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Negative and Positive Regulation of Gene Expression by Mouse Histone Deacetylase 1▽

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Histone deacetylases (HDACs) catalyze the removal of acetyl groups from core histones. Because of their capacity to induce local condensation of chromatin, HDACs are generally considered repressors of transcription. In this report, we analyzed the role of the class I histone deacetylase HDAC1 as a transcriptional regulator by comparing the expression profiles of wild-type and HDAC1-deficient embryonic stem cells. A specific subset of mouse genes (7%) was deregulated in the absence of HDAC1. We identified several putative tumor suppressors (JunB, Prss11, and Plagl1) and imprinted genes (Igf2, H19, and p57) as novel HDAC1 targets. The majority of HDAC1 target genes showed reduced expression accompanied by recruitment of HDAC1 and local reduction in histone acetylation at regulatory regions. At some target genes, the related deacetylase HDAC2 partially masks the loss of HDAC1. A second group of genes was found to be downregulated in HDAC1-deficient cells, predominantly by additional recruitment of HDAC2 in the absence of HDAC1. Finally, a small set of genes (Gja1, Irf1, and Gbp2) was found to require HDAC activity and recruitment of HDAC1 for their transcriptional activation. Our study reveals a regulatory cross talk between HDAC1 and HDAC2 and a novel function for HDAC1 as a transcriptional coactivator.

The DNA of eukaryotic cells is compacted by basic histone proteins in a highly organized structure called chromatin. The nucleosome, the basic unit of chromatin, consists of 147 base pairs of DNA wrapped around the histone octamer, composed of two copies of each of the four core histones, H2A, H2B, H3, and H4 (78). Although the structure of the core nucleosome is well defined, the basic N-terminal histone tails protrude from the core nucleosome and show no defined structure (38, 39). These histone tail domains are subject to posttranslational modifications, such as acetylation, methylation, phosphorylation, and ubiquitination (recently reviewed in reference 41). These modifications affect various biological processes, including the transcription of chromatin-embedded genes. Recent observations indicate that histone modifications occur interdependently and create a pattern that might modulate the affinity of histone-binding proteins. These findings are the basis of the histone code hypothesis (21, 35, 67, 75). An alternative way of explaining the cooperation of multiple histone modifications is the recently proposed chromatin signaling network model (64).

A correlation between histone acetylation and increased gene expression was discovered earlier on (3). According to the current model, the acetylation of lysine residues within the histone tails neutralizes the positive charge of ε-amino groups and thereby reduces the interaction between the N-terminal tails of histones and the negatively charged DNA. Acetylation at the N termini of core histones is therefore believed to induce the local opening of chromatin structures. In addition, acetylated histone tails are specifically recognized and bound by bromodomain-containing proteins, such as components of the basal transcription machinery or histone acetyltransferases (HATs) (85). Reversible histone acetylation is controlled by histone acetyltransferases, which usually act as transcriptional coactivators, and histone deacetylases (HDACs), which repress transcription. Activator complexes containing HAT activity have been shown to contribute to transcriptional activation by recruitment of general transcription factors and RNA polymerase II (7, 74). In contrast, recruitment of repressor complexes with HDAC activity is considered to lead to deacetylation of histones, stabilization of nucleosome structure, and formation of a repressive chromatin state.

During the last decade, more than a dozen histone deacetylases have been identified in mammalian cells. Based on sequence similarities, HDACs are divided into four functional classes: class I (HDAC1, HDAC2, HDAC3, and HDAC8), class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and

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HDAC10), class III (SIRT1 to SIRT7), and the recently described class IV of HDACs, which consists of HDAC11-related enzymes (28, 29). The class I enzyme HDAC1 belongs to an ancient family of highly conserved enzymes and was the first protein shown to have histone deacetylating activity in mammals (reviewed in reference 46). Human HDAC1 was purified and cloned by an affinity purification approach (73) and was shown to share significant homology with the previously identified *Saccharomyces cerevisiae* transcriptional regulator Rpd3/Sdi2/Sds6 (51, 80–82). In mouse cells, expression of the HDAC1 gene is stimulated by growth factors (5) and controlled by its own product in a negative feedback loop (32, 65). The enzyme plays an important role in various biological processes, such as cell cycle progression, cell proliferation, and differentiation (46). The HDAC1 mouse knockout (KO) has also revealed the essential function of this deacetylase in embryonic development (36). HDAC1 is a nuclear protein and can heterodimerize with the closely related deacetylase HDAC2 (31, 72). Both enzymes are found in three major multiprotein complexes, named Sin3, NuRD, and CoREST (2, 29). HDAC1 can repress gene transcription either directly or as part of these multiprotein complexes when recruited by a variety of transcriptional regulators, including SP1/SP3, YY1, unliganded nuclear receptors, the pocket proteins pRB, p107, and p130, and the tumor suppressor p53 (13, 52).

HDAC inhibitors have been shown to induce cell cycle arrest, differentiation, or apoptosis in tumor cells, and some of these compounds are currently tested as antitumor drugs in clinical trials (19, 20, 44). These inhibitors affect the catalytic activity of most class I and class II deacetylases. However, little is known about the individual roles of mammalian deacetylases in transcriptional control and the relevant target enzymes for HDAC inhibitors as antitumor drugs. To unravel the role of HDAC1 as a transcriptional regulator, we identified putative HDAC1 target genes by comparing the gene expression profiles of wild-type and HDAC1-deficient embryonic stem (ES) cells. A restricted subset of mouse genes involved in biological processes, such as growth control, cell communication, and transcriptional regulation, was found to be reversibly deregulated upon the loss of HDAC1. By chromatin immunoprecipitation (ChIP) assays, we observed the presence of the HDAC1 enzyme associated with a reduction in histone acetylation levels at the regulatory regions, thereby providing compelling evidence for a direct regulation of these genes by HDAC1. In addition, our data revealed a delicate balance between the class I enzymes HDAC1 and HDAC2 in the regulation of common target genes. Finally, we also identified a novel function for HDAC1 as a positive regulator of gene expression.

MATERIALS AND METHODS

Cell culture. HDAC1 wild-type and homozygous mutant ES cells (36) were cultivated in M15 medium supplemented with antibiotics and 15% (vol/vol) fetal calf serum and either supplemented with 10^3 U/ml of leukemia inhibitory factor on gelatinized culture dishes or without leukemia inhibitory factor on feeder cell layers. All ES cell experiments were performed with cell lines obtained from littermates (36). To stably express wild-type HDAC1 and the myc-tagged, enzymatically inactive HDAC1-H140/141A mutant in ES cell lines, the ES cell-specific expression vectors pMSCVpuro-HDAC1 and pMSCVpuro-mut were linearized and electroporated into ES cells by using a Bio-Rad Gene Pulser II with 0.4-cm-wide sterile cuvettes (165-2088; Bio-Rad), with a single pulse at 230 V and 500 μF. Electroporated ES cells were incubated for 5 min at room temperature and then plated onto puromycin-resistant feeder cells. After 24 h, puromycin (5 μg/ml) was applied to select for targeted clones. The selection procedure was continued for 14 days, and single clones were picked, expanded, and analyzed for expression of HDAC1. The human osteosarcoma cell line U2OS was kept in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics. U2OS cells were stably transfected with pcDNA3-Prss11 (56) (kindly provided by M. Kawaichi, Takayama) or pcDNA3 empty vector by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were split after 24 h and grown in the presence of 360 μg/ml of G418 for selection. After 10 days, single clones were picked and analyzed for Prss11 expression by real-time reverse transcription (RT)-PCR. Trichostatin A (TSA) was obtained from Wako Pure Chemical Industries.

Affymetrix analysis. Total RNA (approximately 5 μg) was used to synthesize double-stranded cDNA by using a custom Superscript double-stranded cDNA synthesis kit (Invitrogen, Karlsruhe, Germany). Biotin-labeled cRNA was then prepared from this template by using an Enzo BioArray high-yield RNA transcript labeling kit (Affymetrix, High Wycombe, United Kingdom), and unincorporated nucleotides were removed using RNeasy columns (QIAGEN, Hilden, Germany). Hybridization, washing, and fluorescence staining of the Affymetrix GeneChip murine genome MG-U74Av2 array (Affymetrix Inc., Santa Clara, CA) were carried out according to the manufacturer's instructions (GeneChip Expression Analysis Technical Manual; Affymetrix). All experiments were performed in triplicate with independently extracted RNAs. Data analysis was performed by means of a comparison matrix, with control (wild-type) experiments as a background, for generation of expression signal log ratios with basis 2 and subsequently changes (*n*-fold) between wild-type and knockout samples, using Microarray Suite software version 5.0 (Affymetrix). Next, mean changes (*n*-fold) and relative standard deviations between experiments were calculated and genes with changes (*n*-fold) of >2 or <0.5 and relative standard deviations of <0.6 were selected for further analysis. The association of genes with particular functions, pathways, and diseases was analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems). Gene networks are ranked according to their scores. Calculations for network scores are based on the hypergeometric distribution calculated via the computationally efficient Fisher exact test for two-by-two contingency tables. The significance value associated with functions and pathways is a measure of how likely it is that genes from the data set file participate in that function. The significance is expressed as a *P* value, which is calculated using the right-tailed Fisher exact test.

Protein analysis and antibodies. Whole-cell protein extraction, histone isolation, and Western blot analysis were performed as previously described (5, 36, 59). The following antibodies were used for protein detection on immunoblots and for chromatin immunoprecipitation assays: HDAC1 (polyclonal rabbit antibody and monoclonal mouse antibody), HDAC2 (polyclonal rabbit antibody and monoclonal mouse antibody), acetyl histone-H3, acetyl histone-H4, acetyl K9-H3 from Upstate, and the C terminus of histone H3 (Abcam). Polyclonal trimethyl K9-H3 and trimethyl K27-H3 antibodies were kind gifts from T. Jenuwein (59). The β-actin protein was visualized with a monoclonal antibody (AC-74; Sigma). The proliferation marker Ki67 was detected with the monoclonal Ki67 antigen antibody (Novo Castra) by indirect immunofluorescence microscopy (Zeiss Axiovert 135TV microscope) as previously described (72). Nuclear DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI).

