Characterization of the HDAC1 Complex That Regulates the Sensitivity of Cancer Cells to Oxidative Stress

Takuya Kato, Yohei Shimono, Masaki Hasegawa, Mayumi Jijiwa, Atsushi Enomoto, Naoya Asai, Yoshiki Murakumo, and Masahide Takahashi

Abstract

Histone deacetylases (HDAC) are involved in carcinogenesis through their regulation of cell proliferation, differentiation, and survival. The inhibitors of HDAC exhibit profound synergistic effects in cancer treatment when combined with other anticancer drugs. However, the molecular mechanisms underlying this synergy are not fully understood. Here, we show that HDAC1 increases the resistance of cancer cells to oxidative stress by negatively regulating the expression of thioredoxin binding protein 2 (TBP-2). We found that the recruitment of HDAC1 to the TBP-2 promoter is mediated by a protein complex consisting of RET finger protein (RFP; also called TRIM27) and the trimERIC transcription factor NF-Y. Accordingly, RNA interference–mediated depletion of RFP led to the disruption of the protein complex and a marked increase in the sensitivity of cancer cells to cisplatin, a potent inducer of oxidative stress. Furthermore, high levels of RFP expression correlated with down-regulation of TBP-2 in human colon cancers and were associated with poor clinical outcome. These findings reveal the diverse cancer-promoting activities of HDAC1 and identify RFP as a key regulator that provides cancer cells with resistance to anticancer drugs.

Introduction

The acetylation and deacetylation of lysines in the NH2-terminal tails of core histones play crucial roles in the remodeling of chromatin and regulation of transcription. Histone deacetylases (HDAC) catalyze the removal of acetyl moieties from histones that cause chromatin compaction and preclude access by the transcriptional machinery, resulting in transcriptional repression. In addition to histones, HDACs target many other protein substrates and affect their functions in cell proliferation and cell death (1, 2). Mammalian HDACs are divided into several classes based on their sequence similarity to yeast prototypes. Class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8) are primarily nuclear in localization and ubiquitously expressed and act on multiprotein complexes (3). Aberrant expression of several class I HDACs in tumor samples has been reported (4–10). The relationship between altered expression of HDACs and cancer progression remains largely correlative. However, RNA interference (RNAi)–mediated knockdown of individual HDACs, which are overexpressed in certain tumor cell lines, suppresses tumor cell growth and survival (5, 7, 8, 11), suggesting that overexpression of HDACs could contribute to tumor onset and progression.

In vitro studies have revealed that pharmacologic class I and II HDAC inhibitors (HDACi) can arrest cell growth and induce terminal differentiation and cell death in transformed cells. These effects are primarily achieved by induction of antiproliferative, prodifferentiative, or proapoptotic genes (1, 2, 12). HDACi can selectively kill cancer cells and have limited toxicity for normal cells. Based on these in vitro findings, several HDACi are currently being tested in phase I/II clinical trials for cancer treatment. One such inhibitor, suberoylanilide hydroxamic acid (SAHA), has been approved for treatment of cutaneous T-cell lymphoma (12, 13). Whereas their activity in preclinical models has been promising, HDACi have shown relatively modest antitumor activity against solid tumors in human clinical trials (14). More recently, their use in combination with other agents has suggested greater efficacy (2); that is, HDACi have shown synergistic or additive effects with a wide variety of anticancer reagents, including conventional chemotherapeutic drugs (2, 12), leading to the suggestion that HDACi lower the apoptotic threshold of tumor cells. However, the mechanistic basis of the synergistic effect with anticancer agents is not fully understood.

