Histone Deacetylase Inhibitors Repress Tumoral Expression of the Proinvasive Factor RUNX2

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Abstract

Aberrant reactivation of embryonic pathways occurs commonly in cancer. The transcription factor RUNX2 plays a fundamental role during embryogenesis and is aberrantly reactivated during progression and metastasization of different types of human tumors. In this study, we attempted to dissect the molecular mechanisms governing RUNX2 expression and its aberrant reactivation. We identified a new regulatory enhancer element, located within the RUNX2 gene, which is responsible for the activation of the RUNX2 promoter and for the regulation of its expression in cancer cells. Furthermore, we have shown that treatment with the anticaner compounds histone deacetylase inhibitor (HDACi) results in a profound inhibition of RUNX2 expression, which is determined by the disruption of the transcription-activating complex on the identified enhancer. These data envisage a possible targeting strategy to counteract the oncogenic function of RUNX2 in cancer cells and provide evidence that the cytotoxic activity of HDACi in cancer is not only dependent on the reactivation of silenced oncosuppressors but also on the repression of oncogenic factors that are necessary for survival and progression.

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Introduction

RUNX2, a transcription factor belonging to the Runt-related family, is necessary for osteoblast differentiation and skeletal morphogenesis (1, 2). Factors that are crucial during embryogenesis are often hijacked during cancer progression, and RUNX2 is not an exception. RUNX2 is increasingly recognized in cancer biology for its oncogenic properties and a large number of articles linked the deregulation of RUNX2 expression with progression and metastasization of different types of epithelial tumors (1, 3–18). Regulation of RUNX2 may occur at multiple levels and through multiple signaling pathways. However, the mechanisms that regulate the reactivation of this factor in cancer are still unknown. It has been reported that RUNX2 expression is higher in tumor than in normal tissue and that the level of expression of this protein has a negative prognostic value in a number of cancer types (16, 19–22). The RUNX2 gene encodes for two isoforms starting from two separate promoters: the proximal P2 promoter expresses RUNX2 isoform I and the distal P1 promoter expresses isoform II (23, 24). The two isoforms differ for only a few aminoacids at the N-terminal regions, even though differences in their activity were reported. The existence of two alternative promoters suggests that the expression of the two isoforms is regulated through different mechanisms (24–27). Indeed, evidence exists that the use of the two promoters is context dependent. In particular, our group and others have shown that RUNX2 isoform I is the major RUNX2 isoform in tumor cells, while isoform II seems to be restricted to skeletal cells (16, 26). Noticeably, the majority of molecular signals known to control RUNX2 act through the P1 promoter, while the regulation of the P2 promoter remains largely unknown (28–30). Dissecting the mechanisms controlling RUNX2 P2 promoter is a necessary step to elucidate the complex network of molecular determinants that govern RUNX2 expression in tumor and it may help designing an appropriate therapeutic approach to counteract the prometastatic function of RUNX2.

Histone deacetylase inhibitors (HDACi) are a class of chemical compounds that block the activity of Zn-dependent HDAC, inducing the hyperacetylation of a number of proteins (31). Histone acetylation is associated with open chromatin structure and active transcription. Thus, it is believed that the major anticaner effect of HDACi is due to the reactivation of silent oncosuppressor genes. However, increasing evidence indicates that, besides histones, HDACi treatment affects acetylation and function of a number of nonhistonic proteins, opening new explanations to the mechanisms of action of these cancer drugs (32). Recent studies have shown that HDACi suppress the expression of a number of oncogenes that are highly expressed in cancer, supporting the hypothesis that besides reinducing suppressor genes, these drugs may impair cancer cell growth by blocking oncogenic signals that are necessary for tumor survival and progression (33–35).

In this study, we demonstrate for the first time that HDACi profoundly impairs the expression of RUNX2 isoform I in tumor cells. In the attempt to dissect the mechanism responsible for the HDACi-dependent RUNX2 inhibition, we identified a new enhancer within the RUNX2 gene, which is responsible for the activation of the P2 promoter in tumor cells and contains the HDACi-responsive elements. We have shown that c-JUN binds to the EN1H3 and is a positive regulator of RUNX2 expression.
Finally, we have shown that inhibition of HDAC6 is implicated in the repressive effects of HDACi.

Materials and Methods

Cell cultures and treatments

BCPAP and TPC1 cell lines were obtained from Prof. Massimo Santoro (University of Naples, Naples, Italy). MCF7, A549, H1299, HCT116, PC3, DU145, and SAOS2 cell lines were obtained from Dr. Massimo Broggiini (IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy). A375, MDA-MB-231 cell lines and colon H2998 cell line were obtained from Dr. Adriana Albini and Dr. Bruno Casali, respectively (Arcispedale Santa Maria Nuova-IRCCS, Reggio Emilia, Italy). All cell lines were authenticated by SNP profiling at Multiplexion GmbH; dates of last authentication reports are December 9, 2014, and November 12, 2014. All cell lines were obtained from Dr. Massimo Broggiini (IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy). A375, MDA-MB-231 cell lines and colon H2998 cell line were obtained from Dr. Adriana Albini and Dr. Bruno Casali, respectively (Arcispedale Santa Maria Nuova-IRCCS, Reggio Emilia, Italy). All cell lines were authenticated by SNP profiling at Multiplexion GmbH; dates of last authentication reports are December 9, 2014, and November 12, 2014. All cell lines were grown at 37°C/5% CO₂. A549, H1299, PC3, DU145, and H2998 were grown in RPMI with 10% fetal bovine serum. HCT116 were grown in Iscove medium with 10% fetal bovine serum. The remaining lines were grown in DMEM with 10% fetal bovine serum.