Immunoprecipitation assays. Immunoprecipitation assays were performed as previously described (18). For combined analysis of proteins and associated deacetylase activity, the precipitated material was resuspended in 50 μl of extraction buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, Roche Diagnostics complete protease inhibitor cocktail), and 30-μl aliquots were examined for protein expression on Western blots. The remaining 20-μl aliquot was assayed for HDAC activity. Histone deacetylase activity assays were done as described previously (5, 37).

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation assays were carried out as described previously (8, 11, 65) with some modifications. Chromatin was cross-linked for 10 min by using formaldehyde and then sonicated. Equal amounts of sonicated chromatin were diluted 10-fold and precipitated overnight with the following antibodies: HDAC1, HDAC2, acetyl histone-H3, acetyl histone-H4, acetyl K9-H3, trimethyl K9-H3, trimethyl K27-H3, and preimmune serum as a control. The chromatin-antibody complexes were isolated by incubation with 30-μl protein A-Sepharose beads (50% slurry, 100 μg/ml salmon sperm DNA, 500 μg/ml bovine serum albumin) while rocking at 4°C for 2 hours. The beads were harvested and washed as described previously (65). Chromatin-antibody complexes were eluted from the protein A-Sepharose beads

by addition of 2% sodium dodecyl sulfate, 0.1 M $NaHCO_3$, and 10 mM dithiothreitol. Cross-linking was reversed by addition of a 0.05 volume of 4 M NaCl and incubation of the eluted samples for 6 h at 65°C. The DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in water.

PCR analysis of immunoprecipitated DNA. All PCRs were performed on an iCycler (Bio-Rad) by using Promega PCR Master Mix. The linear range for each primer pair was determined empirically using different amounts of genomic DNA. PCRs with 1:40 dilutions of genomic DNA (input) were carried out along with the immunoprecipitated DNA. PCR products were resolved on 2% agarose–Tris-acetate-EDTA gels. Primer sequences are available upon request.

RNA isolation, Northern blotting, and real-time RT-PCR analysis. Total cellular RNA was isolated with TRIzol reagent (GibcoBRL) as recommended by the manufacturer. Northern blot hybridization was performed by the sandwich method as previously described (68). For cDNA, 1 μg of total RNA was reverse transcribed with an iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCRs were performed with 0.5 μl of the RT reaction mixture by the iCycler iQ system (Bio-Rad), using SYBR green (Molecular probes) for labeling. Primer sequences are available upon request.

Microarray data accession number. The microarray data have been deposited in the Gene Expression Omnibus (GEO) public database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE 5583.

RESULTS

HDAC1 regulates a specific subset of genes in mouse ES cells. To understand the role of HDAC1 at the cellular level, we previously generated wild-type and HDAC1-deficient mouse ES cells by blastocyst outgrowth experiments with littermates (36). The total cellular HDAC activity in ES cells is significantly reduced upon the loss of HDAC1, indicating that this enzyme is one of the major deacetylases in these cells. Given the important role of HDAC1 as a chromatin-modifying enzyme, we wanted to determine on a genome-wide basis whether the loss of HDAC1 could affect gene expression in mouse cells. We analyzed total RNA from wild-type and HDAC1 KO ES cells on MGU74Av2 Affymetrix GeneChip microarrays, representing about 10,000 genes with known functions and expressed sequencing tags. Experiments were performed in triplicate with three independently isolated sets of RNA. Significantly differentially regulated genes were identified by comparative analysis using Microarray Suite software version 5.0 (Affymetrix) as described in Materials and Methods. The criterion for the selection of potential target genes was a more-than-twofold difference in expression, based on the mean value for all three independent experiments. Interestingly, only approximately 7% of all genes present on the array were deregulated in the absence of HDAC1, suggesting that HDAC1 controls the transcription of a specific subset of mammalian genes (Fig. 1A). Moreover, of the more than 600 deregulated genes, nearly two-thirds showed increased expression levels in HDAC1 KO cells. This finding supports the generally accepted idea that HDAC1 acts mainly as a transcriptional repressor. However, a significant proportion of genes showed a more-than-twofold decrease in expression in HDAC1-deficient cells, suggesting a potential role for HDAC1 in the activation of these genes.

Potential HDAC1 target genes were analyzed for networks and molecular functions through the use of Ingenuity Pathways Analysis (Ingenuity Systems). In agreement with a proposed role for HDAC1 as a regulator of many biological processes, HDAC1 targets were identified as components of molecular networks for cell-to-cell signaling, cellular movement, cell death, gene expression, cell morphology, and cancer (Fig. 1B and C). Classification according to physiological system development and function revealed a potential role for HDAC1 target genes in the development of the hematological system, skeletal and muscle systems, and immune and nervous systems and in corresponding diseases (data not shown).

Given the growth-inhibitory effects of HDAC inhibitors, the impaired proliferation of HDAC1 KO cells points towards HDAC1 as one of the possible targets for these tumor drugs (36). In this context, it is remarkable that several tumor-related genes, such as those encoding JunB, Prss11, Plagl1, Apc2, metallothionein 1, and metallothionein 2, were found in our screen (see also Discussion). In addition, a relatively high number of imprinted genes were found to be either upregulated (the Peg3, H19, and Plagl1 genes) or downregulated (the Igf2 and p57 genes) in this screen. Of the 35 imprinted genes present on the array, which represent about 55% of all known imprinted genes in the mouse (16, 49), 10 were deregulated in the absence of HDAC1 (data not shown). Most of these genes have been implicated in proliferation and growth control. Intriguingly, chromosome 7, which contains several clusters of imprinted genes, showed a significant deviation from the expected chromosomal distribution of deregulated genes, with 25% more deregulated genes than expected (Fig. 1D). In general, the chromosomal distribution of deregulated genes (i.e., the number of genes found to be deregulated on a specific chromosome relative to the total number of deregulated genes) was similar to the chromosomal distribution of genes present on the array and the chromosomal distribution of genes found in the whole mouse genome (Fig. 1D).

The deregulation of HDAC1 target genes is reversible. As a first step in the analysis of putative HDAC1 target genes, we wanted to validate the results from the DNA array screen. To this end, we isolated total RNA from wild-type and HDAC1 KO ES cells and analyzed a subset of randomly picked potential target genes by real-time RT-PCR and Northern blot analyses. Of the 33 deregulated genes examined, 16 upregulated and 12 downregulated genes were successfully confirmed (Fig. 2). Three genes (the Apobec2, Gata4, and Gjb3 genes) did not show the expected result, while two genes (the Rb1 and Rfx2 genes) were upregulated below the defined threshold level.

Next, we stably transfected HDAC1 KO cells with a retrovirus-derived ES cell expression vector encoding HDAC1, or with the empty expression vector as a control, to test whether the deregulation of putative target genes could be reverted. Among several clones originating from HDAC1-deficient cells transfected with the HDAC1 encoding vector, two cell lines (KO-reA and KO-reB), which expressed HDAC1 protein almost at wild-type levels, were chosen for further analyses. HDAC1-null cells transfected with the empty vector showed no changes in HDAC1 and HDAC2 expression or in total HDAC activity compared to untransfected cells (Fig. 3A and data not shown). We have previously shown that the expression of the closely related deacetylase HDAC2 is increased upon deletion of HDAC1 (36) (Fig. 3A). Interestingly, the reintroduction of HDAC1 into KO cells led to a significant reduction in HDAC2 expression and completely restored total histone deacetylase activity (Fig. 3A). To analyze HDAC2-associated HDAC1 protein levels, we immunoprecipitated HDAC2 from extracts of wild-type, HDAC1 KO, and reintroduced cell lines (KO-reA and KO-reB) and visualized precipitated HDAC1 and HDAC2 on immunoblots. As shown in Fig. 3B, the