HDAC1 is found in multiprotein complexes, such as Sin3 (15), nucleosome remodeling and deacetylase (NuRD; ref. 16), and corepressor of RE1-silencing transcription factor (CoREST; ref. 17), and functions as the catalytic subunit. One common feature of these complexes is their interaction with sequence-specific DNA-binding transcription factors, repressing transcription and cooperating with other chromatin modifications to shape epigenetic programs (18). HDAC1-mediated control of epigenetic regulation plays crucial roles in normal development and tumor progression. For example, in normal development, targeted disruption of HDAC1 causes early embryonic lethality due to increased expression of p21 (19). In cancer cells, HDAC1 represses the expression of tumor suppressive genes such as p21WAF1/CIP1 and Bax (20, 21), leading to aberrant cell proliferation and cell viability. Although it has been well documented that HDAC1 regulates the cell cycle as well as the differentiation and death of cells through its interactions with various proteins, less is known about the effects of HDAC1 on cellular sensitivity to stresses such as anticancer drugs.
The cytotoxicity of some chemotherapeutic agents (such as cisplatin, etoposide, paclitaxel, and doxorubicin) is achieved in part by generation of reactive oxygen species (ROS; refs. 22–25). Consistent with these findings, alterations in antioxidant activities and related gene expression modulate cellular resistance to chemotherapeutic drugs probably through ROS scavenging ability (26–29). It has been shown that inhibition of HDACs with SAHA or related compound induces thioredoxin binding protein 2 (TBP-2) expression in transformed cells associated with down-regulation of thioredoxin and increases sensitivity to ROS-induced cell death (30, 31). These observations prompted us to examine whether HDACs sensitize cancer cells to oxidative stress, resulting in potentiating the effects of chemotherapeutic agents. Here, we show a novel mechanism explaining the synergistic effects between HDACi and anticancer drugs in cancer cells.

Materials and Methods

Antibodies. The RET finger protein (RFP) and NF-YC antibodies and antibodies purchased from commercial sources are detailed in Supplementary data.

Plasmids and RNA interference. All plasmids, RNAi vectors, sequences used, and primers are detailed in Supplementary data.

Cell viability assay. Cells (8 × 10⁴) were seeded in each well of 96-well plates and, 24 h later, were treated for 10 h with H2O2 or for 24 h with cisplatin. Cell viability was measured by WST-1 assay (Roche). The data are representative of three independent experiments done in triplicate.

Reverse transcription-PCR. Total RNA from HeLa cells transfected with small interfering RNA (siRNA) was isolated using the RNeasy Mini Kit (Qiagen). cDNA transcripts were then generated using Superscript II (Invitrogen). Reverse transcription-PCR (RT-PCR) was done with primers specific to each gene (see Supplementary data). GAPDH was used as a loading control.

Immunoprecipitation and Western blotting. Immunoprecipitation experiments were carried out as previously described (33). Briefly, formaldehyde–cross-linked chromatin from the indicated cells was sonicated, and the resulting chromatin fragments were immunoprecipitated with anti-HDAC1, anti–acetylated H3, anti–acetylated H4, anti-RFP or anti-NFYB antibodies. Purified normal rabbit IgG or mouse IgG were used as controls. Formaldehyde cross-links were reversed by adding 5 mol/L NaCl to a final concentration of 0.2 mol/L and incubated for 8 h at 65°C. Purified DNA and input DNA were amplified by PCR for 32 to 37 cycles. Input represents PCR amplification of the total input DNA.

Nude mouse tumor xenografts and treatment. HeLa cells stably expressing short hairpin RNA were s.c. injected into the back of 7-week-old female nude mice (CrljCD1-Foxn1nu, Charles River Laboratories Japan). When the mean tumor volume reached at least 50 mm³, animals were randomly assigned to control and treatment groups before the first treatment with cisplatin. Cisplatin or vehicle (saline; 1 mg/kg) was i.p. administered every 4 d. The tumor volumes were measured by caliper. Mice were maintained in accordance with the institutional guidelines of Nagoya University Graduate School of Medicine and experiments were done according to approved experimental protocols.

Tissues. Formalin-fixed, paraffin-embedded sections of human colon carcinoma tissues were obtained from the Department of Pathology of Nagoya Medical Center (Nagoya, Japan). This study was approved by the ethical committee at Nagoya Medical Center (Ethical Committee Approval No. 2007-86). The patients included 57 men and 57 women (mean age, 59.4 ± range, 40–75 y). The distribution of pathologic stages was as follows: 18 stage I, 47 stage II, 32 stage III, and 15 stage IV.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections from colon carcinoma patients were deparaffinized and rehydrated. Slides were rinsed thoroughly with Protein Blocking Agent (UltraTech HRP) and 3% hydrogen peroxide. Antigen retrieval was done by autoclaving at 121°C for 10 min in 0.1 mol/L citrate buffer (pH 7.0) with 0.3% NP40 (Sigma-Aldrich). The sections were incubated with rabbit polyclonal anti-RFP antibody (1 μg/mL) for 60 min, and then the secondary biotinylated goat polyclonal antibody (Beckman Coulter) was applied for 10 min. The sections were incubated with peroxide-conjugated streptavidin for 10 min and the reaction products were visualized with 3,3’-diaminobenzidine tetrahydrochloride and H2O2. Counterstaining was done using hematoxylin. When >10% of the tumor cells were stained with the antibody, it was categorized as positive for antigen expression.

