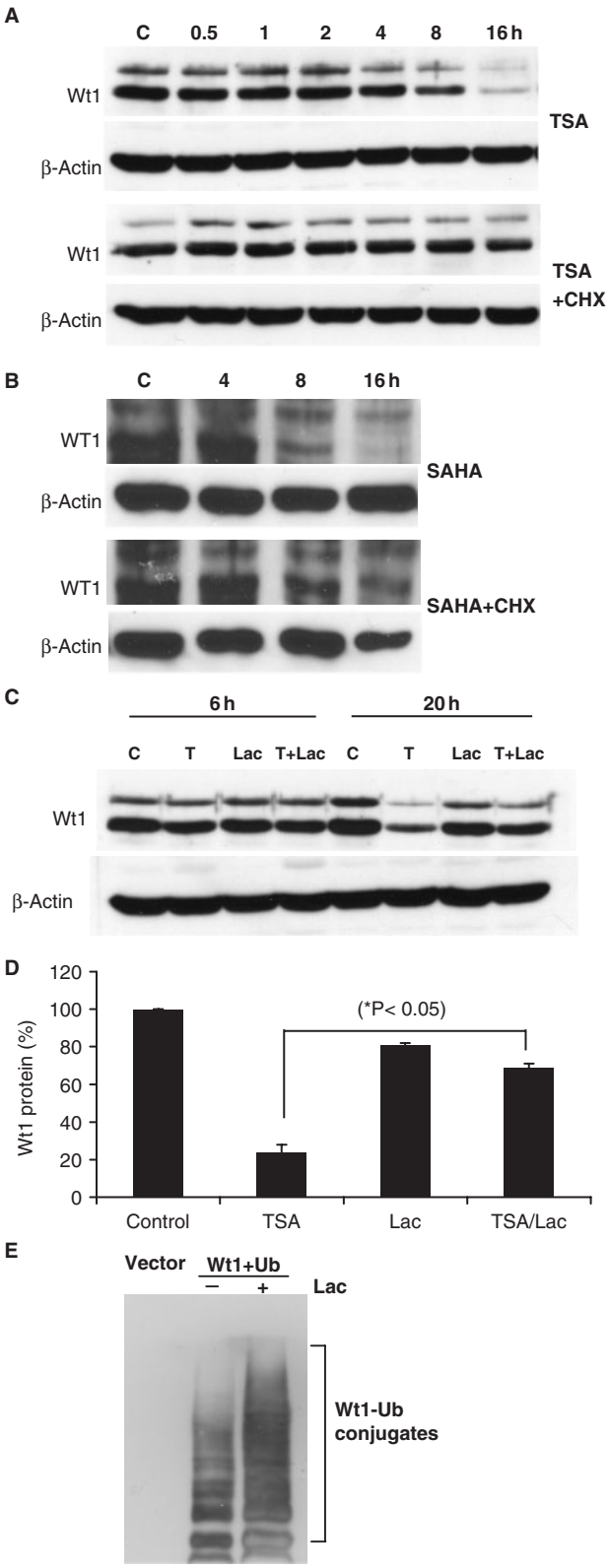

Figure 4. TSA induces Wt1 protein degradation. (**A**) M15 or (**B**) K562 cells were treated with TSA (500 nM) or SAHA (5 μM) alone or in combination with cycloheximide (50 μg/ml); protein extracts were prepared after various time points and subjected to western blot analysis using anti-Wt1 and anti-β-Actin antibodies. (**C**) M15 cells were treated with TSA (500 nM) alone, lactacystine (10 μM) alone or in combination with TSA; protein extracts were prepared after 6 and 20 h and subjected to western blot analysis using anti-Wt1 and anti-β-Actin antibodies. (**D**) Quantification of the western blot analysis shown in (C). Wt1 signals were normalized against β-Actin and the untreated control sample was set as 100%. For the analysis, only the data from the 20 h time-point were used. (**E**) Wt1 protein is ubiquitinated. *Wt1* was coexpressed with His-ubiquitin in 293 cells by transient transfection. His-ubiquitinated proteins were purified and Wt1 was detected by western blotting with anti-HA antibody. C, control; T, TSA; Lac, lactacystine.

this modification can be enhanced by inhibition of the proteasome (Figure 4E).