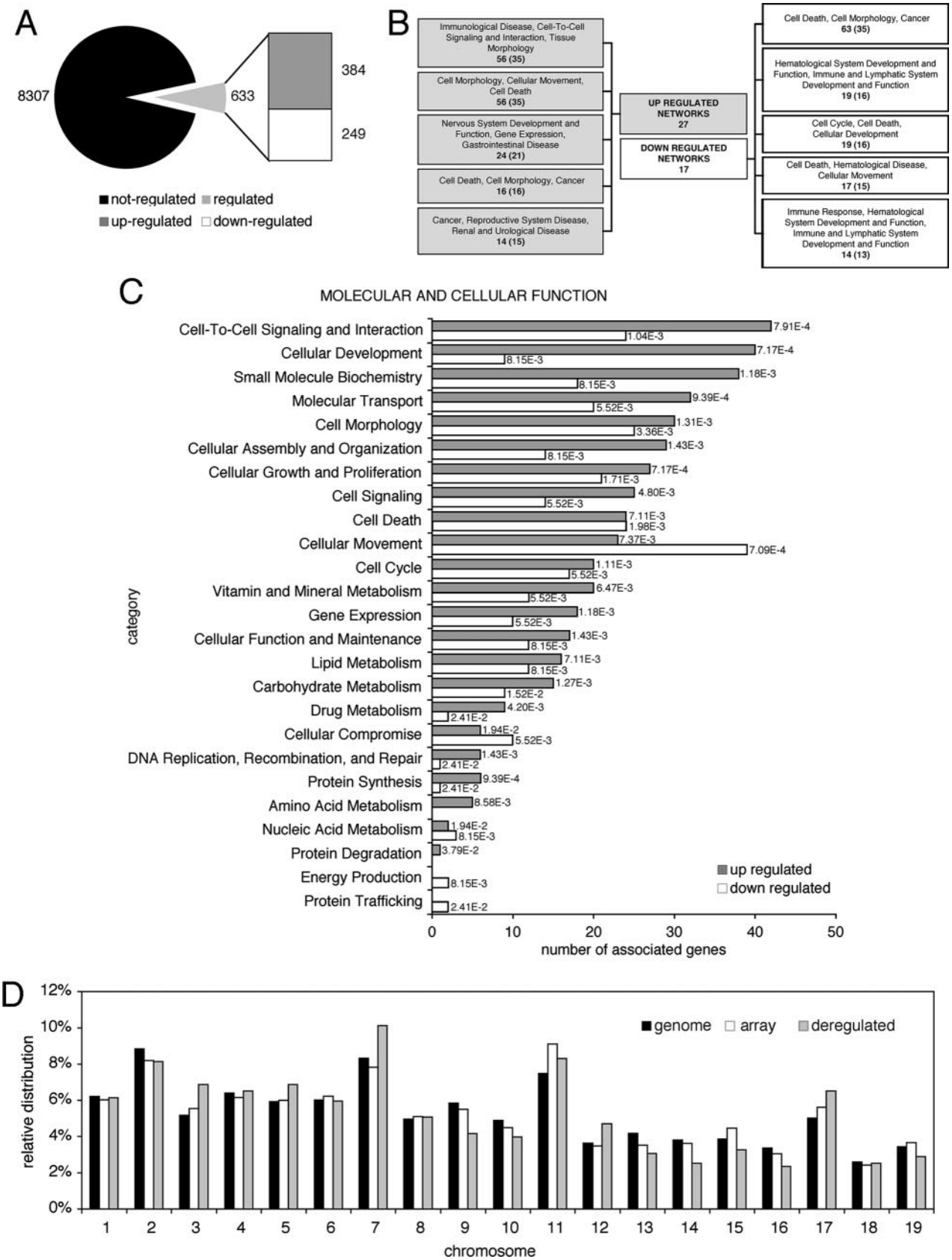

FIG. 1. DNA array analysis of wild-type versus HDAC1-deficient ES cell lines. (A) Summary of DNA array results. Total numbers of genes found on Affymetrix GeneChip Mu74 microarray analysis are presented as nonregulated (black) and deregulated (light gray) genes. The dark gray

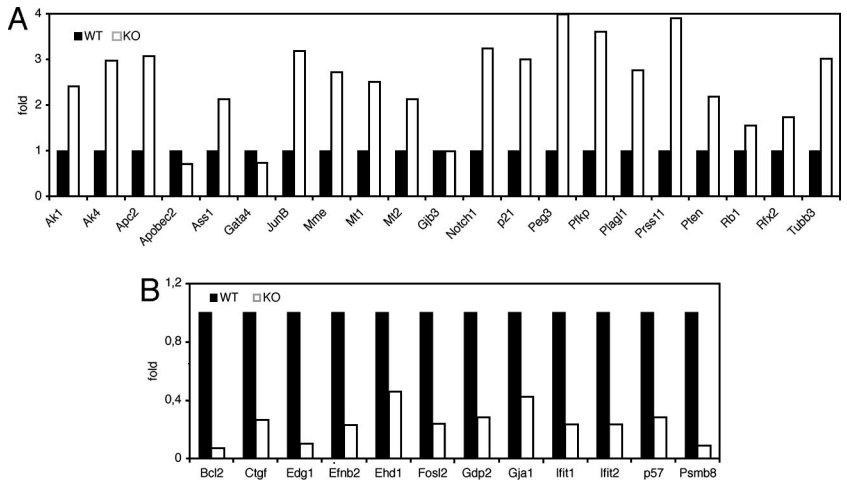

FIG. 2. Validation of the DNA array screen. (A) SYBR green real-time RT-PCR analysis of upregulated genes. Total RNA isolated from wild-type (WT) and HDAC1-deficient ES cells was reverse transcribed, and PCR was performed with primers specific for the cDNA of indicated genes. Gene expression levels were normalized to tubulin α1 levels and shown as expression levels in HDAC1-null cells relative to expression levels in wild-type cells (arbitrarily set to 1). (B) SYBR green real-time RT-PCR analysis of downregulated genes. The analysis was performed as described for panel A.

amounts of HDAC2-associated HDAC1 protein in wild-type, KO-reA, and KO-reB cells were comparable. Similarly, the increased HDAC2-associated deacetylase activities in KO cells were reduced to wild-type-cell levels upon reintroduction of HDAC1. Taken together, these data show that the increase in HDAC2 expression levels and associated activity and the reduction in total deacetylase activity observed in HDAC1 KO cells are reversible.

We compared the mRNA expression levels of potential target genes in reintroduced KO ES cell lines with the respective wild-type and KO controls by real-time RT-PCR and Northern blot analyses (Fig. 3C and D). The expression levels of Prss11, p21, Mt1, Mt2, Ass1, and JunB, which was found to be upregulated in KO ES cells, showed significant decreases upon HDAC1 reintroduction in two independent cell lines (Fig. 3C). The only exception was the Apc2 gene, whose expression was threefold induced in KO cells (Fig. 2) but did not significantly respond to reexpression of HDAC1 (data not shown). At the moment, the reason for this observation is unclear. However, the Apc2 gene was the only gene in this set of HDAC1 targets that misses a CpG island within its promoter region.

Furthermore, mRNA levels of Igf2 and Dnmt3a, which were downregulated in KO cells, were restored almost to wild-type-cell levels after reintroduction of HDAC1 in both cell lines (Fig. 3D). The fact that p57 expression was reestablished in KO-reB cells but not in KO-reA cells might be due to the complex interplay of allele-specific repressive chromatin modifications that is linked to the regulation of imprinted genes (see Discussion). Taken together, these data suggest that HDAC1 can reversibly regulate the expression of a specific subset of mouse genes.

Cross talk between the class I deacetylases HDAC1 and HDAC2. Next, we wanted to know whether the enzymatic activity of HDAC1 is required for the regulation of putative target genes. For that purpose, we expressed an inactive HDAC1 protein in HDAC1-null cells. The mutation of histidines 140/141 into alanines in the catalytic domain leads to a complete loss of HDAC1 enzymatic activity without provoking conformational changes in the protein (31, 72). Wild-type and HDAC1 KO cells were stably transfected with a retroviral expression vector encoding the myc-tagged HDAC1-H140/141A mutant or the empty vector as a control. Interestingly, of several dozens of clones analyzed by indirect immunofluorescence analysis and immunoblotting (data not shown), only one showed a clearly detectable signal for the mutant HDAC1 protein. This clone, referred to as KO-mut, expressed

bar represents the number of positively regulated genes, and the white bar represents the number of negatively regulated genes in HDAC1-deficient ES cells. (B) Molecular networks of putative HDAC1 target genes. Putative HDAC1 target genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of putative HDAC1 target genes were then algorithmically generated based on their connectivity. The networks are ranked according to their scores, and the five highest-ranking networks of upregulated and downregulated genes are displayed. The numbers of genes in each network are shown in brackets. (C) Functional classification of HDAC1 target genes. The probe sets corresponding to all deregulated transcripts were analyzed using Ingenuity Pathways Analysis (Ingenuity Systems) for molecular and cellular functions. Different categories are ranked according to the numbers of associated genes. Gray bars represent genes that are upregulated in HDAC1 KO cells, and white bars represent genes that are downregulated in HDAC1 KO cells. The probability that each biological function and/or disease assigned to the data set is due to chance alone is indicated as a *P* value for each category (shown on the right-hand side of each bar). (D) Chromosomal distribution of deregulated genes. Black bars, total numbers of genes on the indicated chromosomes relative to the total number of genes in the mouse genome; white bars, numbers of genes on the indicated chromosomes present on the array relative to the total number of genes present on the array; gray bars, numbers of deregulated genes on the indicated chromosomes relative to the total number of deregulated genes. Chromosomes X and Y are excluded, since the cell lines were of opposite sexes.

