The Cytoplasmic Deacetylase HDAC6 Is Required for Efficient Oncogenic Tumorigenesis

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Abstract

Histone deacetylase inhibitors (HDACI) are promising antitumor agents. Although transcriptional deregulation is thought to be the main mechanism underlying their therapeutic effects, the exact mechanism and targets by which HDACIs achieve their antitumor effects remain poorly understood. It is not known whether any of the HDAC members support robust tumor growth. In this report, we show that HDAC6, a cytoplasmic-localized and cytoskeleton-associated deacetylase, is required for efficient oncogenic transformation and tumor formation. We found that HDAC6 expression is induced upon oncogenic Ras transformation. Fibroblasts deficient in HDAC6 are more resistant to both oncogenic Ras and ErbB2-dependent transformation, indicating a critical role for HDAC6 in oncogene-induced transformation. Supporting this hypothesis, inactivation of HDAC6 in several cancer cell lines reduces anchorage-independent growth and the ability to form tumors in mice. The loss of anchorage-independent growth is associated with increased anoikis and defects in AKT and extracellular signal-regulated kinase activation upon loss of adhesion. Lastly, HDAC6-null mice are more resistant to chemical carcinogen-induced skin tumors. Our results provide the first experimental evidence that a specific HDAC member is required for efficient oncogenic transformation and indicate that HDAC6 is an important component underlying the antitumor effects of HDACIs. [Cancer Res 2008;68(18):7561–9]

Introduction

HDACs are a family of enzymes that were initially characterized as histone deacetylases (thus named HDAC). Reversible histone acetylation on lysine residues is a dynamic and highly regulated posttranslational modification, which plays a central role in gene regulation and chromatin remodeling (1). Accordingly, HDACs have been extensively and almost exclusively studied for their roles in transcriptional regulation and chromatin remodeling. HDACs have drawn intense research interest as inhibitors for these enzymes display potent antitumor activities and induce cancer growth arrest or cell death (2). Many HDAC inhibitors (HDACI) are at various stages of clinical trials for cancer patients (3–5), and suberoylancide hydroxamic acid has been approved for the clinical use in advanced, refractory cutaneous T-cell lymphoma (6). Despite the potent activity of these compounds, the fundamental question of how HDACIs achieve their antitumor effect remains poorly understood. This is due in part to the existence of at least 11 HDAC family members in the human genome (7). To date, it is not known which of the HDAC family members are the critical targets that underlie the antitumor activity of HDACIs. This knowledge not only would pave ways to the development of more selective and less toxic inhibitors by targeting specific HDAC member(s), but will also elucidate the biological pathway(s) critical for HDACIs to treat tumors, thereby facilitating rational design for future cancer therapy.

The well-established role for HDACs in histone acetylation and gene transcription has led to a general assumption that HDACIs achieve their therapeutic effects by affecting specific transcriptional programs important for proliferation and apoptosis (8). Indeed, HDAC2, which acts as a potent transcriptional corepressor, has been shown to play an important tumor-promoting role in a murine colon cancer model caused by the adenomatous polyposis coli mutation (9). However, the model that HDACIs work solely by affecting gene transcription is likely an oversimplified one. Recent studies have clearly shown that some HDAC members are localized to the cytoplasm and regulate acetylation of nonnuclear proteins (10–14). Whether the nongenomic functions regulated by HDACs are important in oncogenesis, however, remains unknown.