Ubiquitin-conjugating enzyme UBCH8 is involved in WT1 degradation

Ubc8 (named Ubce8 in mouse and UBCH8 in humans) is an ubiquitin-conjugating enzyme, which is 4–5-fold upregulated in F9 cells upon TSA treatment (37). In order to analyze whether this enzyme might also be involved in TSA-induced WT1 degradation, we investigated the expression of *UBCH8* in TSA-treated 293 cells by semi-quantitative PCR and further quantification by real-time PCR in a dose-dependent manner. At a concentration of 100 nM TSA, 6-fold *UBCH8* induction was observed that was further increased with 500 nM and 1 μM TSA after 20 h (Figure 5A). A similar effect was observed in M15 cells (data not shown). The increase in *UBCH8* expression was also reflected at the protein level (Figure 5B). *Ubc8*/*UBCH8* induction was not only exerted by TSA but also by VPA as well as SAHA in M15 as well as K562 cells (Supplementary Figure 3). To analyze whether WT1 is a substrate of UBCH8, overexpression studies were performed. The 293 cells were transiently transfected with increasing concentrations of an UBCH8 encoding plasmid in absence or presence of a construct encoding HA-tagged Wt1. The levels of endogenous as well as transfected WT1 were significantly decreased upon increasing concentrations of UBCH8 (Figure 5C and D). Constant levels of β-Actin indicated that the effect on WT1 is specific and not due to general protein degradation.

The functional significance of *UBCH8* expression levels for WT1 degradation was further substantiated by siRNA experiments in 293 cells. Application of a siRNA pool against *UBCH8* mRNA resulted in a dose-dependent and significant reduction in *UBCH8* expression level (Figure 5D). Transfection of 293 cells with *UBCH8* siRNA prevented degradation of the endogenous WT1 protein upon TSA treatment (Figure 5E). These experiments support the involvement of UBCH8 in the degradation of WT1 upon TSA treatment.

Thus, we conclude that in addition to an effect of the HDAC inhibitor TSA on transcription of the *Wt1* gene, TSA also leads to the induction of proteasomal degradation of WT1 at the protein level. This is at least in part mediated by induction of *UBCH8* expression, which encodes an ubiquitin-conjugating enzyme.

DISCUSSION

Conceptually, hyperacetylation of histones following inhibition of HDAC activity would be predicted to promote a general increase in gene expression. As chromatin