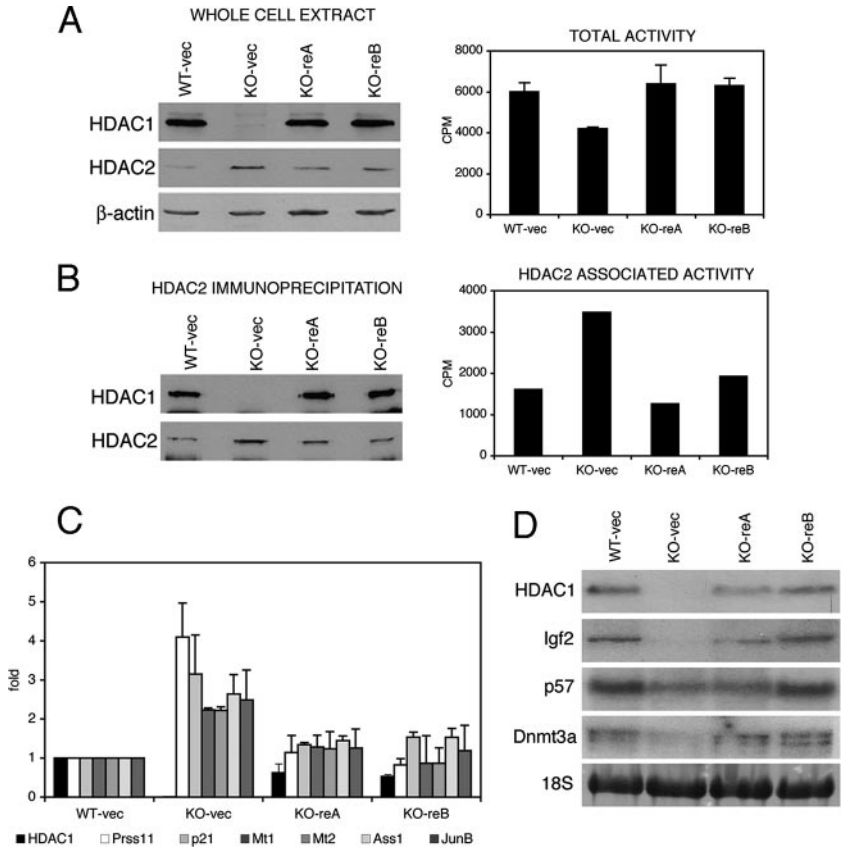

FIG. 3. The deregulation of HDAC1 target genes in HDAC1-null cells is reversible. (A) Whole-cell extracts were prepared from two different clones of HDAC1-transfected KO ES cells (KO-reA and KO-reB) and respective wild-type (WT-vec) and KO (KO-vec) controls. Extracts were analyzed for expression of HDAC1, HDAC2, and β-actin on a Western blot (left panel) and for deacetylase activity as described in Materials and Methods (right panel). Data presented for relative HDAC activities per hour per 10 μg protein are mean values for three independent experiments. (B) Equal amounts of the extracts described in panel A were precipitated with HDAC2-specific antibodies, and the immunoprecipitates were examined for the presence of HDAC1 and HDAC2 (left panel) and associated deacetylase activity (right panel). Data presented are representative of three independent experiments. (C) SYBR green real-time RT-PCR analysis of mRNA expression in the cell lines described for panel A. Gene expression levels were normalized to tubulin α1 levels and are shown relative to expression levels in the wild-type control. Data presented are mean values for three independent experiments. (D) Northern blot analysis of total RNA isolated from the same cells as used for panel A probed with cDNA fragments of the indicated genes. Methylene blue staining of 18S RNA illustrates the equal loading of the samples.

HDAC1-H140/141A at a relatively low level compared to the levels of HDAC1 in the wild-type control (Fig. 4A, left panel), suggesting that constant high expression of the inactive HDAC1 mutant might interfere with proliferation of ES cells. Hence, this clone was taken for further analyses. Remarkably, the total HDAC activity in the HDAC1 mutant-expressing cells was about 10% lower than the activity in KO control cells (Fig. 4A, right panel). This might be due to the slightly reduced HDAC2 expression in HDAC1-H140/141A-expressing cells and/or to a negative effect of the mutant HDAC1 protein on HDAC2 activity. Alternatively, HDAC2 might act as an impostor in the absence of HDAC1, a mechanism that has previously been described for certain mitogen-activated protein kinases (40).

Since HDAC1 and HDAC2 proteins are known to coprecipitate and are usually found in the same complexes (2, 29), we wanted to test whether the HDAC1-H140/141A mutant also interacted with HDAC2. Thus, HDAC2 was precipitated from extracts prepared from vector-transfected wild-type cells, vector-transfected KO cells, and KO cells expressing HDAC1 mutant protein. Subsequent immunoblot analysis with the HDAC2 antibody showed an expression pattern similar to that observed for whole-cell lysates (Fig. 4A), with a weak HDAC2 signal in wild-type cells, a strong HDAC2 signal in KO cells, and slightly reduced amounts of precipitated HDAC2 upon expression of the H140/141A mutant in KO cells (Fig. 4B). Both wild-type and mutant HDAC1 proteins efficiently associated with HDAC2, as shown by immunoblot analysis of HDAC2 immunoprecipitates with the HDAC1 antibody (Fig. 4B). Consistent with previous findings (36), HDAC2-associated activity was significantly increased in HDAC1 KO cells, indicating that despite its habitual heterodimerization with HDAC1, HDAC2 can also act as an active deacetylase in the absence of HDAC1 (Fig. 4B, right panel). Upon the expression of the HDAC1-H140/141A mutant, HDAC2 activity levels in HDAC1-null ES cells dropped by 20% (Fig. 4B, right panel). All together, these data imply that HDAC1-H140/141A expression in HDAC1 KO cells leads to reduced HDAC2 expression and HDAC2-associated activity and to a decrease in total HDAC activity.

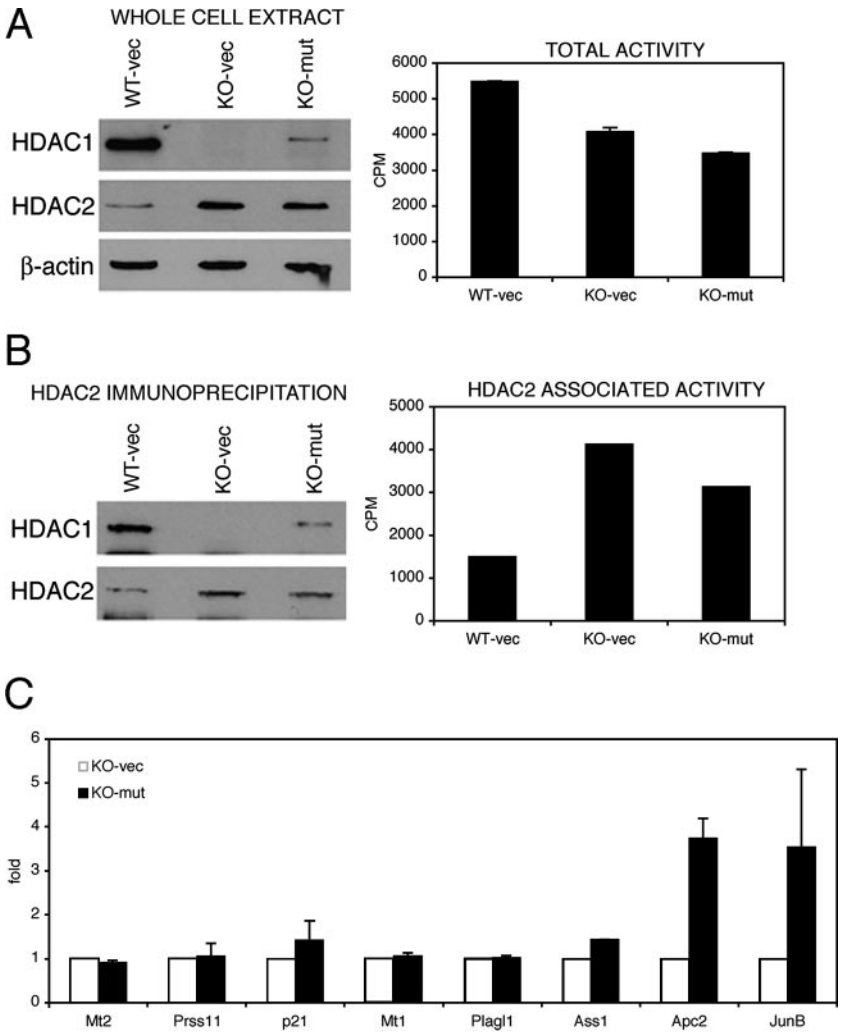

FIG. 4. Expression of an enzymatically inactive HDAC1 mutant induces a subset of HDAC1 target genes. (A) Left panel, Western blot analysis of whole-cell extracts prepared from HDAC1-H140/141A mutant-expressing HDAC1 KO cells (KO-mut) and respective wild-type (WT-vec) and KO (KO-vec) controls. The blot was incubated sequentially with antibodies specific for HDAC1, HDAC2, and β-actin, respectively. Right panel, equal amounts of the extracts described for the left panel were analyzed for total deacetylase activity. Data presented for relative HDAC activities are mean values for three independent experiments. (B) Left panel, Western blot analysis of HDAC2 immunoprecipitates prepared from the protein extracts described for panel A with antibodies specific for HDAC1 and HDAC2. Right panel, comparison of HDAC2-associated deacetylase activities after immunoprecipitation with an HDAC2-specific antibody from the whole-cell extracts described for panel A. Data presented are representative of three independent experiments. (C) SYBR green real-time RT-PCR analysis of target genes. Total RNA isolated from KO-mut cells and vector-transfected KO cells was analyzed by quantitative RT-PCR for the expression of the indicated genes. Expression levels (*n*-fold) were normalized to expression levels in KO-vec cells and presented as mean values for three experiments.

To analyze how the HDAC1-H140/141A mutant affects the transcription of putative HDAC1 target genes, we examined the expression of selected target genes by quantitative real-time RT-PCR (Fig. 4C). In contrast to that of the HDAC1 wild-type protein, expression of the inactive mutant did not restore normal expression of putative HDAC1 target genes. Most of the genes analyzed, e.g., those encoding Mt2, Prss11, p21, Mt1, Plagl1, and Ass1, showed no change or a moderate increase in mRNA levels in HDAC1-H140/141A-expressing cells compared to those in the KO control. Strikingly, two of the genes analyzed, the Apc2 and JunB genes, had three- to four-times-higher expression levels in KO-mut cells. Given the fact that the inactive HDAC1 mutant reduces the enzymatic activity of HDAC2, the strong increases in the expression levels of these two genes suggest that, in the absence of HDAC1, HDAC2 might be involved in their regulation.