The significance of correlation between RFP signals and histopathologic factors was determined using Pearson’s χ² test. Kaplan-Meier plots were
used to estimate the prognostic relevance of RFP in univariate analysis using StatView.

**Immunofluorescence.** Formalin-fixed, paraffin-embedded sections from colon carcinoma patients were deparaffinized and dehydrated. Antigen retrieval was done by microwave heating for 10 min in 0.1 mol/L citrate buffer (pH 6). Samples were blocked with 1% bovine serum albumin in PBS and incubated with anti-RFP polyclonal antibody (2 μg/mL) and anti-TBP-2 monoclonal antibody (1:250). Then, samples were stained with Alexa Fluor 594-conjugated anti-rabbit IgG antibody, Alexa Fluor 488-conjugated anti-mouse IgG antibody, and 4′,6-diamidino-2-phenylindole (DAPI). Slides were mounted in PermaFluor (Shandon) and observed using a confocal microscope (Olympus).

**Results**

**Physical and functional association between HDAC1 and RFP.** We first tested whether HDACi affected cellular sensitivity to oxidative stress using the HeLa human cervical cancer cell line. Treatment with trichostatin A (TSA), a conventional HDACi, sensitized HeLa cells to H$_2$O$_2$, a potent oxidative stress inducer (Fig. 1A). To investigate the molecular mechanism underlying sensitization of cancer cells to oxidative stress, we focused on the roles of HDAC1, which is a well-studied member of HDACs. Knockdown of HDAC1 greatly increased the cytotoxicity of H$_2$O$_2$ as well as cisplatin (CDDP) in HeLa cells (Fig. 1B and C). Although it has been proposed that p53 associates with HDAC1 and thereby regulates cellular sensitivity to oxidative stress (21, 34–36), other mechanisms may be important in HeLa cells because p53 is inactivated by the papillomavirus protein E6 in these cells.

We previously found that the RFP, which is highly expressed in most cancer cell lines, is associated and colocalized with HDAC1 (37). Therefore, we asked whether RFP was involved in the HDAC1 modulation of cell sensitivity to oxidative stress. First, we determined binding domains between RFP and HDAC1. Coimmunoprecipitation experiments using HDAC1 or RFP deletion constructs revealed that the NH$_2$-terminal region of HDAC1 bound to both the coiled-coil and Rp domains of RFP (Supplementary Fig. 2).

**Mechanism of Anticancer Drug Resistance by HDAC1**

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![Figure 2. TBP-2 is a target of both HDAC1 and RFP and is responsible for sensitization of cancer cells to H$_2$O$_2$ and cisplatin. A, knockdown of HDAC1 or RFP causes up-regulation of TBP-2 expression. HeLa cells were transfected with control, HDAC1, or RFP siRNAs and incubated for 72 h. Expression levels of indicated genes in these cells were analyzed by RT-PCR (top) and Western blotting (bottom). B, effects of TBP-2 overexpression on cellular sensitivity to H$_2$O$_2$ and cisplatin. HeLa cells were transfected with vector control or TBP-2-V5 and incubated for 48 h. Expression of V5-TBP-2 was detected with anti-V5 antibody (top). Transfected cells were treated with H$_2$O$_2$ (bottom left) or cisplatin (bottom right). Cell viability was measured with the WST-1 assay and the cell survival index of the nontreated group was defined as 1. C, HeLa cells were transfected with control, HDAC1, RFP, or TBP-2 siRNAs and incubated for 72 h. Total cell lysates were analyzed by Western blotting with the indicated antibodies. D, HeLa cells were transfected as described in C. After 48-h incubation, cells were treated with H$_2$O$_2$ (left) or cisplatin (right). Cell viability was measured and calculated as described in B. Columns, mean of three independent experiments done in triplicate; bars, SD. *, P < 0.05 (Student’s t test).](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-08-4368)
knockdown of HDAC1 (Fig. 1B). These data suggest that HDAC1 is functionally associated with RFP.