Among the expanding nongenomic functions regulated by HDACs, those controlled by HDAC6 are best characterized (15, 16). Unlike the extensively studied nuclear HDACs involved in gene regulation, HDAC6 is localized exclusively to the cytoplasm where it associates with microtubule and actin cytoskeleton (14, 17). Studies have shown that HDAC6 affects cytoskeleton-dependent processes, including actin remodeling, fluid phase endocytosis (macropinocytosis), dynamics of cell adhesion, and motility (13, 14, 17, 18). HDAC6 has also been shown to regulate microtubule-dependent transport and processing of toxic misfolded proteins, thereby protecting cells from the toxicity of protein aggregates, a common cause of neurodegenerative diseases (10, 19). Consistent with its function in the cytosol, the activities of HDAC6 are independent of histones but instead involve cytoplasmic substrates, such as tubulin and Hsp90 (17, 20, 21). Acetylation modulated by HDAC6 has been shown to regulate the formation of chaperone complexes and maturation of Hsp90 client proteins, including glucocorticoid receptor (21) and several oncogenic kinases (12, 22). Taken together, HDAC6 seems to be involved in the regulation of several critical cellular functions intimately linked to cancer. A specific role for HDAC6 in oncogenic transformation, however, is not yet established. In this report, we...
present evidence that HDAC6 is required for efficient oncogenic transformation, anchorage-independent proliferation, and tumor growth. Inactivation of HDAC6 by genetic ablation or specific short hairpin RNA (shRNA) renders cells more resistant to oncogenic transformation and markedly reduces tumor cell growth in vitro and in vivo. HDAC6 deficiency also sensitizes cancer cells to anoikis, cell death induced by loss of adhesion. Supporting these observations, we found that HDAC6-null mice are more resistant to chemical carcinogen-induced skin tumor formation. Importantly, we found that deacetylase activity is required for HDAC6 to support malignant tumorigenic growth, suggesting that pharmacologic inhibition of HDAC6 enzymatic activity could potentially confer an antitumor effect. Our study provides the first experimental evidence that a specific HDAC member is required for autonomous cancer cell growth of solid tumors both in vitro and in vivo and indicates that HDAC6 could be a therapeutic target for cancer treatment.

Materials and Methods

Retroviral vectors. HDAC6 small interfering RNA (siRNA) sequence (ref. 17; and as an alternate, 5'-AGACCTAATCTGGGAGACTGC-3') scramble controls (23) were cloned into pSUPER-RETRO-PURO or pSUPER-RETRO-GFP/NEO (Oligoengine). cDNAs encoding human ErbB2/HER2/neu, wild-type HDAC6, siRNA-resistant catalytically inactive HDAC6 mutants (for A431) supplemented with 10% fetal bovine serum (FBS). Mammary epithelial cells (HMEC; refs. 25, 26) and prostate epithelial cells (PrEC; refs. 27) were maintained in defined media as recommended. The indicated cell lines were serum starved overnight, trypsinized, washed, and transferred to polyHEMA-coated Petri dishes for 1 h, then stimulated with 50 μg/mL epidermal growth factor (Upstate) for indicated time.

Levels of endogenous activated Ras-GTP were assayed as described previously (29). Briefly, tumor cell lysates were incubated with recombinant glutathione 5-transferase-RaBID. Total level of Ras was detected by SDS-PAGE and immunoblotting with the aforementioned anti-Ras antibody.

Soft-agar assay. Five thousand cells per 3-cm plate were suspended in soft agar as described (30), and colonies >30 cells were scored after 3 to 4 wk. Assays were done in triplicate and thrice independently.

Proliferation assay. Five thousand cells per 24-well plate were seeded and maintained in RPMI 1640 with 10% FBS in quadruplicate. Viable trypan blue-negative cells were counted daily with hemocytometer for 5 d.

Anoikis assay. Anoikis was induced by cultured the cells in Petri dishes coated with poly(hydroxyethyl methacryl) acid (polyHEMA) as described previously (31). In brief, subconfluent cells were trypsinized, washed, and transferred to polyHEMA-coated Petri dishes at 25,000 cells/ml in serum-free media for indicated time. Apoptotic cells were then examined using Annexin V Apoptosis Detection kit (BD Pharmingen), and cells negative for both 7-AAD and Annexin V were considered to be resistant to anoikis.

Tumor growth. All procedures with mice were done under an Institutional Animal Care and Use Committee–approved protocol. For xenograft tumorigenesis assay, 10 million cells of the indicated cell lines were mixed with Matrigel and injected s.c. into both flanks of two immunocompromised SCID/Beige mice (Charles River Laboratory). Tumor volumes were determined approximately twice per week and calculated as 1/2 length × width in unit of mm3. For resection of xenograft tumors, tumors were harvested, minced, and trypsinized for 2 h at 37°C and then passed through 18G needles, washed, and plated in RPMI/10% FBS plus puromycin at 4°C to eliminate all murine cells before immunoblot analysis was performed. For chemical carcinogenesis, the backs of five male control (wild type) Sv/Eve/Black Swiss mice and 10 male experimental (HDAC6-null; ref. 14) Sv/Eve/Black Swiss mice were shaved, and the following day, 150 μL of 125 μg/ml 7,12-dimethylbenz(a)anthracene (DMBA, Sigma) in DMSO was applied topically, followed 1 wk later by twice-weekly topical applications of 150 μL of 10−6 mol/L 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma) in DMSO for 20 wk. Tumor number and size were recorded weekly. Student’s t test was used to compare tumor growth in the various models. The differences between means were considered significant if P < 0.05.