HDAC inhibitor treatment reveals two distinct groups of HDAC1 target genes. In order to determine whether other histone deacetylases are involved in the regulation of putative HDAC1 target genes, we next analyzed the responsiveness of putative HDAC1 target genes to the deacetylase inhibitor TSA, a general inhibitor of most class I and class II HDACs (43, 86). To this end, wild-type and HDAC1-deficient ES cells were treated for 12 h with the solvent dimethyl sulfoxide (DMSO) or increasing concentrations of TSA. The expression of a panel of putative HDAC1 target genes was then analyzed by real-time RT-PCR. The known TSA responsive gene HDAC1 (26, 32, 65) showed a dose-dependent increase in

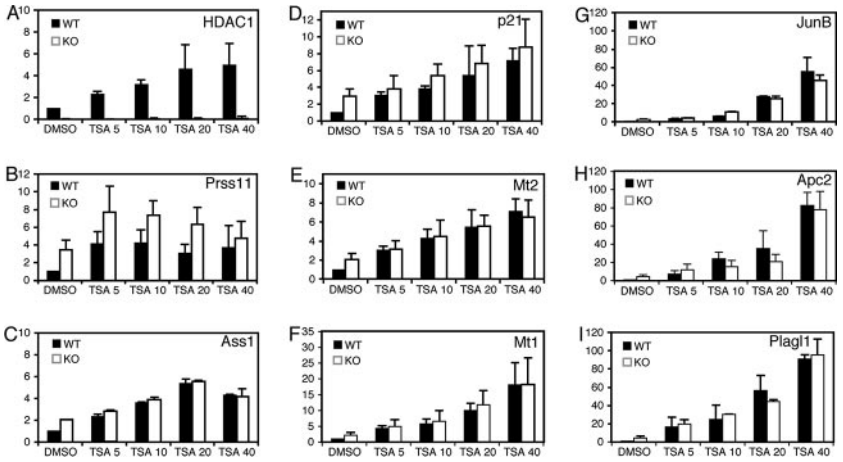

FIG. 5. TSA positively regulates the expression of potential HDAC1 target genes. SYBR green real-time RT-PCR analysis of wild-type (WT) and KO ES cells treated for 12 h with DMSO or 5, 10, 20, and 40 ng/ml (16.5, 33.1, 66.1, and 132.3 nM, respectively) of TSA. Target gene expression levels, normalized to tubulin α1 expression levels, are shown relative to expression levels in DMSO-treated wild-type cells (arbitrarily set to 1). The data presented are mean values for three independent experiments.

expression in wild-type ES cells (Fig. 5A). As expected for HDAC1 target genes, all tested genes displayed increased expression in response to TSA treatment in wild-type cells (Fig. 5B-I). Furthermore, in the absence of HDAC1, all tested target genes were less sensitive to the deacetylase inhibitor. These results confirm a crucial role for HDAC1 and its enzymatic activity in the regulation of these genes. Interestingly, in HDAC1 KO cells, genes such as those encoding Prss11, p21, Ass1, and Mt2 (Fig. 5B to E) showed only moderate response (two- to threefold) to TSA, suggesting that the major histone deacetylase involved in their regulation is HDAC1. In contrast, four other genes, the Mt1, JunB, Apc2, and Plagl1 genes, displayed a strong response to TSA (8- to 22-fold) in HDAC1 KO cells (Fig. 5F to I), indicating that these genes are regulated by other HDACs as well. As shown above, the expression of Apc2 and JunB was significantly enhanced in HDAC1 KO cells upon the expression of the enzymatically inactive mutant, i.e., under conditions where HDAC2-associated deacetylase activity was reduced (Fig. 4C). These findings strongly suggest that HDAC2 is involved in the regulation of Apc2 and JunB. Another putative HDAC1 target gene, the Plagl1 gene, showed a different response; it was not induced by the inactive HDAC1 mutant but showed a dramatic increase in expression upon TSA treatment. A possible explanation is that Plagl1 might be regulated by other histone deacetylases, which are sensitive to TSA but unaffected by the expression of the inactive HDAC1 mutant. Moreover, since the Plagl1 gene is an imprinted gene, the regulation of its expression might be more complex. Taken together, these data imply that HDAC1 putative target genes can be divided into two groups: genes that are regulated mainly by HDAC1 (e.g., the Prss11 and Ass1 genes) and genes, such as the JunB and Apc2 genes, that are regulated by HDAC1 and other histone deacetylases.

Repression of target genes is associated with the presence of HDAC1 and histone deacetylation. To test whether the regulation of the identified target genes involves direct recruitment of HDAC1, we analyzed two genes of each group in ChIP assays. Chromatin isolated from wild-type and HDAC1-deficient ES cells was immunoprecipitated with antibodies specific for HDAC1 and HDAC2 or with an unspecific antibody as a control. The amount of immunoprecipitated DNA was analyzed by semiquantitative PCR using primers specific for the proximal promoters (within the first 500 bp upstream of the first exon) and intragenic regions (within 1,000 to 1,500 bp downstream of the first exon) of individual genes. In wild-type ES cells, HDAC1 was present on the analyzed regulatory regions of all tested genes, with the exception of the proximal promoter region of Apc2 (Fig. 6). However, it is likely that other regions within the Apc2 gene are important for the HDAC1-dependent regulation of this gene, since HDAC1 was detected at the Apc2 intragenic region within 1,000 bp of exon 1. In contrast, HDAC1 was absent from control genes, such as those encoding β-actin and GAPDH (data not shown). All together, these data provide strong evidence for a direct involvement of HDAC1 in the regulation of Prss11, Ass1, JunB, and Apc2.

In addition, we analyzed the presence of the HDAC1 homologue HDAC2 at the promoter of the putative target genes. Here, it is important to note that the HDAC2 antibody is more efficient than the HDAC1 antibody in ChIP assays and gives stronger signals. Therefore, it is not possible to directly compare the amounts of recruited HDAC1 and HDAC2. As shown in Fig. 6, HDAC2 was present on all genes tested in this experiment. The amounts of recruited HDAC2 stayed unchanged on the Prss11 and Ass1 promoters in the absence of HDAC1. These data suggest that HDAC1 is the predominant deacetylase regulating Prss11 and Ass1 and that HDAC2 cannot replace HDAC1 as a repressor of these genes. HDAC1 and HDAC2 are probably recruited by different transcription factors and/or by complexes in which HDAC2 does not compensate for the loss of HDAC1. This idea is also supported by the weak responses of Prss11 and Ass1 to TSA in HDAC1-null cells (Fig. 5B and C).

In contrast, HDAC2 recruitment was highly increased at the JunB and Apc2 promoters in HDAC1 knockout cells. This is consistent with the finding that these genes respond robustly to

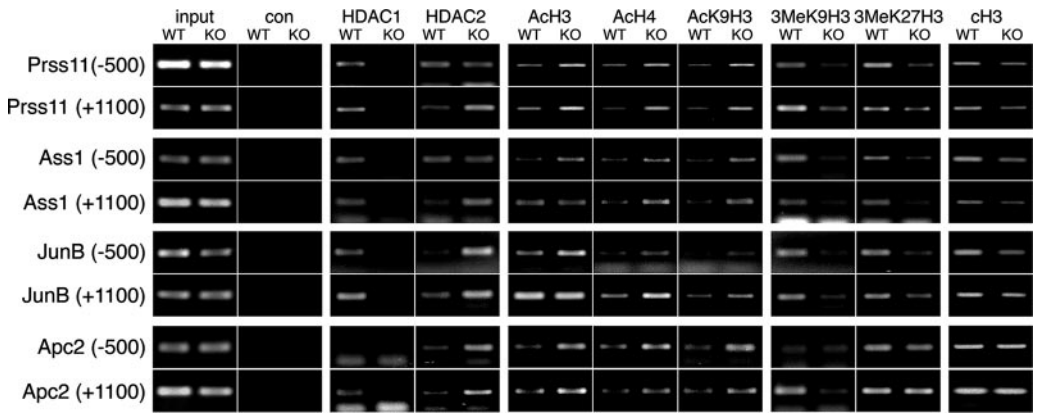

FIG. 6. Absence of HDAC1 on the regulatory regions of target genes is associated with hyperacetylation of histones. Formaldehyde-cross-linked chromatin from wild-type (WT) and HDAC1 KO cells was immunoprecipitated with control (con), HDAC1, HDAC2, acetylated H3 (AcH3), acetylated H4 (AcH4), acetylated lysine 9 H3 (AcK9H3), trimethylated lysine 9 H3 (3MeK9H3), trimethylated lysine 27 H3 (3MeK27H3), and C-terminal H3 antibodies (cH3). DNA isolated from immunoprecipitated fractions and total input chromatin was analyzed by semiquantitative PCR specific for the indicated regions. These results are representative of three independent experiments.

TSA even in the absence of HDAC1 (Fig. 5G and H). We therefore conclude that both HDAC1 and HDAC2 are involved in the repression of these genes, probably within the same complexes, where the increased presence of HDAC2 can partially compensate for the loss of HDAC1 or act as an impostor. This idea is also supported by the fact that the expression of the inactive HDAC1 mutant activates JunB and Apc2 in KO cells, either by negatively affecting HDAC2 activity or by replacing HDAC2 in putative regulatory complexes.

To examine the effect of the loss of HDAC1 activity on local histone modifications, we next examined the acetylation levels of histones H3 and H4 at the chosen HDAC1 target genes in wild-type and HDAC1-deficient cells. In addition, we analyzed these genes for the presence of the repressive marks trimethyl K9 and trimethyl K27 at histone H3. To test whether the absence of HDAC1 and increased transcription affect the nucleosome density, we also performed ChIP analyses with an antibody that recognizes the C terminus of histone H3. As shown in Fig. 6, most of the target genes (with the exception of the Apc2 promoter) showed slightly reduced nucleosome densities at both the promoters and the intragenic regions in HDAC1 KO cells. Despite the small reduction in associated histone proteins upon the loss of HDAC1, we consistently observed increased acetylation levels of histones H3 and H4 on nucleosomes associated with the promoters and intragenic regions of all tested genes (Fig. 6). Furthermore, the analysis of modifications of specific lysine residues on the HDAC1 target gene promoters revealed a strong hyperacetylation of lysine 9 on histone H3 within all tested target gene regions. Taking into account the slight reduction in nucleosome density, we observed at the same time the loss of the trimethylation marks on both lysine 9 and lysine 27 on histone H3 in KO cells.

Taken together, these data show a strong correlation between the presence of HDAC1 and decreased histone acetylation at specific genes, indicating that these are indeed direct HDAC1 target genes. Our results also emphasize the role of HDAC1 as an epigenetic regulator, by showing that the loss of this enzyme can provoke changes not only on its direct substrates, acetylated histones, but also on substrates of other histone-modifying enzymes.