Unless otherwise specified, cells were treated for 24 hours with trichostatin A (TSA) 1 μmol/L or suberoylanilide hydroxamic acid (SAHA; Vorinostat) 10 μmol/L (Sigma-Aldrich). Tubacin, 5-Aza-2′-deoxycytidine (5AZA), actinomycin D, cycloheximide, and DMSO were purchased from Sigma-Aldrich.

Quantitative real time-PCR

Total RNA was purified with RNAeasy Mini Kit (Qiagen) and retrotranscribed using the iScript cDNA kit (Bio-Rad). Quantitative real time-PCR (qRT-PCR) was conducted using Sso Fast EvaGreen Super Mix (Bio-Rad) in the CFX96 Real Time PCR Detection System (Bio-Rad).

Western blot analysis

Western blot analysis was performed as previously described (16). Antibodies used were goat anti-RUNX2 (AF2006; R&D Systems), rabbit anti-c-JUN (H-79, sc-1694; Santa Cruz Biotechnology, DBA Italia), mouse anti-actin (AC-15; Sigma-Aldrich), horseradish peroxidase–conjugated anti-goat (sc-2350; Santa Cruz Biotechnology), anti-rabbit and anti-mouse (GE Healthcare).

Transfection and luciferase assay

Cells were cotransfected with a pGL3 reporter vector and the pRL-TK vector (Promega) as a control, using Lipofectamine LTX (Life Technologies). Forty-eight hours after transfection, cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) in a GloMax 20/20 Luminometer (Promega), according to the manufacturer's instructions. For each sample, firefly luciferase activity was normalized on Renilla luciferase activity and transactivation of the various reporter constructs was expressed as fold induction on pGL3-basic activity.

Bioinformatic analysis

For the identification of putative enhancers, we analyzed a 300-kb genomic region spanning the RUNX2 gene using ENCODE project annotations (36), integrated in Genome Browser (http://genome.ucsc.edu/; refs. 37, 38). Putative enhancer regions were identified on the basis of conservation in mammals, the presence of DNAseI hypersensitivity clusters, and the presence of specific histone modifications associated with enhancer regions (H3K27ac and H3K4Me1). Transcription factor–binding sites analysis on the ENH3 core was performed by merging the data from the open-source JASPAR database (http://jaspar.genereg.net/) and by the data retrieved from the TRANSFAC Professional database (http://www.biorbase-international.com/) using the Match algorithm.

DNase sensitivity assay

Chromatin accessibility of the P2 promoter and ENH1, ENH2, and ENH3 was assessed using the EpiQuik Chromatin Analysis Kit (Bio-Rad) according to the manufacturer's protocol.

Chromatin immunoprecipitation

After cross-linking with 1% formaldehyde, chromatin was sonicated using a Sonopuls HD2070 sonicator (Bandelin) and precipitated with anti-RNA-PolII antibodies (ab817; Abcam), rabbit anti-c-JUN antibodies (H-79, sc-1694X; Santa Cruz Biotechnology), or with mouse and rabbit IgG (Santa Cruz Biotechnology) as control, and the immunoprecipitated DNA fragments were quantified by qPCR.

Electromobility shift assay

S3A wild-type (WT) and mutant probes were obtained by Integrated DNA Technologies. Probe was radiolabeled with [γ-32P]-ATP (PerkinElmer) using T4 polynucleotide kinase (New England Biolabs) and purified on a Chromaspin-30 column (Clontech Laboratories). Radiolabeled probe was incubated with or without nuclear extract in the presence of 1 μg of dl-dC (Sigma-Aldrich). Binding reactions were resolved on a nondenaturant polyacrylamide gel.

DNA pulldown

Fifty microliters of streptavidin-conjugated Dynabeads M-280 (Life Technologies) was incubated with 20 pmol of biotinylated WT or Mut1 + 2 S3A probe (Integrated DNA Technologies). Bound proteins were eluted in SDS sample buffer and resolved on SDS-PAGE.

siRNA transfections

c-JUN Stealth RNAi (20 nmol/L) and control oligos (Life Technologies) or 30 nmol/L of TrTrFECTa RNAi against HDAC6 and control oligos (Integrated DNA Technologies) were transfected using RNAiMax Lipofectamine (Life Technologies). For cotransfection of plasmids and siRNA, Lipofectamine 2000 (Life Technologies) was used and the cells were harvested 48 hours after transfection for luciferase assay.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software (GraphPad). Statistical significance was determined using the Student t test. Each experiment was replicated from a minimum of two times to a maximum of five times. Cooperation between Mut1 and Mut2 was assessed by F statistics in an ANOVA setting.

Additional materials and methods are provided in the Supplementary Data.
Results

HDACi inhibit RUNX2 expression in thyroid cancer cell lines

Reactivation of crucial factors during cancer progression may be determined by changes in chromatin organization. We evaluated the effect of chromatin remodeling agents on RUNX2 expression in two different papillary thyroid cancer (PTC) cell lines. TPC1 and BCPAP cells were treated with 5AZA, inhibitor of DNA methyltransferases, and with TSA and SAHA, inhibitors of HDACs (Fig. 1A–D). In both cell lines, treatment with TSA and SAHA resulted in a strong inhibition of RUNX2 expression at the mRNA and protein level, whereas 5AZA displayed only marginal effects as compared with control.