Immunohistochemistry. Excised tumors were fixed in formalin embedded in paraffin and sectioned. Ki67 (α-Ki67 antibody; Vector Laboratories) was performed on the indicated tumor sections by Duke Pathology Core Facility using standard assays.

Reverse transcription–PCR. Total RNA was isolated using RNeasy Mini kit (Qiagen), DiNase-treated using DNA-free kit (Ambion), and 1 μg RNA was used for cDNA synthesis reaction using iScript Reverse transcriptase kit (Bio-Rad). cDNA (10%) was used per reverse transcription–PCR (RT-PCR) reaction. RT-PCR program was 94°C 20 s, 48°C 20 s, 72°C 20 s for 40 cycles. RT-PCR primer sequences are as follows: HDAC6 forward 5′-TCA GGT CTA CTG TGG TCG TT, reverse 5′-TCT TCA CAT CTA GGA GAG CC; G-actin forward 5′-ACCAGGGATTGCTGACAGGATGC, reverse 5′-CATCTAGA AGCATCTGG GTGGGACG.

Results

HDAC6 is required for malignant growth of transformed cells in vitro. To explore a possible role for HDAC6 in malignant transformation, we examined whether HDAC6 expression is induced in transformed cells versus their nontransformed counterparts. Specifically, primary human cells from various origins were transformed into tumor cells by serial introduction of human telomerase (hTERT), SV40 early region, which contains large T antigen and small t antigen, and finally oncogenic Ras (32). As shown in Fig. 1A, introduction of SV40 early region and oncogenic Ras induces HDAC6 proteins level by 2-fold to 4-fold in human embryonic kidney cells (HEK), PrEC, and mammary epithelial cells

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Figure 1. HDAC6 is required for malignant growth of transformed cells. A, detection of HDAC6 by immunoblot and RT-PCR in transformed HEK, PrEC, and MEF cells compared with their nontransformed counterparts. Actin serves as a loading control. The band intensity of the HDAC6 levels was normalized to the actin levels in each lane. B, detection of mouse HDAC6, Ras, and tubulin by immunoblot in the transformed wild-type or HDAC6-null MEFs stably expressing early region of SV40 and oncogenic Ras<sup>G12V</sup>. Tubulin serves as a loading control. Photographs demonstrate anchorage-independent growth of indicated polyclonal aforementioned MEFs. The bottom labels show the average and SF of the percentage of the colonies growing in an anchorage-independent fashion compared with wild-type transformed MEF (normalized to 100%) as calculated from triplicate plates. Data are from two independent assays. C, detection of mouse HDAC6, ErbB2/HER2/neu, large T antigen, acetylated tubulin, and tubulin by immunoblot in the transformed wild-type or HDAC6-null MEFs stably expressing early region of SV40 and human ErbB2/HER2/neu. Tubulin serves as a loading control. Photographs demonstrate anchorage-independent growth of indicated polyclonal aforementioned MEFs. The bottom labels show the average and SD of the percentage of the colonies growing in an anchorage-independent fashion compared with wild-type transformed MEF (normalized to 100%) as calculated from triplicate plates. Data are from two independent assays.

(HMEC; data not shown). Similarly, introduction of oncogenic Ras<sup>G12V</sup> mutant also modestly induces HDAC6 in MEFs, when compared with control MEFs infected with empty vector. RT-PCR analysis showed that HDAC6 mRNA was up-regulated in PrEC and HEK (Fig. 1A and data not shown), indicating the oncogenic Ras can induce HDAC6. This up-regulation of HDAC6 in response to oncogenic transformation suggests a potential regulatory role of HDAC6 in the malignant transformation process.