An HDAC1 target gene, the Prss11 gene, encodes a regulator of cell proliferation. An HDAC1 target gene, the Prss11 gene, is a putative tumor suppressor gene in ovarian cancer (12) and was shown to be consistently upregulated upon the loss of HDAC1 expression in a variety of cell types, including mouse fibroblasts and human tumor cells (G. Zupkovitz, S. Chiocca, and C. Seiser, unpublished observations). Remarkably, small interfering RNA-mediated ablation of HDAC1 impaired the proliferation of the human osteosarcoma cell line U2OS, suggesting that HDAC1 might be a relevant target for HDAC inhibitors as tumor drugs (S. Senese, K. Zaragoza-Dorr, S. Minardi, L. Bernard, G. F. Draetta, M. Alcalay, C. Seiser, and S. Chiocca, submitted for publication). To test whether enhanced expression of Prss11 would affect the proliferation rates of human tumor cells, U2OS cells were stably transfected with a Prss11 expression vector. Vector-transfected control cells and Prss11-overexpressing single clones were analyzed in proliferation assays and for expression of the proliferation marker Ki67. As shown in Fig. 7, both cell numbers and percentages of Ki67 positive cells were significantly reduced in Prss11-overexpressing tumor cells. Thus, the HDAC1 target gene Prss11 gene encodes a negative regulator of cell proliferation and its overexpression in the absence of HDAC1 might contribute to the proliferation phenotype observed in HDAC1 KO ES cells and HDAC1-deficient tumor cells.

HDAC1 as a positive regulator of gene expression. Our analysis of gene expression in wild-type and HDAC1 KO ES cells revealed that in addition to the 4% of genes upregulated, 3% of genes were downregulated in the absence of HDAC1. These data suggest that HDAC1 can positively regulate a significant portion of murine genes (Fig. 1A). The activating effect of HDAC1 on gene expression can be a direct consequence of HDAC1 recruitment to a target gene or an indirect effect due to the increased expression and/or activity of transcriptional repressors upon the loss of HDAC1. For instance, overexpression of HDAC2 in HDAC1 KO ES cells might

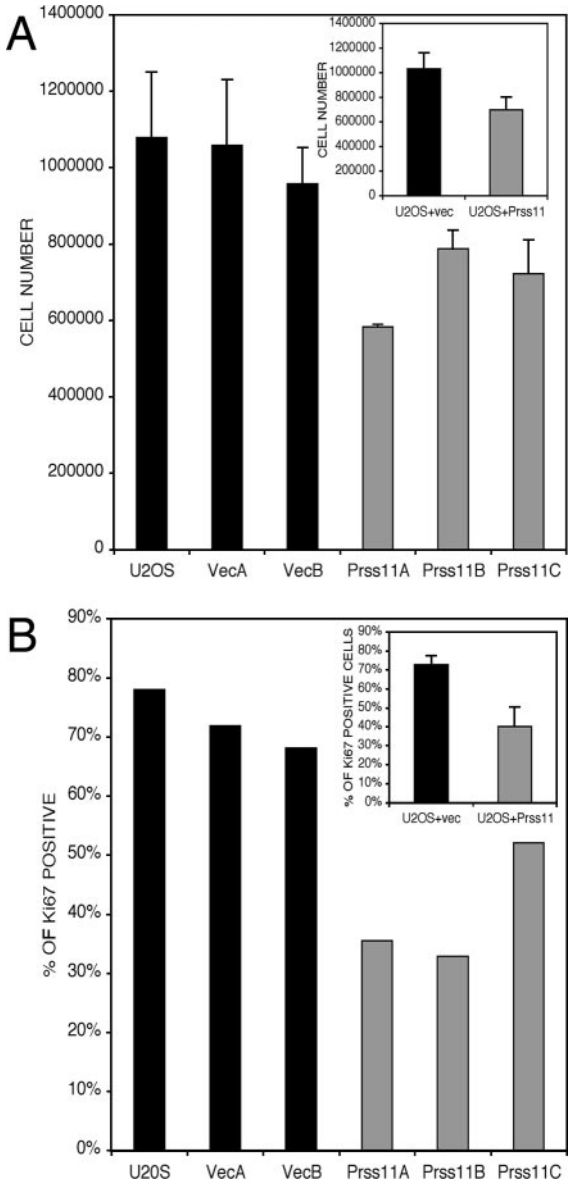

FIG. 7. The HDAC1 target gene Prss1 gene negatively affects proliferation in human tumor cells. (A) Equal numbers (120,000) of untransfected, empty-vector-transfected (VecA and VecB), and Prss11-overexpressing (Prss11A, Prss11B, and Prss11C) U2OS cells were plated in triplicate on 10-cm dishes. Cell numbers were determined after 3 days by using a Casy cell counter. The insert shows mean values for three vector-transfected cell lines (U2OS+vec) and three Prss11-overexpressing cell lines (U2OS+Prss11). (B) Ki67 staining of the same cell lines as used for panel A. Ki67 was visualized by indirect fluorescence microscopy, and nuclear DNA was stained with DAPI. Data are presented as percentages of Ki67-positive cells per 100 counted cells. The insert shows mean values for Ki67-positive cells for cell lines transfected with empty-vector (U2OS+vec) and Prss11-overexpressing (U2OS+Prss11) cell lines.

cause the repression of specific genes in the absence of HDAC1 by a compensatory mechanism. To distinguish between these possibilities, we analyzed several putative target genes that were downregulated in HDAC1 KO cells for their responsiveness to TSA. Genes that require HDAC1 for their activation should be negatively regulated by the deacetylase inhibitor in wild-type cells. In contrast, genes that are repressed by increased levels of HDAC2 in HDAC1 KO cells should be reactivated by TSA.

Four putative target genes, the Edg1, Efnb2, Ehd1, and Gja1 genes, showed significant downregulation in HDAC1 KO cells (2.5- to 5-fold) when tested by real-time RT-PCR analysis (Fig. 8A). The expression of three of these genes (the Efnb2, Ehd1, and Edg1 genes) was induced by TSA in HDAC1-null cells, indicating that these genes are negatively regulated by HDACs in the absence of HDAC1. Efnb2 and Ehd1 showed no significant response to the deacetylase inhibitor in wild-type cells, suggesting that their expression is normally not controlled by TSA-sensitive deacetylases. In contrast, higher concentrations of TSA stimulated Edg1 expression in wild-type cells and HDAC1 KO ES cells, indicating that this gene is negatively regulated by HDACs in both cell types.

Therefore, we examined the Edg1 gene for the presence of HDAC1 and HDAC2 in ChIP assays. As shown in Fig. 8B, HDAC1 and small amounts of HDAC2 were detectable at the Edg1 promoter in wild-type ES cells. The recruitment of HDAC2 was significantly increased upon the loss of HDAC1, which correlated with the almost complete loss of Edg1 expression. Surprisingly, histone acetylation at the Edg1 promoter was not affected by the absence of HDAC1, as signals for both acetylated histone H3 and acetylated histone H4 and the C terminus of histone H3 (nucleosome density) were all reduced to similar extents (Fig. 8B). These results indicate that additional deacetylase substrates might participate in the regulation of Edg1. Interestingly, K9 trimethylation on histone H3 seemed to be linked to the presence of HDAC1, whereas K27 trimethylation on histone H3 was not significantly changed in HDAC1 KO cells. Taken together, these data suggest that the expression levels of Edg1, Efnb2, and Ehd1 are reduced in HDAC1-null cells by a compensatory mechanism that involves the activity of other histone deacetylases, such as HDAC2.

In contrast to those of the target genes described above, expression levels of Gja1 were reduced not only upon the loss of HDAC1 but also in a dose-dependent manner by TSA (Fig. 8A). As demonstrated by ChIP analyses, HDAC1 was present at the Gja1 promoter in wild-type cells, while HDAC2 was recruited mainly in HDAC1-null cells (Fig. 8B). Given that Gja1 expression is further reduced by TSA treatment of HDAC1 KO cells, HDAC2 might to some extent compensate for the loss of HDAC1. Analysis of chromatin modifications at the Gja1 promoter revealed that the acetylations of histone H4 and K9 at histone H3 were slightly reduced in the absence of HDAC1 (Fig. 8B), while the trimethylation of H3-K9 was increased in HDAC1 KO cells (Fig. 8B). Thus, the reduced expression of Gja1 correlated with the increased presence of the repressive K9 trimethylation mark on histone H3. The fact that histone acetylation on the Gja1 promoter is only slightly affected suggests that for the regulation of this gene, other proteins are the relevant targets for HDAC1. In summary, the Gja1 gene represents an unusual HDAC1 target gene that requires the presence of HDAC1 and its enzymatic activity for activation.