We further explored if HDAC2 and HDAC3, which are frequently found in association with HDAC1 in transcription factor protein complexes, interact with RFP. Immunoprecipitation experiments showed that HDAC2, but not HDAC3, was associated with RFP (Supplementary Fig. S2A). In HDAC1-knockdown cells, HDAC2 expression and its association with RFP did not alter, implying that HDAC2 did not compensate for the HDAC1 function that regulates transcriptional repressor activity (1, 32, 37), we hypothesized that HDAC1 and RFP may cooperatively repress shared target genes that are responsible for the sensitization of cells to cytotoxic agents. To identify such target genes, we generated stable HeLa cells expressing control or RFP short hairpin RNA and compared the gene expression profiles by DNA microarray (Supplementary Fig. S3; Supplementary Table S1). Among genes up-regulated in RFP-knockdown cells, we focused on the TBP-2 gene because it is well known that TBP-2 inhibits thioredoxin (38, 39), a scavenger of ROS, and sensitizes cells to oxidative stress and cisplatin (40, 41). From the results of RT-PCR and Western blot analysis using HDAC1 or RFP siRNA, we found that TBP-2 expression was transcriptionally regulated by HDAC1 and RFP (Fig. 2A). To test whether TBP-2 indeed sensitized the cells to H2O2 and cisplatin, TBP-2 was overexpressed in HeLa cells (Fig. 2B). Overexpression of TBP-2 markedly increased the sensitivity of cells to these agents (Fig. 2B). These data suggest that the enhanced expression of TBP-2 in HeLa cells was responsible for the increased sensitivity to H2O2 and cisplatin in HDAC1- or RFP-knockdown cells. In addition, neither overexpression nor knockdown of TBP-2 significantly affected the protein levels of thioredoxin (Supplementary Fig. S4). We further asked if the induction of TBP-2 expression by siHDAC1 or siRFP accounted for the increased sensitivity to toxic agents. Following knockdown of TBP-2, cells recovered their resistance to H2O2 and cisplatin, which was decreased by knockdown of HDAC1 or RFP (Fig. 2C and D). These results showed that HDAC1 and RFP increased cellular resistance to these agents in part by repressing expression of TBP-2.

We also knocked down HDAC1 or RFP in three colon and breast cancer cell lines. In all three lines, results confirmed that knockdown of HDAC1 or RFP up-regulated TBP-2 expression and increased sensitivity to H2O2 and cisplatin (Supplementary Fig. S5A–C).

**HDAC1 and RFP associate with NF-Y to form a repressive complex on the TBP-2 promoter.** We performed chromatin immunoprecipitation to examine whether HDAC1 and RFP were recruited to the promoter region of the TBP-2 gene and directly regulated its expression. The TBP-2 promoter region has been reported to be ~1.5 kb in length (42). We designed one set of primers within the promoter region proximal to the TBP-2 gene (proximal) and another set upstream from the TBP-2 promoter region (distal). As shown in Fig. 3A, both HDAC1 and RFP were recruited to the TBP-2 promoter by NF-Y, A–D). In addition, knockdown of RFP enhanced the sensitivity of cancer cells to H2O2 and cisplatin as observed for knockdown of HDAC1 (Fig. 1B and D), suggesting the possibility that HDAC1 is functionally associated with RFP.

**HDAC1 and RFP regulate TBP-2 expression, sensitizing cancer cells to oxidative stress.** Because both HDAC1 and RFP have transcriptional repressor activity (1, 32, 37), we hypothesized that HDAC1 and RFP may cooperatively repress shared target genes that are responsible for the sensitization of cells to cytotoxic agents. To identify such target genes, we generated stable HeLa cells expressing control or RFP short hairpin RNA and compared the gene expression profiles by DNA microarray (Supplementary Fig. S3; Supplementary Table S1). Among genes up-regulated in RFP-knockdown cells, we focused on the TBP-2 gene because it is well known that TBP-2 inhibits thioredoxin (38, 39), a scavenger of ROS, and sensitizes cells to oxidative stress and cisplatin (40, 41). From the results of RT-PCR and Western blot analysis using HDAC1 or RFP siRNA, we found that TBP-2 expression was transcriptionally regulated by HDAC1 and RFP (Fig. 2A). To test whether TBP-2 indeed sensitized the cells to H2O2 and cisplatin, TBP-2 was overexpressed in HeLa cells (Fig. 2B). Overexpression of TBP-2 markedly increased the sensitivity of cells to these agents (Fig. 2B). These data suggest that the enhanced expression of TBP-2 in HeLa cells was responsible for the increased sensitivity to H2O2 and cisplatin in HDAC1- or RFP-knockdown cells. In addition, neither overexpression nor knockdown of TBP-2 significantly affected the protein levels of thioredoxin (Supplementary Fig. S4). We further asked if the induction of TBP-2 expression by siHDAC1 or siRFP accounted for the increased sensitivity to toxic agents. Following knockdown of TBP-2, cells recovered their resistance to H2O2 and cisplatin, which was decreased by knockdown of HDAC1 or RFP (Fig. 2C and D). These results showed that HDAC1 and RFP increased cellular resistance to these agents in part by repressing expression of TBP-2.