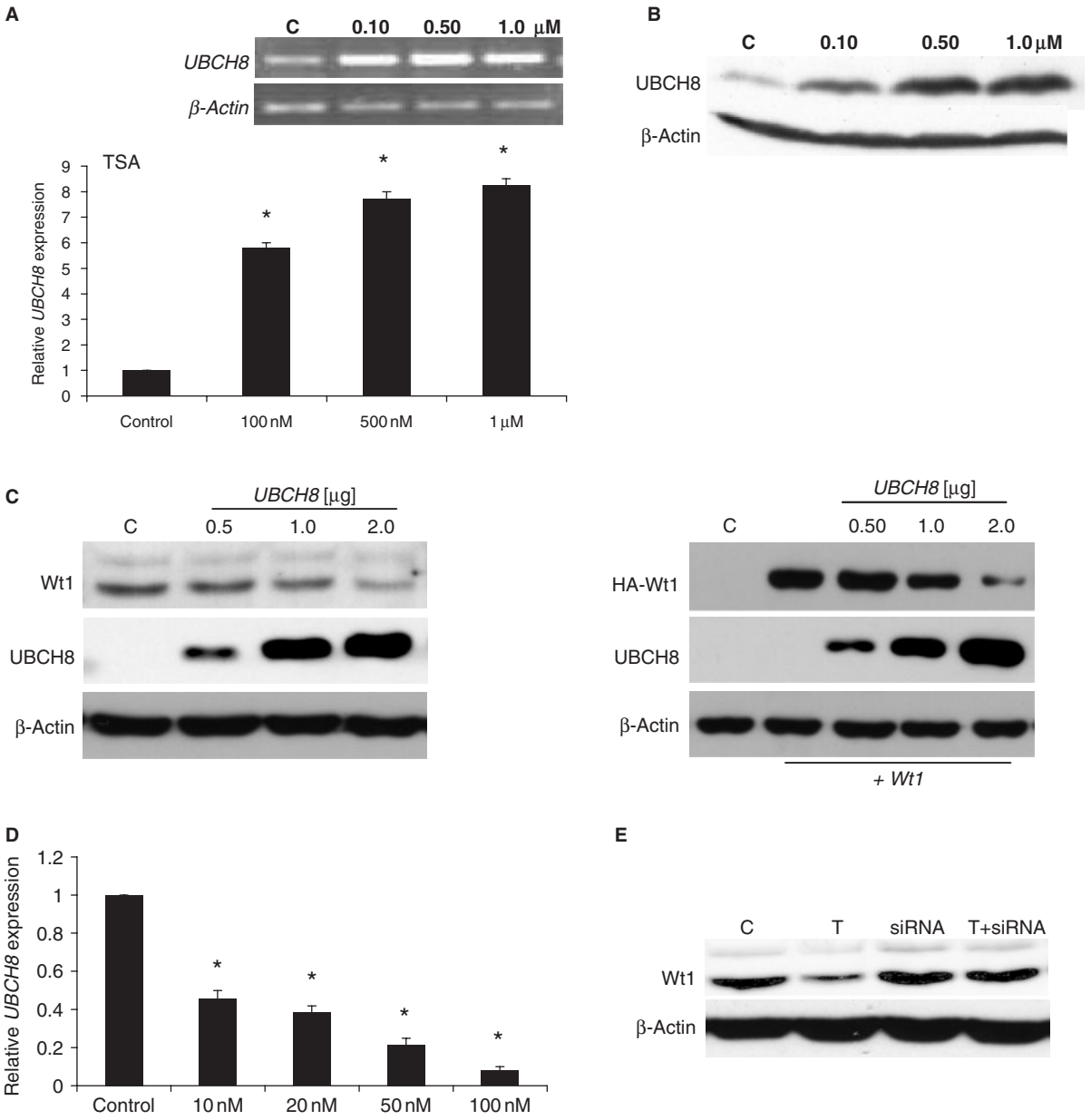

Figure 5. The ubiquitin-conjugating enzyme UBCH8 is induced by TSA and is involved in WT1 protein degradation. (**A**) 293 cells were treated with the indicated concentrations of TSA for 20 h. Cells were harvested and *UBCH8* mRNA message was determined by semiquantitative PCR (inset) and quantitative real time PCR. (**B**) Analysis of UBCH8 protein level upon treatment of 293 cells with different concentrations of TSA. (**C**) 293 cells were transfected with the indicated concentrations of *UBCH8* encoding plasmid either without or together with 1 μg of a construct encoding HA-tagged *Wt1*; 24 h posttransfection endogenous WT1 (*left*) or plasmid-encoded HA-tagged Wt1 (*right*) was analyzed by western blot analysis using an anti-Wt1 or an anti-HA antibody. (**D**) 293 cells were transfected with indicated concentrations of *UBCH8* siRNA. Forty-eight hours posttransfection, *UBCH8* mRNA levels were measured by real-time PCR. (**E**) 293 cells were transfected with 50 nM *UBCH8* siRNA; 60 h posttransfection cells were treated with 500 nM TSA for additional 20 h. Mock transfected cells treated with vehicle or TSA alone or *UBCH8* siRNA alone were used as controls. Subsequently, protein extracts were prepared and subjected to western blot analysis using anti-Wt1 and anti-β-Actin antibodies, respectively. $^{*}P < 0.05$; paired Student's *t*-test.