We analyzed the kinetic of HDACi effect on RUNX2 expression (Fig. 1E and F). In both cell lines, RUNX2 mRNA levels decreased rapidly after TSA exposure and the effect was already detectable after 3 hours. To assess the reversibility of the HDACi effect on RUNX2 expression, TPC1 and BCPAP cells were treated with TSA for 6 hours and then moved to TSA-free medium (Fig. 1G and H). In both cell lines, inhibition of RUNX2 was completely rescued after 6 hours from TSA withdrawal. The rapidity of the TSA mediated inhibition and the ready reversibility of the effect suggested that HDACi control RUNX2 expression through a direct mechanism. To test this hypothesis, BCPAP and TPC1 cells were treated with TSA in the presence or absence of actinomycin D that inhibits transcription, and cycloheximide that blocks protein synthesis (Fig. 1I and J). Blockade of transcription by actinomycin D completely abolishes the effect of TSA on RUNX2 expression in both cell lines, demonstrating that repression of RUNX2 by TSA requires active transcription. In contrast, the inhibition of protein synthesis by cycloheximide did not block the TSA-mediated RUNX2 repression, demonstrating that the HDACi effect was mostly direct and did not require synthesis of new factors. However, in BCPAP cells, cycloheximide treatment slightly reduced the repressive effect of TSA, suggesting the possible involvement of additional indirect events in this mechanism.

Next, we investigated the effect of HDACi on RUNX2 mRNA stability. TPC1 and BCPAP cells were pretreated with TSA for 30 minutes and then actinomycin D was added to halt transcription (Fig. 1K and L). In both cell lines, degradation rate was similar, independently from the TSA pretreatment, indicating that HDACi do not have significant effect on RUNX2 mRNA stability. In addition, luciferase assay has shown that TSA did not target the RUNX2 3'-UTR, thus ruling out the possibility that HDACi induced the expression of noncoding RNAs that may affect RUNX2 stability (Supplementary Fig. S1A and S1B).

Next, we tested the effect of TSA on RUNX2 promoter activity. We previously have shown that isoform I is the major RUNX2 isoform expressed in thyroid cancer patients (16). RT-PCR analysis confirmed that isoform II was not detectable in either TPC1 or BCPAP untreated cells and that TSA did not affect its expression. In contrast, isoform I was strongly repressed by TSA in both cell lines (Fig. 2A and B). Thus, we focused our analysis on the P2 promoter. The full-length RUNX2 P2 promoter, and a shorter region of about 400 bp encompassing the transcriptional starting site (SS; sp2) were cloned upstream of a luciferase reporter gene and their transcriptional activity was assessed in the presence or absence of TSA (Fig. 2C). Surprisingly, TSA treatment induced a strong activation of both the full-length and sp2 promoter in both cell lines. This observation was in conflict with the expression data and suggested that RUNX2-response elements involved in this mechanism were not located within the P2 promoter. We also noticed that the P2 promoter was not able to induce a significant activation of the reporter gene in untreated cells, and that there was no significant difference in the activity of the full-length and of the short version of the P2 promoter.

A new enhancer controls RUNX2 expression in thyroid cancer cells

On the basis of this evidence, we hypothesized the existence of unknown DNA regulatory regions outside the P2 promoter. We analyzed the annotation data of the ENCODE project to identify DNA regions within the RUNX2 locus with features of regulatory elements (36–38). Three potential enhancers (ENH) were identified and named ENH1, ENH2, and ENH3 (Fig. 2D). Figure 2E shows the chromatin accessibility of these regions assessed by DNase sensitivity assay. In both cell lines, ENH1 and ENH2 showed partial accessibility, whereas ENH3 displayed a fully accessible chromatin structure, comparable with the P2 promoter. Next, the DNA fragments corresponding to ENH1, 2, and 3 were cloned into the pGL3-P2 vector downstream of the luciferase reporter gene and their ability to activate the P2 promoter was assessed by luciferase assay (Fig. 2F). Only ENH3 determined a strong induction of the reporter gene, suggesting a potent activity of this element on the P2 promoter. Noticeably, the activating effect of ENH3 on the P2 promoter was stronger in TPC1 than in BCPAP cells, in line with the higher level of RUNX2 expression detected in TPC1 as compared with BCPAP cells (Fig. 2B; ref. 16).

Figure 1.