HDAC1 and its activity are required for the activation of IFN target genes. Next, we asked whether the positive role of HDAC1 in the regulation of gene expression is a more general

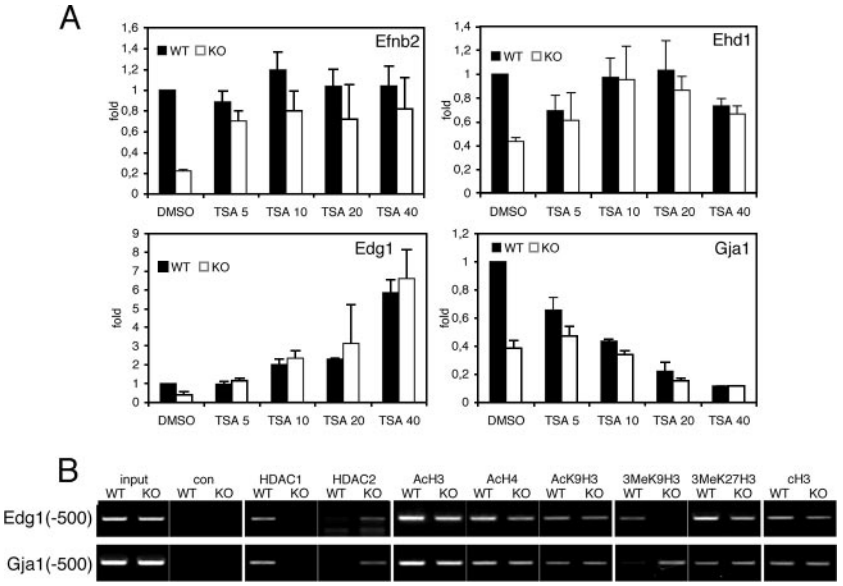

FIG. 8. Positive impact of HDAC1 on the expression of a subset of target genes. (A) Real-time RT-PCR analysis of the expression of Edg1, Efnb2, Ehd1, and Gja1 in wild-type (WT) and HDAC1 KO ES cells upon treatment for 12 h with solvent (DMSO) or different concentrations of TSA. Normalized gene expression levels are shown relative to expression levels in DMSO-treated cells. (B) ChIP analysis of the Edg1 and Gja1 promoters in wild-type and HDAC1 KO cells. Formaldehyde-cross-linked chromatin was immunoprecipitated with control antibodies (con) or antibodies specific for HDAC1, HDAC2, acetylated H3 (AcH3), acetylated H4 (AcH4), or the C terminus of histone H3 (cH3). DNA isolated from immunoprecipitated material was analyzed by semiquantitative PCR with primers specific for the respective promoter regions. These results are representative of three independent experiments.

phenomenon. Several reports suggest that histone deacetylase activity is necessary for activation of interferon (IFN) target genes, probably through involvement of HDAC1 (9, 54, 63). Interferon stimulation leads to activation of the JAK/STAT pathway, resulting in tyrosine phosphorylation of STAT1 and STAT2. Homodimerization of phosphorylated STAT1 or heterodimerization of phosphorylated STAT1 and STAT2 induces their translocation into the nucleus and the activation of their target genes (for a review, see reference 15). To test a direct involvement for HDAC1 in the interferon response, we analyzed the expression of IFN target genes in wild-type and HDAC1 KO ES cells. Pilot experiments showed that IFN treatment induced STAT1 phosphorylation in both wild-type and HDAC1-null ES cells (data not shown). We next stimulated wild-type and HDAC1 KO ES cells with IFN-γ in the presence or absence of TSA and analyzed the expression of two known IFN-γ target genes, the Irf1 and Gbp2 genes. Real-time RT-PCR analysis demonstrated the transcriptional activation of both genes in response to IFN-γ in wild-type cells (Fig. 9A). In contrast, the presence of the deacetylase inhibitor TSA during IFN-γ stimulation inhibited the induction of both genes. Interestingly, the interferon-dependent activation levels of both Irf1 and Gbp2 were significantly reduced in HDAC1 KO ES cells. These data strongly support the idea that histone deacetylase activity, and in particular HDAC1 activity, is important for the induction of certain interferon target genes.

To test whether the activation of specific IFN target genes requires direct recruitment of HDAC1 to their promoters, we performed ChIP assays with wild-type and HDAC1 KO ES cells before and after stimulation with IFN-γ and in the presence or absence of TSA, using specific antibodies for HDAC1, the C terminus of histone H3, and acetylated histones H3 and H4. In addition, we monitored the presence of trimethylated K9 and K27 at histone H3 at both target promoters. The immunoprecipitated DNA was further analyzed by semiquantitative PCR or real-time PCR with primers specific for the IFN-γ-activated site of the Irf1 promoter and the proximal promoter region of Gbp2. As shown in Fig. 9B, HDAC1 was absent from both promoters in unstimulated wild-type cells but was recruited in response to IFN-γ stimulation. While TSA treatment abolished the IFN-γ-dependent induction of both genes, it did not affect the presence of HDAC1 on the Irf1 promoter in IFN-γ-treated wild-type cells, suggesting that the activity of recruited HDAC1 is essential for gene activation. Small amounts of HDAC1 were also found on both promoters upon treatment with TSA alone. This might be due to the previously demonstrated induction of HDAC1 expression by TSA (32, 65) or to increased recruitment caused by TSA-mediated acetylation of HDAC1 (60).

As shown in ChIP assays with the C-terminal H3 antibody, the nucleosome density at the Irf1 promoter was slightly reduced in the presence of TSA and was unchanged at the Gbp2 promoter in both wild-type and HDAC1 KO cells under all conditions tested. Histone acetylation was mostly unaffected upon IFN treatment, with the exception of H4 acetylation at the Irf1 promoter in wild-type cells, which was decreased upon recruitment of HDAC1. Treatment with TSA led uniformly to hyperacetylation of histone H4 on both promoters. Since there was no consistent reduction of histone acetylation during the activation of these target genes (Fig. 9B), we conclude that the crucial substrates for HDAC1 seem to be nonhistone proteins.

Remarkably, IFN-dependent induction of both genes in wild-type cells correlated with reduced K9 trimethylation of histone H3 (Fig. 9B). In HDAC1 KO cells, levels of H3-K9

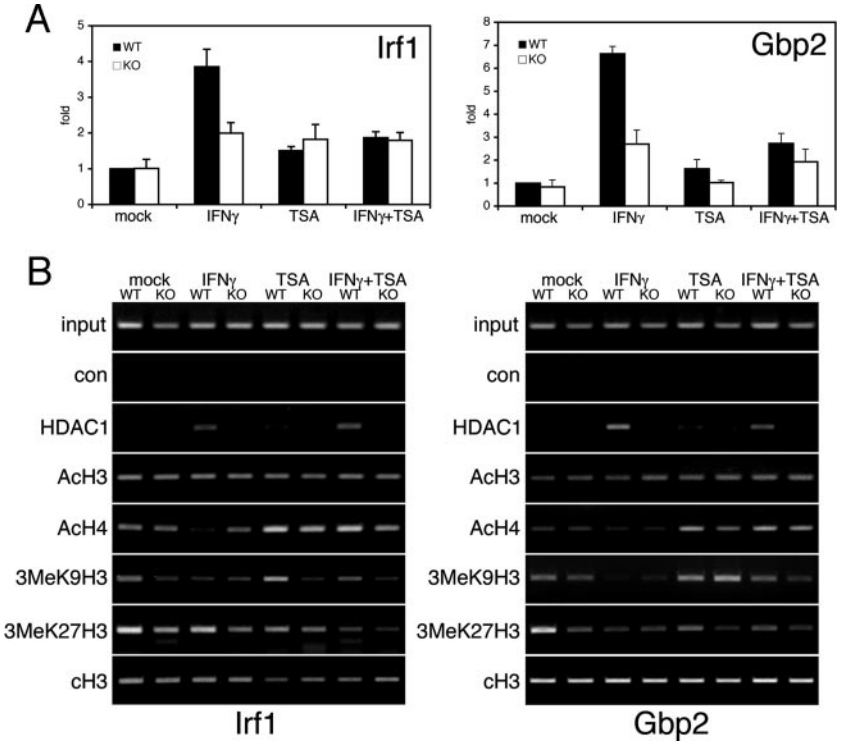

FIG. 9. HDAC1 activity is required for the induction of specific IFN target genes in ES cells. (A) Real-time RT-PCR analysis of the expression of Irf1 and Gbp2 in wild-type (WT) and HDAC1 KO ES cells. Cells were treated for 1 h with solvent (mock) or with 10 μg/ml IFN-γ (IFNγ), 20 ng/ml (33.1 nM) TSA alone (TSA), or IFN-γ and TSA together (IFN+TSA). Expression levels of Irf1 and Gbp2 were normalized to tubulin α1 levels and are shown relative to the expression levels in wild-type ES cells. Data presented are mean values for three independent experiments. (B) Presence of HDAC1 and hypoacetylation of histone H4 on Irf1 promoter are associated with its IFN-γ induction. Formaldehyde-cross-linked chromatin from wild-type and HDAC1 KO ES cells treated as described for panel A was immunoprecipitated with control antibodies (con) or antibodies specific for HDAC1, acetyl-H3 (AcH3), acetyl-H4 (AcH4), or the C terminus of histone H3 (cH3). DNA isolated from immunoprecipitated fractions was analyzed by semiquantitative PCR specific for proximal promoter regions of the Irf1 (left) and Gbp2 (right) genes.

trimethylation at both promoters were relatively low and not affected by IFN or TSA treatments, with the exception of the Gbp2 promoter under TSA treatment. Methylation of H3-K27 was reduced on both promoters upon IFN or TSA treatment of wild-type cells and was generally lower in HDAC1 KO cells. Thus, activation of both target genes was consistently accompanied by the gradual loss of repressive chromatin marks. All together, these data strongly suggest that HDAC1 and histone deacetylase activity are necessary for the induction of certain IFN target genes.

DISCUSSION

HDAC1 as transcriptional regulator of a specific subset of mouse genes. In this study, we have analyzed the role of HDAC1 as transcriptional regulator in mouse ES cells. Several studies have investigated the effect of histone deacetylase inhibitors on gene expression (19, 20), but this is to our knowledge the first analysis of the regulatory function of a single mammalian deacetylase using a genome-wide approach. We show here that a specific subset (7%) of mouse genes is deregulated in the absence of HDAC1. The number of putative HDAC1 targets might be in fact underestimated, since HDAC2 can partially compensate for the loss of HDAC1 as a transcriptional regulator (see below). Compared to the numbers of genes found to be deregulated by deacetylase inhibitors (2 to 8%), the number of HDAC1 target genes is relatively high (24, 42, 48, 79). This fact indicates that HDAC1 is a key player in the regulation of gene expression in ES cells and is in accordance with the changes observed in HDAC1-deficient ES cells, such as significantly decreased cellular HDAC activity and hyperacetylation of a subset of core histones (36). Furthermore, all analyzed HDAC1 target genes were sensitive to the deacetylase inhibitor TSA, thus supporting the idea that the enzymatic activity of HDAC1 is important for the regulation of target genes.