structure opens, transcription factors should gain easier access to their cognate-binding sites, thus enhancing the recruitment of cofactors and the basal transcription machinery. However, treatment of cells with HDAC inhibitors like TSA does not lead to a general increase in gene expression and changes the activity state of only a limited number of genes (24). Genes with upregulated activity upon HDAC inhibitor treatment comprise the cell cycle inhibitor encoding gene *p21*, *p53* and the *von Hippel–Lindau* tumor suppressor genes as well as the pro-apoptotic genes *bax* and *bad* (28,38–40). Conversely, a smaller number of genes have been shown to be downregulated by HDAC inhibitors; these include *bcl-2*, *HIF-1α*, *VEGF* and *p16* (28,41,42). The reasons for this selectivity are not yet understood. In one case, HDAC inhibitor-mediated downregulation of a particular set of genes has been

shown to result from physical interaction of a transcription factor, namely NF-κB p65 with the acetylated form of a signaling molecule, Stat1, and subsequent reduction of p65 DNA binding (43).

We report here that the *Wilms tumor suppressor gene Wt1* also belongs to the class of genes that are downregulated by HDAC inhibitors. Downregulation was observed with TSA as well as with SAHA, both inhibitors of class I and II HDACs. VPA, an inhibitor of class I HDACs only (34) did not cause a reduction in *Wt1* expression, while MS-275, another class I-selective HDAC inhibitor also downregulated *Wt1* expression. The different behavior of MS-275 and VPA on *Wt1* expression might be explained by their different biological potency (the respective EC_{50} values depend on the specific substrates and differ by a factor of 10^3–10^4) or by their different target profile. It has been shown that while VPA can be classified as a specific class I HDAC inhibitor, MS-275 is only class I-selective and can, e.g. also efficiently inhibit the class II HDAC9 (35). Thus, from these data, we cannot conclude which specific HDACs mediate downregulation of *Wt1* mRNA. The latter was observed in the murine cell lines M15 and TM3 as well as in human K562 and 293 cells. This indicates that reduction of *Wt1* expression by HDAC inhibitors is a general and not a cell-line-specific phenomenon.

Strikingly, downregulation of *Wt1* expression was very rapid; within 3 h of TSA treatment *Wt1* mRNA was reduced to almost 50%. This suggests that repression of *Wt1* mRNA expression is an early event and that the half-life of *Wt1* mRNA must be ≤ 3 h. Mechanistically, loss of *Wt1* mRNA could be mediated by two possible mechanisms. First, TSA could affect the rate of *Wt1* transcription or second, *Wt1* mRNA stability. Our experiments employing the transcriptional (RNA polymerase) inhibitor actinomycin D indicate that TSA has no effect on *Wt1* mRNA stability and downregulation of *Wt1* mRNA upon TSA treatment requires ongoing RNA synthesis. Moreover, we were able to show by nuclear run-off assay that TSA strongly affected *Wt1* transcription.