HDACi repress RUNX2 expression. A and B, qRT-PCR analysis of RUNX2 mRNA levels in TPC1 and BCPAP cells treated with two concentrations of 5AZA for the indicated time. The bars represent the average fold change of RUNX2 in treated cells as compared with untreated cells (DMSO, Mock). C and D, qRT-PCR analysis of RUNX2 mRNA levels in TPC1 and BCPAP cells treated with three concentrations of TSA and SAHA for 24 hours. The bars represent the average fold change of RUNX2 in treated cells as compared with untreated cells (DMSO, Mock). E and F, qRT-PCR analysis of RUNX2 mRNA levels in TPC1 and BCPAP cells treated with 1 μmol/L TSA for the indicated time. The bars represent the average fold change of RUNX2 in TPC1-treated cells as compared with untreated cells (Mock). G and H, qRT-PCR analysis of RUNX2 mRNA levels in TPC1 and BCPAP cells treated with 1 μmol/L TSA for the indicated time. The bars represent the average fold change of RUNX2 in TPC1-treated cells as compared with untreated cells (Mock). I, Western blot analysis of RUNX2 and actin levels in TPC1 and BCPAP cells untreated (Mock) or treated with TSA or SAHA. E and F, qRT-PCR analysis of RUNX2 mRNA levels in TPC1 and BCPAP cells treated with 1 μmol/L TSA for the indicated time. The bars represent the average fold change of RUNX2 in TPC1-treated cells as compared with untreated cells (Mock). I, Western blot analysis of RUNX2 and actin levels in TPC1 and BCPAP cells untreated (black bars) or treated with TSA (gray bars) for 24 hours, in the presence or absence of actinomycin D (ActD; 5 μg/mL) or cycloheximide (CHX; 50 μg/mL). NT, cells that were not treated either with actinomycin D or cycloheximide. Bars represent the average fold change of RUNX2 in treated cells as compared with cells that did not receive any treatment (Mock NT). RUNX2 levels in TSA-treated BCPAP cells subjected to actinomycin D and cycloheximide are significantly different as compared with NT cells (P < 0.01). I and L, degradation curve of RUNX2 mRNA in TPC1 and BCPAP cells in the presence or absence of TSA. Cells were left untreated (Mock) or pretreated for 30 minutes with TSA before actinomycin D was added to halt transcription (10). RUNX2 mRNA levels were measured by qRT-PCR after 3 and 6 hours from actinomycin D addition. The curves represent the average fold change of RUNX2 mRNA at each time point as compared with RUNX2 levels at 0. All expression data were normalized to cyclophilin A levels and are expressed as mean values ± SEM (n = 3). * * * , P ≤ 0.000; ** * , P ≤ 0.01; * * , P ≤ 0.05.
A new ENH element is required to induce activation of the P2 promoter in thyroid cancer cells. 

A, schematic representation of RUNX2 isoform I and isoform II. 

B, RT-PCR analysis of RUNX2 isoform I and isoform II expression in BCPAP and TPC1 cells treated or untreated with TSA. GAPDH expression was analyzed as a control. M, molecular weight marker. 

C, luciferase analysis of RUNX2 P2 promoter activity in TPC1 and BCPAP cells. (Continued on the following page.)
Furthermore, ENH3 maintained the ability of activating the reporter gene when cloned in the inverted orientation and when cloned in the pGL3 vector containing the sP2 promoter. Finally, we have shown by chromatin immunoprecipitation (ChIP) experiments that the binding of RNA Polymerase II (RNA PolII) on the ENH3 was significantly enriched as compared with two downstream RUNX2 exons (exons 5 and 7; Fig. 3A and B). This observation is in agreement with the hypothesis that enhancer elements may function as RNA PolII reservoir, facilitating the access of the enzyme on the target promoter (39, 40).

A minimal 30-bp element represents the ENH3 core

With the intent of identifying the minimal DNA regulatory elements (ENH3 core) of ENH3, we performed a functional dissection of this region (Fig. 3C). We generated sequential deletion mutants of the ENH3 and their activity on the sP2 promoter was assessed by luciferase assay (Fig. 3D–F). This analysis restricted the ENH3 activity to the ENH3.C3 fragment in the central region of the ENH3 (Fig. 3D and E). This region was further divided into four fragments (Fig. 3F). Two overlapping fragments ENH3.C3A and ENH3.C3B retained most of the activity. We reasoned that the 30-bp overlapping region contained the functional sites of ENH3 and represented the ENH3 core. Noticeably, this region has shown high-grade conservation across species, suggesting a selective pressure to preserve this element during evolution (Supplementary Fig. S1C). In order to understand which nucleotides had a relevant function for the activity of ENH3, we introduced three independent point mutations within the ENH3 core in the full-length ENH3.C3 fragment (Fig. 4A and B). G>T substitution in position 1149 (Mut1) and A>T substitution in position 1156 (Mut2) strongly repressed the activity of ENH3.C3. In contrast, C>G mutation in position 1157 (Mut3) showed a trend (even if not statistically significant) toward induction, suggesting the possibility that this site negatively control ENH3 activity. Double mutation G>T and A>T (Mut1 + Mut2) further decreased the activity of the ENH3.C3 with a significant interaction effect, suggesting a positive cooperation between these two sites.

Next, we performed electromobility shift assay (EMSA) using a probe of 47 bp containing the ENH3 core (S3A probe). Figure 4C shows that the radiolabeled S3A probe was bound by a protein complex in both TPC1 and BCPAP cells and that the interaction was specific because the addition of increasing amounts of a cold WT competitor abolished the binding. We also performed competition assays using increasing amounts of Mut1, Mut2, and Mut1+2 cold competitors. Mut1 displayed similar competition efficiency as the WT competitor in both cell lines. In contrast, Mut2 displayed partial efficiency in competing for the binding as compared with the WT competitor and the competition efficiency was even further decreased when both position were mutated in the Mut1+2 double-mutant competitor. Also, Mut3 had reduced competition efficiency as compared with the WT probe, suggesting the possibility that this site participate to the binding of the protein complex to ENH3 (Fig. 4D and Supplementary Fig. S1D).