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recruited to the proximal but not distal region of the TBP-2 promoter. We also found that HDAC2, but not HDAC3, was recruited to the proximal region of the TBP-2 promoter (Supplementary Fig. S6).

 Luciferase reporter assays showed that RFP repressed TBP-2 promoter activity, and transcriptional repression by RFP was almost completely inhibited by TSA (Supplementary Fig. S7A). Expression of HDAC1 also repressed TBP-2 promoter activity, and cotransfection of HDAC1 with RFP showed enhanced repression of the promoter (Supplementary Fig. S7B and C). Consistent with the up-regulation of TBP-2 expression, knockdown of RFP attenuated the recruitment of HDAC1 to the TBP-2 promoter and increased the acetylation levels of histones H3 and H4 at the promoter (Fig. 3B). These results strongly suggest that RFP recruits HDAC1 to the TBP-2 promoter region and represses its transcription through HDAC activity. Moreover, treatment with TSA caused dissociation of both HDAC1 and RFP from the TBP-2 promoter (Supplementary Fig. S8), suggesting that HDAC1 may impair the formation of HDAC1-RFP protein complex and/or its recruitment to the TBP-2 promoter.

Because neither HDAC1 nor RFP has been reported to directly bind to DNA, the HDAC1-RFP complex might be recruited to the promoter by DNA binding proteins. Previous studies indicated that the proximal region of TBP-2 promoter includes an NF-Y binding sequence and that the derepression of TBP-2 expression by HDACi was regulated by NF-Y (30). Hence, we speculated that NF-Y might recruit the HDAC1-RFP complex to the TBP-2 promoter. NF-Y is a trimeric transcription factor consisting of NF-YA, NF-YB, and NF-YC. Coimmunoprecipitation studies revealed that RFP can specifically interact with NF-YC (Fig. 3C; Supplementary Fig. S9A), but not with NF-YA or NF-YB (data not shown), through its RFP domain (Supplementary Fig. S9B). These data indicated that NF-Y protein might be responsible for recruiting HDAC1 and RFP, thereby forming the repressive complex on the TBP-2 promoter.

To investigate whether HDAC1, RFP, and NF-YC formed a complex, NF-YC-V5, HDAC1-Flag, and RFP-Flag constructs were transiently cotransfected into HEK293 cells and immunoprecipitated with anti-V5 antibody, followed by immunoblotting with anti-V5 or anti-Flag antibody. As shown in Supplementary Fig. S10A, HDAC1 and RFP were coimmunoprecipitated with NF-YC, indicating that NF-YC interacted with both HDAC1 and RFP in vivo. To further elucidate whether the three proteins formed a protein complex, we performed gel filtration chromatography using HeLa cell lysates. HDAC1, RFP, and NF-YC were detected in the same fractions (corresponding to ~ 400 kDa; Supplementary Fig. 10F). These data strongly support the idea that HDAC1, RFP, and NF-YC are components of the same protein complex. Chromatin immunoprecipitation assays revealed that NF-Y is associated with the proximal region of the TBP-2 promoter (Fig. 3A) as observed for HDAC1 and RFP, and knockdown of NF-YC markedly attenuated recruitment of both HDAC1 and RFP to the TBP-2 promoter (Fig. 3D). These results imply that HDAC1, RFP, and NF-Y form a protein complex on the TBP-2 promoter.