In order to explore the possible mechanisms, we investigated whether ongoing protein synthesis was required for TSA-mediated downregulation of *Wt1*. For this, we used the protein translation inhibitor cycloheximide and observed that this inhibitor did not prevent downregulation of *Wt1* mRNA. Thus, *Wt1* mRNA loss does not require *de novo* protein synthesis. Conceivably, loss of *Wt1* expression by blocking HDAC activity could be mediated by histones or by nonhistone proteins. Increasing acetylation of histones associated with *Wt1* regulatory regions could cause the movement of nucleosomes on the *Wt1* locus and thus mask critical *cis*-acting sites. Nucleosomes have been shown to move and be remodeled on specific loci, including the *lysozyme* gene and the *IL-12p40* (44,45). Alternatively, as a consequence of HDAC inhibition a transcriptional activator of *Wt1* expression could loose its function or a potential repressor could be activated as a consequence of hyperacetylation. Acetylation/deacetylation of several transcription factors including p53, NF-κB, E2f1 and Stat1 have been described (43,46–48). Further studies will be required to identify the *trans*-acting factors mediating the TSA effect on *Wt1* expression.

We also wanted to identify the *cis*-element that is responsible for the TSA effect on *Wt1* expression. Considerable work has been done regarding the tissue- and temporal-specific expression of *Wt1*; however, the *Wt1* locus is remarkably complex and our knowledge of its regulation is still quite limited. In terms of elements regulating *Wt1* expression in particular tissues, a 258 bp hematopoietic cell-specific enhancer that is located in intron 3 and bound by GATA-1 and c-Myb has been described (49). The same intron harbors a 460 bp silencer that represses *WT1* transcription in nonrenal cells such as leukemic (K562 and HL60) and cervical carcinoma cells (HeLa) (50). Moreover, a construct including the promoter, two enhancer elements located in intron 3 and at the 3′ end of the *WT1* gene could induce robust expression of a reporter gene in respective transgenic leukemia cells (51).

We reasoned that transient transfections would not be well suited for the identification of regulatory elements mediating the TSA effects, since the chromatin configuration might not be physiological. We have therefore employed short-term stably transfected cells for these experiments. When we used a luciferase construct linked to a 3.3 kb *Wt1* promoter fragment, TSA did not have any effect. Conversely, a reporter construct harboring intron 3 of *Wt1* led to more than 60% downregulation in reporter activity upon TSA treatment. Unexpectedly, we did see a TSA-mediated enhancement in intron 1 stimulated reporter gene activity. While no enhancers have been identified in intron 1 of *Wt1*, a promoter that controls expression of an antisense *Wt1* transcript has been reported to reside in the first intron (36). It might be possible that TSA exerts an effect on the activity of this promoter. By ChIP, we could also show that histones H4 in a region in the 5′ part of intron 3 are differentially acetylated in the presence and absence of TSA, respectively. Other regions within intron 3 did not seem to be affected. This is in agreement with the existence of a regulatory region in intron 3 of the human *WT1* locus that has been mentioned above. Whether the sequences that respond to TSA overlap with the enhancer and silencer sequences in the human *WT1* gene remains to be explored.

In addition to downregulation of *Wt1* mRNA, TSA also led to enhanced degradation of the WT1 protein. This effect was at least in part mediated by the ubiquitin-conjugating enzyme Ubc8, which has been reported to be induced by HDAC inhibitors (36). Of note, induction of Ubc8 could also be observed by VPA as well as SAHA and in a variety of different cell lines. The observation that VPA causes Ubc8 induction and subsequent WT1 degradation, while not affecting *Wt1's* transcription indicates that the two phenomena are mediated by different molecular mechanisms. Overexpression of *UBCH8* resulted in the enhanced destabilization of both endogenous and transfected WT1 protein and knockdown of *UBCH8* rescued TSA-mediated degradation of WT1. This shows that Ubc8 is a rate-limiting factor in WT1 degradation, while the corresponding E3 ligase is not.

This is the first demonstration that WT1 degradation is controlled by the proteasomal machinery.

In summary, we provide evidence that HDAC inhibitors like TSA lead to a dramatic reduction of *Wt1* expression, both at the level of transcription and at the protein level. Given that WT1 levels are high not only in many hematopoietic malignancies but also in a variety of solid tumors, that high WT1 levels are associated with poor prognosis, and that various cancer cells depend on high *WT1* expression levels downregulation of this protein could be an anticancer strategy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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