We used two independent databases (TRANSFAC and JASPAR) to predict which transcription factors were likely to bind this region. Thirty-five and 39 binding sites were predicted respectively. From these lists, we sorted transcription factors whose binding was abolished by Mut1 and Mut2 and that was not affected by Mut3. Noticeably, Mut1 and Mut2 affected the binding of two completely distinct sets of transcription factors and none of the predicted sites was commonly abolished by both mutations. Therefore, we concluded that the ENH3 core is composed of two adjacent functional sites (sites 1 and 2), which cooperate in controlling ENH3 activity and RUNX2 expression (Fig. 4E and F). This model was strongly supported by luciferase and EMSA data, in which simultaneous mutation of both sites (Mut1 +2) had a stronger effect on the ENH3 activity than the single Mut1 and Mut2 substitutions (Fig. 4B and C). Next, we crossed the results obtained by TRANSFAC and JASPAR databases in order to find binding sites that were predicted by both approaches. From this search, we obtained a list of five transcription factors (ETS1, ELK1, ERG, FLI1 belonging to the ETS family, and NFAT1 belonging to the Rel family) that were predicted to bind to site 1, and AP1 that was predicted to bind to site 2.

We performed supershift assay using anti-c-JUN antibodies to confirm that AP1 binds to site 2. In both cell lines, the addition of c-JUN antibodies determined a reduction of the binding of the protein complex to the S3A probe (Fig. 4G). Furthermore, ChIP experiments using anti-c-JUN antibodies showed that c-JUN actively binds to ENH3 in a context of structured chromatin (Fig. 4H). Finally, we used the S3A probe or the Mut1 +2 double-mutant probe to precipitate binding proteins in a DNA pulldown assay. Western blot analysis using anti-c-JUN antibodies showed that c-JUN is actively precipitated by the S3A probe while, double mutation strongly inhibited the binding (Fig. 4I).

All together, these experiments indicate that the ENH3 transcriptional activity is controlled by a multiprotein complex that binds to a bipartite site within the ENH3 core and showed that AP1 is one of the components of this functional complex.

The ENH3 contains the HDACi-responsive elements

We tested whether ENH3 was able to mediate the HDACi-repressive effect on RUNX2 expression (Fig. 5A). Noticeably, in...
Figure 3.
Mapping of the ENH3 core. A and B, ChIP analysis of RUNX2 P2, ENH3, exon 5, and exon 7 regions with anti-RNA PolII antibodies in TPC1 and BCPAP cells. ChIP with mouse IgG was performed as a control. An unrelated DNA region upstream of the P2 promoter was used as a negative control. The bars represent the average enrichment of the indicated genomic regions in the immunoprecipitated DNA expressed as percentage of the input. C, schematic representation of ENH3 deletion mutants. The ENH3.C3 fragment is highlighted in black. D–F, luciferase analysis of ENH3 deletion mutants in TPC1 and BCPAP cells. Cells were transfected with the indicated pGL3 constructs. The bars represent the average fold change of luciferase activity in cells transfected with the pGL3-sP2/ENH3 mutants vectors as compared with cells transfected with the pGL3-sP2 vector. All the luciferase data (D–F) were normalized to Renilla luciferase activity as transfection efficiency control and are expressed as mean values ± SEM (n = 3). ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05.
both cell lines, TSA determined a strong inhibition of the reporter gene in the presence of the ENH3. The ENH3.C3 region that contains the ENH3 core was sufficient to mediate the HDACi repression, while single mutation in site 1 or site 2 or double mutation in both sites abolished the response to HDACi, most likely as a consequence of their deleterious effect on the ENH3 activity (Fig. 5A and Supplemental Fig. S1E). In contrast, Mut3 did not affect the repressive effect of HDACi on ENH3, suggesting that this site is not directly involved in the HDACi-mediated RUNX2 regulation. These results indicated that the ENH3 and, in particular, the ENH3 core contains the elements necessary to mediate the HDACi-repressive effect on RUNX2 expression.

To test the possibility that HDACi alter the formation of the transcriptional complex on the ENH3, we performed EMSA with the S3A probe in both TPC1 and BCPAP cells in the presence or absence of TSA (Fig. 5B). Noticeably, the addition of TSA abolished the binding of the protein complex on the ENH3 core, pointing to a possible mechanism to explain the HDACi-mediated RUNX2 repression. On the basis of these observations, we propose the following model. In unperturbed cancer cells, RUNX2 expression is sustained by ENH3 that stimulates the transcriptional activity of the otherwise inert P2 promoter. When cells are exposed to HDACi, these drugs alter the formation of the transcriptional complex on ENH3, likely by changing the acetylation status of one or more of its components, thus shortcutting the ENH3 effect on the P2 promoter and abolishing RUNX2 expression (Fig. 5C). Indeed, when we silenced c-JUN using specific siRNAs, we observed a significant reduction of RUNX2 levels in both TPC and BCPAP cells, demonstrating that c-JUN is a positive regulator of RUNX2 expression (Fig. 5D). This observation strongly supports the proposed model. However, we cannot exclude that additional mechanisms may impinge on the HDACi-mediated effect on ENH3.