**RFP oligomer mediates HDAC1/RFP/NF-Y complex formation.** The results shown in Fig. 3 suggested that RFP recruits HDAC1 to the TBP-2 promoter by mediating the interaction between HDAC1 and NF-YC. To support this hypothesis, we performed coimmunoprecipitation analysis using HEK293 cells in which NF-YC-V5 and HDAC1-myc were cotransfected with Flag vector or Flag-RFP. The association between HDAC1 and NF-YC was enhanced in RFP-transfected cells (Fig. 4A). Conversely, the interaction was greatly diminished in HeLa cells transfected with siRFP compared with control cells (Fig. 4B). In addition, HDAC1 knockdown lowered the association between RFP and NF-YC (Fig. 4C), indicating that both RFP and HDAC1 are required for the complex formation with NF-YC.

We further analyzed the detailed role of RFP in complex formation. Because RFP forms oligomers (43, 44), we speculated that RFP oligomerization could be important for the complex. First, we determined the domain of RFP required for its oligomerization. Coimmunoprecipitation experiments using GFP-RFP and Flag-RFP constructs confirmed that RFP forms oligomers (Fig. 5A). In addition, we found that the Ring-B-box and coiled-coil domains, but not the Rfp domain, of RFP are required for RFP-RFP interaction (Fig. 5B).

We next examined the importance of RFP oligomerization in the enhanced association between HDAC1 and NF-YC. HEK293 cells were cotransfected with NF-YC-V5, HDAC1-myc, and Flag-tagged RFP deletion constructs and resultant lysates were...
RFP expression increases cancer cell resistance to cisplatin and is associated with poor clinical outcome of cancer patients. To further investigate whether RFP expression affects the toxicity of cisplatin in cancer treatment, control and RFP-knockdown HeLa cells were injected s.c. into nude mice. When tumors grew to volumes larger than 50 mm³, cisplatin was injected i.p. every 4 days. As shown in Fig. 6A, treatment with cisplatin suppressed tumor growth of RFP-knockdown cells to a markedly greater extent than control cells, which was consistent with the in vitro data (Fig. 1D).

To analyze the effect of RFP expression on the prognosis of colon carcinoma patients, we obtained specimens and medical histories of 112 patients. Colon cancer was defined as RFP positive when more than 10% of tumor cells were clearly stained with anti-RFP antibody. Based on this criterion, 70 of 112 colon carcinoma patients were RFP positive (Fig. 2B). Consistent with in vitro data (Fig. 2A and B), TBP-2 expression was clearly down-regulated in RFP-expressing cells in colon cancer specimens (Fig. 6C). Kaplan-Meier survival analysis of these cases revealed a significant correlation between RFP expression and shorter overall survival (Fig. 6D). These results suggest that the level of RFP expression in colon cancer affects both the efficacy of anticancer drugs and long-term clinical outcome.

Discussion
Whereas the synergistic antitumor effects of HDACi and chemotherapeutic agents have received considerable attention in cancer research, the mechanistic basis of the synergy has not been clear. Elucidation of this mechanism could enhance our understanding of underlying cancer biology and promote effective therapeutic strategies. In the present study, we showed that HDACi sensitize cancer cells to oxidative stress by affecting TBP-2 expression repressed by the HDAC1/RFP/NF-Y complex. This results in reexpression of TBP-2, which sensitizes neoplastic cells to anticancer agents. We also showed that RFP has a crucial role in HDAC1/RFP/NF-Y complex formation, which increases the resistance of neoplastic cells to anticancer drugs. Thus, RFP could be a novel target for cancer therapeutics.

Several studies have suggested that HDAC1 regulates (oxidative) stress-mediated apoptosis through its association with p35 (21, 34–36). In this study, we present direct evidence that HDAC1 forms a complex with RFP and NF-Y and immunoprecipitated with anti-Flag antibody, followed by immunoblotting with anti-Flag and anti-GFP antibodies. Whole cell lysates. B, the Ring-B-box (RB) and coiled-coil domains (CC), but not the Rfp domain (RD), of RFP were required for its oligomerization. HEK293 cells were cotransfected with GFP-RFP and Flag-tagged RFP and immunoprecipitated with anti-Flag antibody. Based on this criterion, 70 of 112 colon carcinoma patients, we obtained specimens and medical histories of 112 patients. Colon cancer was defined as RFP positive when more than 10% of tumor cells were clearly stained with anti-RFP antibody. Based on this criterion, 70 of 112 colon carcinoma samples were RFP positive (Fig. 6B). Consistent with in vitro data (Fig. 2A and B), TBP-2 expression was clearly down-regulated in RFP-expressing cells in colon cancer specimens (Fig. 6C). Kaplan-Meier survival analysis of these cases revealed a significant correlation between RFP expression and shorter overall survival (Fig. 6D). These results suggest that the level of RFP expression in colon cancer affects both the efficacy of anticancer drugs and long-term clinical outcome.