To directly assess a role of HDAC6 in oncogenic transformation, we used MEFs derived from wild-type or HDAC6-null embryos. These MEFs provide a well-established and genetically defined model to examine the requirement of HDAC6 in malignant transformation. To this end, wild-type and HDAC6-null MEFs were transduced with retrovirus expressing SV40 early region and Ras<sup>G12V</sup> (Fig. 1B) and subsequently assayed for anchorage-independent growth in soft agar, a standard and stringent assay for malignant transformation in vitro. As shown in Fig. 1B, wild-type MEFs gave rise to a large number of colonies after retroviral transduction of Large T and small t antigens and Ras<sup>G12V</sup> oncogenes. In contrast, transduction of the same oncogenes at similar protein expression levels into HDAC6-null MEFs results in 10-fold fewer colonies (Fig. 1B), revealing that HDAC6 is required for Ras-induced oncogenic transformation. To investigate whether HDAC6 plays a broader role in oncogene-induced transformation, we also examined ErbB2-dependent transformation in wild-type and HDAC6-deficient MEFs. As shown in Fig. 1C, the transforming activity of ErbB2 is also impaired in HDAC6-null MEFs, as shown by a reduction in soft agar colony formation. Together, these results show that HDAC6 is required for efficient oncogene-induced transformation.

After establishing that HDAC6 is required for oncogenic transformation of primary murine fibroblasts, we asked if HDAC6 is critical to maintain the transformed phenotypes of established human cancer cell lines. To this end, we stably expressed two sets of small hairpin RNA targeting different regions of HDAC6 mRNA in three well-characterized lines: ovarian cancer cell line SKOV3 and breast cancer cell lines SKBR3 and MCF-7. These specific HDAC6 shRNA, but not a scramble shRNA, efficiently reduced HDAC6 levels and caused an increase in the level of acetylated α-tubulin in all three cancer cell lines (Fig. 2A). An increase in acetylated Hsp90 is also observed (Fig. 2B), demonstrating that HDAC6 is efficiently knocked down. The control and HDAC6 knockdown tumor cell lines were then subject to soft-agar growth assay. As shown in Fig. 2C, knockdown of HDAC6 by either of the two shRNA significantly inhibits the anchorage-independent growth of these cancer cells by 5-fold to 30-fold. Therefore, HDAC6 is not only important for oncogenic transformation of primary cell but is also required for maintaining the anchorage-independent growth of established cancer cell lines.
Deacetylase activity of HDAC6 is required for malignant growth of cancer cells. We next determined whether deacetylase activity of HDAC6 is required for malignant growth of cancer cells. To this end, we reconstituted the HDAC6 knockdown cancer cell lines with shRNA-resistant wild-type or catalytically inactive mutant HDAC6 (Fig. 2A) and determined their ability to grow in soft agar. As shown in Fig. 2C, reintroduction of wild-type HDAC6 effectively restored colony formation of HDAC6 knockdown cancer cells, demonstrating that the requirement of HDAC6 for anchorage-independent growth is specific. In contrast, HDAC6 knockdown cell lines reconstituted with a catalytically inactive mutant HDAC6 remain defective in forming colony in soft agar. These results show that deacetylase activity of HDAC6 is required to support malignant growth of cancer cells in vitro.

HDAC6 regulates tumor cell proliferation and survival. To form colonies in soft agar, cells need to both grow and escape anoikis, a specific form of cell death caused by the lack of proper adhesion to basement membrane or extracellular matrix. Resistance to anoikis is one of the most important cellular mechanisms underlying anchorage-independent growth and a hallmark of malignant transformation (33). Given the possible effect of HDAC6 on cell adhesion (18), we examined whether HDAC6 status affects anoikis. To this end, we grew control and HDAC6 knockdown SKVO3 cells on culture plates coated with polyHEMA. PolyHEMA prevents cells from adhering to the plates and therefore allows one to test the ability of cells to survive in an anchorage-independent fashion (31). As shown in Fig. 3A, while scramble control cells retained the resistance to anoikis induction and survived, HDAC6 knockdown SKVO3 cells were significantly more susceptible to anoikis. This result indicates that HDAC6 contributes to anoikis resistance in cancer cells.

It has been shown that activation of PI3K/AKT and MAPK/ERK signaling pathways are critical for anoikis resistance in cancer cells (34). To examine whether HDAC6 modulates these pathways during anchorage-independent growth, we compared the phosphorylation...
status of AKT and ERK1/2 in scramble control cells or HDAC6 knockdown cells grown in the polyHEMA-coated plates. As shown in Fig. 3B, HDAC6 knockout cells showed a significant decrease in phosphorylation of AKT and ERK1/2 in response to growth factor stimulation, compared with scramble control cells, indicating that HDAC6 is required for growth factor induced activation of MAPK and PI3K signaling cascades that could contribute to anchorage-independent growth.