**HDAC6 contributed to ENH3 activity and to RUNX2 expression regulation**

Recently, a functional link between HDAC6 and RUNX2 in cancer cells has been proposed (41). To assess the possibility that HDAC6 controls RUNX2 expression in cancer cells, we treated TPC1 and BCPAP cells with increasing amounts of tubacin, which is a selective inhibitor of HDAC6. Tubacin strongly repressed RUNX2 expression in TPC1 cells in a dose-dependent manner. In contrast, this drug had only minimal effect on RUNX2 levels in BCPAP cells (Fig. 6A and B). We confirmed this observation by siRNA-mediated inhibition of HDAC6 expression. As expected, anti-HDAC6 siRNA determined a profound reduction of RUNX2 expression in TPC1 cells, but not in BCPAP cells, confirming the results obtained with the chemical inhibitor (Fig. 6C and D). In order to measure the effect of HDAC6 knockdown on the activity of the ENH3, we performed luciferase assay in TPC1 and BCPAP cells transfected with anti-HDAC6 siRNA or control siRNA. Indeed, while in BCPAP cells no effect was detected, in TPC1 cells, the siRNA-mediated HDAC6 inhibition resulted in a significant reduction of the transcriptional activity of both the full-length ENH3 and the ENH3.C3 fragment, indicating that HDAC6 participates to the regulation of the transcriptional complex controlling ENH3 (Fig. 6E and F). However, ChIP experiments using anti-HDAC6 antibodies failed to detect HDAC6 on either the RUNX2 P2 promoter or the ENH3, suggesting that this deacetylase controls RUNX2 expression without physically interacting with its regulatory regions (data not shown). These observations demonstrate for the first time that HDCA6 has a function in controlling RUNX2 expression in thyroid cancer cells and that its inhibition accounts for most of the repressive effect of the HDACi in TPC1 cells. However, these data also point toward the existence of cell-specific mechanisms, involving other HDACs, in controlling RUNX2 expression.

**Inhibition of ENH3 activity is a general mechanism of action of HDACi in different types of cancer**

Besides thyroid cancer, RUNX2 has also been proposed to sustain progression in other types of cancer. Thus, we investigated the HDACi effect on RUNX2 expression in 10 cancer cell lines from different tumors. In six cell lines (A549, H1299, MCF7, MDA-MB-231, PC3, and SAOS2), the TSA treatment determined a significant repression of RUNX2 expression (>1.5-fold; Fig. 7A). Comparable results were obtained with SAHA (data not shown). Recent evidence indicates that the cytotoxic effect of HDACi on cancer cells is also mediated by the repression of highly expressed oncogenes. Thus, we analyzed whether the degree of HDACi-mediated RUNX2 repression was associated with the basal level of RUNX2 expression in cancer cells (Fig. 7B). Regression analysis showed a striking correlation between the expression levels of RUNX2 and the strength of TSA-repressing effect. This observation suggests that the HDACi-mediated RUNX2 repression takes place only in those tumors in which this transcription factor is highly expressed. Finally, we tested whether the TSA-repressive effect was mediated by repression of ENH3 as we have shown in thyroid cancer cells. In all cell lines tested, the presence of ENH3 downstream of the sP2 promoter determined the activation of the reporter gene as already observed in TPC1 and BCPAP cells (Fig. 7C). Similarly, upon TSA treatment, the ENH3 activity was repressed in all cell lines, suggesting the possibility to extend to other cancer types the model of ENH3 functioning that we proposed in thyroid cancer.

**Discussion**

Tumor development and progression are the consequence of the deregulation of many pathways, which are usually orchestrated by the aberrant function of transcription factors. RUNX2 has been described as driver of aggressiveness and major determinant of metastatic spreading in cancer (1, 3–7, 9, 12, 13, 16). However, the regulatory mechanisms that lead to RUNX2 reactivation during cancer development and progression are still to be unraveled. In this respect, this study provides a completely new model of RUNX2 regulation and opens new perspectives. We have shown that the P2 promoter, which is responsible for the expression of the major RUNX2 isoform in cancer, is not sufficient to drive RUNX2 expression. This observation is in agreement with other articles, which investigated the function of RUNX2 promoters and described a highly regulated P1 promoter and a rather constitutive P2
promoter with a constant basal activity, which is not altered by the presence of regulatory stimuli (24, 28–30). Our results demonstrate that the major transcriptional activity resides in a newly identified enhancer element, located in an intronic region of the RUNX2 gene, which is able to enhance the activity of the P2 promoter and to respond to external regulatory stimuli. Our data indicate that this element is active in several types of tumors in which RUNX2 is overexpressed. It is known that the expression of genes involved in critical processes is extremely refined and it may be regulated by the precise spatiotemporal activation of multiple regulatory elements. Thus, it is likely that, besides ENH3, other elements may contribute to the complex regulation of RUNX2 expression.

Being the point of convergence of multiple oncogenic processes, RUNX2 appears to be a promising target for developing therapeutic strategies to combat cancer. Several transcription factors have been implicated in tumor progression and targeting transcription factors in cancer has been proven to be extremely challenging (42). In this study, we demonstrated for the first time that the HDACi strongly repress the expression of RUNX2 in cancer cells, suggesting a possible therapeutic strategy to counteract the prooncogenic effect of this factor in cancer patients.