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Based on these reports, up-regulated TBP-2 expression may reduce the ROS scavenging ability of thioredoxin and increase the ROS-induced oxidative stresses in cancer cells. Taken together, HDAC1 renders the cancer cells resistant to anticancer agents by regulating the state of the antioxidant system.

HDAC1 has been reported to interact with various transcription factors to repress specific genes. NF-Y has been shown to be associated with HDAC1 and the association is mediated by other proteins such as p73 (45). In this study, we showed that the interaction between HDAC1 and NF-Y is enhanced by RFP in transformed cells. RFP oligomer interacts with both HDAC1 and NF-YC through the COOH-terminal Rfp domain, and then HDAC1 is recruited to the TBP-2 promoter, which binds NF-Y (Fig. 5D). In addition, gel filtration chromatography showed that HDAC1, RFP, and NF-Y were present in the same fractions. Thus, RFP plays a crucial role in the formation of this protein complex, which represses TBP-2 expression.

We also found that HDAC1 is required for the association of RFP with NF-YC, and that inhibition of deacetylase activity by HDACi (TSA) causes dissociation of HDAC1 and RFP from the TBP-2 promoter. This finding suggests that the deacetylase activity of HDAC1 may facilitate the association of RFP with NF-YC. However, the precise mechanism how the complex formation of these three proteins is regulated remains elusive.

Moreover, HDAC2 was associated with RFP and recruited to the TBP-2 promoter, although HDAC2 does not seem to compensate for the function of HDAC1, which regulates the sensitivity of cancer cells to oxidative stress. Further investigation is necessary to elucidate the exact role of HDAC2 in the protein complex.

We showed that knockdown of RFP significantly sensitized cancer cells to cisplatin in vitro and in vivo. This effect correlated with the up-regulated expression of TBP-2, which inhibits the activity of thioredoxin. Because many anticancer drugs other than cisplatin induce ROS generation (23–25), knockdown of RFP could sensitize cancer cells to those drugs by up-regulation of TBP-2 expression. Interestingly, we found that RFP expression significantly correlates with poor clinical outcome of colon cancer patients. In addition, TBP-2 expression was clearly down-regulated in RFP-expressing colon cancer cells. These findings suggest that RFP expression renders colon cancer cells resistant to chemotherapy by repressing TBP-2 expression. Thus, RFP could become a novel target for anticancer therapy in combination with conventional anticancer drugs. Although HDACi have potent anticancer activities at concentrations that are minimally toxic to the host,

Figure 6. RFP is a novel target for cancer therapy. A, knockdown of RFP enhances the sensitivity of tumors to cisplatin in vivo. Mice were s.c. injected with shNC1, shRFP23, or shRFP26 cell lines (Supplementary Fig. S3). When the mean tumor volume reached at least 50 mm³, animals were randomly assigned to control and treatment groups before the first treatment with cisplatin. Cisplatin or vehicle (saline; 1 mg/kg) was i.p. administered every 4 d. Left, photographs of representative tumors. Middle, tumor growth curve. The mean tumor volume of the nontreated group (vehicle) was defined as 100% and compared with that of the treated group on days 1 and 21 (right). *, P < 0.05, compared with control (Student’s t test). Columns, mean (CDDP treatment group, n = 3; vehicle treatment group, n = 4); bars, SD. B, RFP expression in representative tumor tissues from colon carcinoma patients. Brown, staining with an RFP polyclonal antibody. C, RFP represses the expression of TBP-2 in colon carcinoma cells. Tumor tissues were stained with anti-RFP polyclonal antibody (red) and anti-TBP-2 monoclonal antibody (green). Cell nuclei were labeled with DAPI. D, RFP expression correlates with poor prognosis in colon carcinoma patients. Overall survival of 112 patients with colon carcinomas was stratified by RFP expression (cutoff, 10%) and analyzed by Kaplan-Meier plot. A log-rank test showed significant differences between RFP+ and RFP− groups.
dose-limiting toxicities have been observed (46). Given the tissue-restricted expression of RFP in normal cells (47) and no apparent induction of HDAC1 mRNA expression in invasive carcinoma of the breast. Breast Cancer Res Treat 2005;94:11–6.


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