Biological function of HDAC1 target genes. Database analysis of HDAC1 target genes showed that the deacetylase controls the expression of genes involved in a variety of biological processes. Based on the phenotype of HDAC1-deficient embryos and ES cells, HDAC1 has been implicated in proliferation control. Along this line, a significant fraction of HDAC1 target genes is involved in growth control and cell communication. In particular, several genes with proposed tumor suppressor activity (the JunB, Plagl1, Apc2, metallothionein 1, metallothionein 2, and Prss11 genes) are regulated by HDAC1. For instance, JunB is downregulated in several human tumors (10, 57) and was shown to suppress cell proliferation by transcriptional activation of p16 (58). The Plagl1 gene, which encodes a growth suppressor, is frequently silenced in ovarian

and breast cancer cells (1, 6), the adenomatous polyposis coli (APC)-like APC2 gene encodes a putative tumor suppressor (34), and the genes for Mt1 and Mt2 are repressed in some metastatic tumors (87).

The Prss11 gene was originally isolated as a gene whose expression was downregulated in a human fibroblast cell line after transformation with simian virus 40 (89). Repression of human Prss11 has been repeatedly observed in ovarian cancers (66) and melanomas (4), in close correlation with the malignant progression and metastasis of these tumors. We show here that Prss11 is negatively regulated by HDAC1 and that its overexpression significantly impairs proliferation in human tumor cells. These findings suggest that HDAC1 is one of the relevant target enzymes for HDAC inhibitors as tumor drugs. This idea is also supported by the finding that HDAC1 inactivation induced apoptosis in human tumor cells (Senese et al., submitted). We have recently established an HDAC1 mouse tumor model and will test the function of some of the abovementioned genes for their relevance as HDAC1-regulated tumor suppressors.

Another interesting finding is the identification of several imprinted genes as HDAC1 targets. It is important to note that the allele-specific silencing of some of these imprinted genes occurs only upon differentiation. However, the observed deregulation of specific imprinted genes is not due to unscheduled ES cell differentiation in the absence of HDAC1, since virtually all wild-type and HDAC1 KO cells are positive for Oct4, a marker for undifferentiated ES cells (R. Brunmeir and C. Seiser, unpublished results). A hallmark of imprinted genes is the presence of differentially methylated CpG islands known as differentially methylated regions. Preliminary data suggest that HDAC1 might be required for the methylation of specific differentially methylated regions (G. Egger, unpublished observations). DNA methylation is closely linked to histone deacetylation, since class I deacetylases, including HDAC1, were found to associate with both methyl-binding proteins and methyltransferases (22, 23, 50, 88). In accordance with these findings, deacetylase inhibitors have been previously shown to affect the expression of several imprinted HDAC1 target genes, including the H19, Igf2, and p57 genes (25, 26). To better understand the role of HDAC1 in the regulation of imprinted genes, it will be necessary to establish a system suitable for the study of allele-specific gene expression in the presence and absence of HDAC1.

Consequences of HDAC1 recruitment. ChIP analysis revealed that HDAC1 is recruited to a specific set of target promoters. Similarly, the homologous yeast deacetylase Rpd3p was found to be associated with a specific class of target genes, while the HATs Gcn5 and Esa1 are generally recruited to the promoters of active protein-coding genes (61). In addition, HDAC1 was recruited to the 5′ intragenic regions of all investigated target genes. A detailed ChIP analysis throughout the murine HDAC1 gene, whose expression is under the control of HDAC1, showed a predominant localization of HDAC1 at the 5′ region of this gene (65; G. Zupkovitz, unpublished results). These findings corroborate the results from many laboratories showing the recruitment of HDAC1 by specific transcription factors to promoters and enhancers of target genes (46). Noticeably, the loss of HDAC1 led to reduced nucleosome density on most of the HDAC1 target promoters, suggesting a crucial role for the deacetylase for chromatin condensation at these genes. Accordingly, the occupancy of target genes by HDAC1, with the exception of positively regulated target genes (see below), resulted in the reduced acetylation of histones H3 and H4.

Interestingly, the presence of other epigenetic marks, namely, trimethylation at K9 and K27 of histone H3, was reduced at HDAC1 target genes in the absence of HDAC1. Concerning histone modifications on target promoters, these results are in agreement with several reports describing the association of HDAC1 with histone H3 methyltransferases, including the K9 methyltransferase Suv39h1 and the histone H3-modifying polycomb complex PRC2 (14, 77). However, in contrast to a previous report that showed a link between K9 trimethylation and transcriptional elongation (76), we observed reduced K9 trimethylation at intragenic regions at genes that were induced in the absence of HDAC1. This might be explained by the complex cross talk between enzymes that control histone acetylation and methylation. For instance, HDAC1 was shown to interact not only with a K9 methyltransferase but also with the recently identified K9-demethylating enzyme JMJD2A (83). In addition, the recruitment and expression of JMJD2A might be regulated in an HDAC-dependent manner (27). We are currently performing a chromosome-wide ChIP-on-chip analysis for mouse HDAC1 to understand in more detail the consequences of HDAC1 recruitment on histone acetylation and other chromatin modifications.

Functional links between HDAC1 and HDAC2. Expression of the related class I enzyme HDAC2 was reliably upregulated upon inactivation of HDAC1 in all cell systems that we tested, including mouse ES cells, fibroblasts, T cells, and human tumor cells (36; unpublished data; Senese et al., submitted). Interestingly, only 7% of the mouse HDAC1 target genes are found to be deregulated in human tumor cells missing HDAC1 (Senese et al., submitted). However, when HDAC1 and HDAC2 were simultaneously inactivated, more than 20% of the murine HDAC1 targets were differentially expressed in U2OS cells. Increased amounts of HDAC2 might partially compensate for the loss of HDAC1 as a transcriptional repressor. This idea is supported by several findings in the present study. For instance, a group of genes, including the JunB and Apc2 genes, showed a significant sensitivity towards the HDAC inhibitor TSA even in the absence of HDAC1 (Fig. 5), suggesting that they are regulated also by other HDACs. Accordingly, ChIP assays demonstrated increased recruitment of HDAC2 as a consequence of the loss of HDAC1 (Fig. 6). Such genes are most probably regulated by the recruitment of repressor complexes that contain increased amounts of HDAC2 in the absence of HDAC1, which mask the actual repressive capacity of HDAC1 for these target genes. In fact, HDAC2 might act as an impostor (40) by replacing HDAC1 as a component of certain repressor complexes in HDAC1 KO cells. In agreement with this idea, the expression of an enzymatically inactive HDAC1 mutant led to increased expression of JunB and Apc2 in HDAC1-null cells (Fig. 4C). It is likely that for some other genes, HDAC2 can fully compensate for the lost repressor function of HDAC1 in KO cells. These genes were not detected in our screen but might be identified in an HDAC1 ChIP-on-chip approach.

HDAC1 as a positive regulator of transcription. Finally, we have also characterized genes that display reduced expression levels in HDAC1-deficient cells. For one group of genes, the decrease in expression in the absence of HDAC1 was rescued by TSA treatment (Fig. 8A, Efnb2, Ehd1, and Edg1), indicating that these genes are repressed by other deacetylases in HDAC1 KO cells. In accordance with this idea, large amounts of HDAC2 were associated with these genes in the absence of HDAC1 but not in wild-type cells. In parallel with reduced expression levels, the acetylation levels of associated histones were also decreased. Thus, the loss of HDAC1 indirectly leads to the repression of this group of genes due to the enhanced presence of HDAC2. Efnb2, a ligand of the ephrin receptor tyrosine kinase that is required for hippocampal plasticity (30), was found to be a prognostic marker for neuroblastomas (70, 71). Ehd1, a member of the eps15 homology domain-containing family (47), was shown to participate in the endocytosis of the insulin-like growth factor 1 receptor (62), and Edg1 is a G-protein-coupled receptor for sphingosine-1-phosphate (33) that seems to stimulate cell migration and metastasis (84).

The most surprising finding of this study was the identification of genes that require HDAC1 directly for their activation. In contrast to all the other HDAC1 target genes, these genes showed a negative response to TSA (Fig. 8A, Gja1, and Fig. 9A). HDAC1 was recruited either constitutively (Gja1) or, in the case of the Irf1 and Gbp2 IFN-responsive genes, in response to the IFN signal, strongly suggesting that these genes are direct HDAC1 targets. Gja1 is the major protein of gap junctions in the heart and seems to display differential responses to deacetylase inhibitors in normal versus transformed cells (55). The Irf1 gene was shown to be a tumor susceptibility gene which encodes a protein with tumor suppressor-like function (reviewed in reference 69). In contrast to those of the tumor suppressors p21 and Prss11, which are negatively regulated by HDAC1, Irf1 expression levels seem to be dependent on HDAC1. The HDAC1 homologue Rpd3p was originally identified as a factor required for both activation and repression of yeast genes (82) and plays a positive role in the activation of osmoresponsive promoters (17). In mammalian cells, HDAC1 was recently identified as a coactivator of the glucocorticoid receptor (60). Furthermore, histone deacetylase activity was shown to be required for the proper activation of IFN-responsive genes and HDAC1 was implicated in this process (reviewed in reference 53). We provide the first evidence for the regulated HDAC1 recruitment to the promoters of Irf1 and Gbp2 during transcriptional induction by IFN-γ. The mechanism of HDAC1-dependent activation of target genes is not entirely clarified. On the one hand, HDAC1 might be required predominantly for the deacetylation of specific transcription regulators that trigger their activation. This idea is supported by the fact that histone acetylation at the positively regulated Gja1, Irf1, and Gbp2 target genes is mostly unaffected by the presence of HDAC1 (Fig. 8 and 9). On the other hand, the stimulation of HDAC1 target genes could be accompanied by waves of histone acetylation and deacetylation that are controlled by both HATs and HDACs. Similar cyclic changes in the acetylation of core histones have been previously observed during the hormone-dependent activation of genes (45, 60). Taken together, the results presented in our study demonstrate a major function for HDAC1 as a transcriptional regulator in mouse ES cells.

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