To our knowledge, this is one of the first works describing the effect of HDACi on RUNX2 expression in cancer. Nevertheless, our observations appear to be in contrast with previous works in which a positive effect of these drugs on RUNX2 expression was reported in skeletal cells (43, 44). Currently, there are no experimental data to explain such discrepancy, but the different cellular context is likely to play a relevant role. In bone and cartilage cells, both RUNX2 isoforms are expressed, whereas in cancer, only isoform 1 is observed. It is possible that the increased expression of RUNX2 detected upon HDACi treatment in skeletal cells is the result of a combinatorial effect on the expression of both isoforms. It is also reasonable to suppose that in a nontumoral context, such as the skeletal tissue, the ENH3 is not active and RUNX2 expression is under the control of different elements on which HDACi exert a different regulation.

One of the major effects of HDACi on cancer cells is the remodeling of chromatin, which usually leads to the reexpression of epigenetically silenced oncosuppressor. For many years, this has been considered as the main mechanism of action of these drugs as anticancer therapies (31). Reversible acetylation is a widespread phenomenon that affects many signaling pathways and thereby many cellular processes. The growing number of identified acetylatable targets beyond chromatin provides a whole new world of potential regulatory effects of these drugs, even though most of these are likely to converge on controlling gene expression (32). Indeed, it has been demonstrated that HDACi treatment induces expression changes in 2% to 10% of all human genes with almost an equal amount of induced and repressed genes (45, 46). This effect cannot be explained without taking into consideration the role of HDACi as fine modulators of transcription through modification of nonhistonic proteins. We observed that treatment with HDACi abolishes the formation of a protein complex on the ENH3 core, thus abrogating its activating effect on the P2 promoter. We mapped a bipartite binding site, in the ENH3 core, in which three families of transcription factors are predicted to bind. The ETS family and the Rel family, would bind to site 1, making complex with AP1, which is predicted to bind site 2. All three transcription factors were also predicted in cancer progression, in line with our data that suggest their possible role in supporting the expression of the prooncogenic factor RUNX2. Noteworthy, the ability of AP1 to form transcriptional complexes with members of both ETS and NFAT families is well established, and all these factors have been shown to be directly or indirectly controlled by HDACi (47, 48).

We also demonstrated that c-JUN, component of the AP1 complex physically interacts with ENH3 and is a positive regulator of RUNX2 expression. To our knowledge, this is the first report describing a direct link between c-JUN activity and RUNX2 expression. Acetylation changes the electrostatic state of lysine residues of c-JUN, positive to neutral, thus reducing the affinity for negatively charged molecules like DNA. Similarly, acetylation of lysine residues can create new docking sites for protein–protein interaction. Therefore, it is likely that changes in the acetylation profile of one or more of the factors of the transcriptional complex binding the ENH3 affect the affinity of the interactions disassembling the complex. Previous studies have shown that c-JUN is acetylated and that its acetylation status affects transcriptional activity. Vries and colleagues (49, 50) observed that the hyperacetylation of Lys271 of c-JUN is necessary for the repression of the collagenase promoter in the presence of the EIA protein.

Many HDACi have been shown to bind to RUNX2 and to control its transcriptional activity. Of these, HDAC6 is the only HDAC that has been described to cooperate with RUNX2 and to mediate its prooncogenic role in cancer (41). In this work, we demonstrated for the first time that HDAC6 is required for the

Figure 4. Functional analysis of the ENH3 core elements. A, schematic representation of the point mutations in the ENH3 core. Gray bar, the position of the S3A probe. B, luciferase analysis of the effect of targeted point mutations in the ENH3 core. Cells were transfected with the indicated pGL3 constructs. The bars represent the average fold change of luciferase activity in cells transfected with the pGL3-S2 vector as compared with cells transfected with the pGL3-S2/ENH3 mutants vectors. The bars represent the average fold change of luciferase activity in the indicated genomic regions in the P2 promoter and ENH3 with anti-c-JUN antibodies in TPC1 and BCPAP cells. ChIP analysis of histone H3 acetylation in TPC1 and BCPAP cells. The black arrow, the position of the S3A probe. C and D, EMSA analysis of the transcriptional complex bound to ENH3 core region. Nuclear extracts from TPC1 and BCPAP cells were incubated with 32P-labeled S3A probe in the absence or presence of increasing amount of the indicated competitor DNA sequences. Consensus diagram of the predicted transcription factor binding sites is reported above the alignment and matrix scores (TRANSFAC) are indicated in brackets. E, supershift analysis of c-JUN binding to S3A probe. Nuclear extract from TPC1 and BCPAP cells was incubated with biotin-labeled S3A probe in the presence or absence of 4 µg of c-JUN antibodies or control IgG. Arrowhead, the specific complex bound to the probe. F, sequences of the WT and mutated ENH3 core in which the mutated nucleotides are displayed. Boxes highlight the site 1 and site 2 sequences. Consensus diagram of the predicted transcription factor binding sites is reported above the alignment and matrix scores (TRANSFAC) are indicated in brackets.
RUNX2 transcription in thyroid cancer, because its inhibition resulted in the repression of the ENH3 activity and of RUNX2 levels. This observation implies the existence of an additional level of positive regulation of HDAC6 on RUNX2 in cancer cells, besides the functional cooperation between these two proteins.

We have shown that the inhibitory effect of HDACi on RUNX2 expression is not restricted to thyroid cancer cells but is common to other cancer types in which RUNX2 has been implicated. We have also shown that the mechanism through which this inhibition takes place is similar in all cell lines tested and involves the inhibition of ENH3 transcription activity. In contrast, the observation that HDAC6 inhibition does not affect expression of RUNX2 in BCPAP seems to suggest that different HDACs may contribute to this regulation depending on the cellular context or on the genetic background of the tumors.

**Figure 5.**
HDACi repress RUNX2 expression by inhibiting the ENH3 function. A, luciferase analysis of the effect of TSA on ENH3 activity in TPC1 and BCPAP cells. Cells were transfected with the indicated pGL3 constructs and left untreated (black bars) or treated with TSA (gray bars). For each set of samples (TSA treated or Mock), the bars represent the average fold change of luciferase activity in cells transfected with indicated pGL3-ENH3.C3 vectors relative to pGL3-sP2 vector transfected cells. Data were normalized to Renilla luciferase activity as transfection efficiency control and are expressed as mean values ± SEM (n = 3). P value indicates the statistical significance of the difference between TSA treated and untreated samples. ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05. B, EMSA analysis of the effect of TSA on the binding of the transcriptional complex to the ENH3 core. Nuclear extracts from TPC1 and BCPAP cells treated or untreated with TSA were incubated with 32P-labeled S3A probe. Black arrow, the specific protein complex bound to the probe. *, unspecific bands. C, putative model of HDACi mechanism of action on RUNX2 gene expression. D, qRT-PCR analysis of c-JUN and RUNX2 mRNA levels in TPC1 and BCPAP cells transfected with siRNA against c-JUN (gray bar) or scramble oligos (black bars). The bars represent the average fold change of c-JUN and RUNX2 expression in c-JUN siRNA-transfected cells as compared with control. Expression data were normalized to cyclophilin A.

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Figure 6.
HDAC6 controls ENH3-mediated RUNX2 expression in TPC1 cells. A and B, qRT-PCR analysis of RUNX2 mRNA levels in TPC1 and BCPAP cells treated with tubacin (TUB) for 24 hours. The bars represent the average fold change of RUNX2 in tubacin-treated cells as compared with untreated cells (Mock). C and D, qRT-PCR analysis of HDAC6 and RUNX2 mRNA levels in TPC1 and BCPAP cells transfected with siRNA against HDAC6 (gray bar) or scramble oligos (black bars). The bars represent the average fold change of HDAC6 and RUNX2 expression in HDAC6 siRNA-transfected cells as compared with control. Expression data were normalized to cyclophilin A (A and B) or GUSB (C and D) levels and are expressed as mean values \( \pm \) SEM (\( n = 3 \)). E and F, luciferase analysis of the effect of HDAC6 siRNA on ENH3 activity in TPC1 and BCPAP cells. Cells were transfected with the indicated pGL3-sP2 constructs together with HDAC6 siRNA (gray bars) or scramble oligos (black bars). The bars represent the average fold change of luciferase activity normalized to cells transfected with the pGL3-sP2 vector together with scramble oligo. Luciferase data were normalized to Renilla luciferase activity as transfection efficiency control and are expressed as mean values \( \pm \) SEM (\( n = 3 \)). G and H, proliferation curve of TPC1 and BCPAP cells untreated or treated with TSA or tubacin. Mean values \( \pm \) SEM (\( n = 3 \)). * \( P \leq 0.01 \), ** \( P \leq 0.001 \), *** \( P \leq 0.01 \), **** \( P \leq 0.001 \).
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: V. Sancisi, A. Ciarrocchi
Development of methodology: V. Sancisi, D.C. Ambrosetti

Figure 7.
ENH3 mediates HDACi-repressive effect in different types of cancer. A, qRT-PCR analysis of RUNX2 mRNA levels in the indicated cancer cell lines. Cell lines from lung (A549 and H1299), breast (MCF7 and MDA-MB-231), colon (HTC116 and HCC2998), prostate (PC3 and DU145) carcinoma, osteosarcoma (SAOS2), and melanoma (A375) were treated with TSA for 24 hours. The bars represent the average fold change of RUNX2 in TSA-treated (gray bars) as compared with untreated cells (Mock, black bars). Expression data were normalized to cyclophilin A levels and are expressed as mean values ± SEM (n = 3). *** P ≤ 0.001; ** P ≤ 0.01; * P ≤ 0.05. The induction observed in HTC116 upon TSA treatment is most likely a normalization artefact due to the basal low level of RUNX2 expression in these cells. B, correlation analysis between RUNX2 expression levels and TSA-induced repression expressed as fold change in a panel of cancer cell lines. C, luciferase analysis of the effect of TSA on ENH3 activity in the indicated cell lines. Cells were transfected with pGL3-sP2 or with pGL3-sP2/ENH3 construct in the presence (gray bars) or absence (Mock, black bars) of TSA. For each set of samples (TSA treated or Mock), the bars represent the average fold change of luciferase activity in cells transfected with indicated pGL3-sP2/ENH3 vector relative to pGL3-sP2 vector transfected cells. Luciferase data were normalized to Renilla luciferase activity as transfection efficiency control and are expressed as mean values ± SEM (n = 3). *** P ≤ 0.001; ** P ≤ 0.01; * P ≤ 0.05.
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