**HDAC6 is required for robust tumorigenic growth of human cancer cells in vivo.** These analyses support an important role of HDAC6 in promoting oncogenic phenotypes in vitro. To determine whether HDAC6 is critical for tumorigenic growth of cancer cells in vivo, SKOV3 stably expressing either an HDAC6-specific shRNA or a scramble control were s.c. injected into immunocompromised SCID-Beige mice, and tumor growth was followed over time. As shown in Fig. 4A, tumorigenic growth of HDAC6 knockout cells was significantly retarded when compared with that of control cancer cells by a 2-fold increase in latency and significant reduction in tumor volume (~4-fold at 28 days). This effect is specific as HDAC6 knockout cells reconstituted with wild-type HDAC6 but not catalytically inactive mutant regained the robust tumorigenic growth comparable with the parental cell lines (Fig. 4A). A similar requirement of HDAC6 for xenograft tumor growth was found in another cancer cell line A431 (Supplementary Fig. S1). Importantly, tumors that eventually grew from HDAC6 knockout cells restored HDAC6 expression (Fig. 4B), suggesting a strong selective pressure against tumor cells deficient in HDAC6.

Analyses of tumor samples revealed that tumors derived from HDAC6 knockout cells showed ~2-fold reduction in Ki-67 staining, a marker for mitotic cells, indicating a reduction in tumor proliferation caused by a loss of HDAC6. Consistent with this conclusion, re-expression of wild-type, but not catalytically inactive, HDAC6 mutant fully restored the number of Ki-67–positive cells in tumor sections (Fig. 4C). To directly assess if HDAC6 affects tumor cell growth, control and HDAC6 knockout SKOV3 cancer cells were analyzed in monolayer culture. As shown in Fig. 4D, the cell number is reduced in HDAC6 knockout cells compared with a scramble control cell lines. Together, these results show that HDAC6 is required for robust tumor cell growth.

Finally, we determined whether HDAC6 is required for carcinogen-induced spontaneous tumorigenesis. Topical application of the carcinogen 7,12-DMBA, followed by repetitive application of TPA induces papillomas with high rate of Ras mutation (35). We treated wild type and HDAC6-null mice with topical DMBA and then TPA for 20 weeks and monitored tumor growth. Consistent with previously published results (36, 37), skin tumors appeared within 9 weeks after initiation of treatment in wild-type mice with an average of six tumors per mouse by termination of the experiment (Fig. 5A and B). Significantly, in HDAC6-null mice, the appearance of tumors was delayed by 2 weeks with an average number of three tumors per mouse at the termination of the experiment. Furthermore, the average volume of the tumors at week 20 was ~3-fold smaller in HDAC6-null mice when compared with the wild-type mice (P < 0.05; Fig. 5C). We conclude that loss of HDAC6 impedes spontaneous formation of carcinogen-induced tumors.

Consistent with in vitro results from established cancer cells (Fig. 3B), a significant decrease in the phosphorylation of AKT and ERK1/2 was also observed in tumor derived from HDAC6 knockout mice (Fig. 6A), supporting the notion that HDAC6 is required for the proper activation of MAPK and PI3K signaling cascades required for optimal tumor cell growth in vivo. In the light of our observation that loss of HDAC6 renders cells more resistant to Ras oncogene-driven transformation and blunting of activation of downstream effectors ERK and AKT, we further examined the activation status of Ras (29) in tumor specimens from DMBA-TPA–induced skin tumors. As shown in Fig. 6B, tumor lysates from HDAC6 knockout mice contain much lower levels of activated Ras than those derived from wild-type littermates. Taken together, these results indicate that HDAC6 is required for efficient activation of the oncogenic Ras signaling pathway induced by DMBA-TPA.

**Discussion**

In this report, we present evidence that HDAC6, a cytoplasmic deacetylase, is required for efficient oncogenic tumorigenesis in both cultured cells and mouse tumor model. HDAC6 is induced during malignant transformation, whereas loss of HDAC6 impedes anchorage-independent growth, resistance to anoikis, xenograft tumor growth, and chemical carcinogen-induced skin tumors formation. The requirement of HDAC6 in maintaining tumor phenotypes in established cancer lines reveals the potential of HDAC6 as a therapeutic target and suggests a new mechanism by which HDACIs achieve their antitumor activity.

![Figure 3](https://example.com/figure3.png)
Although the analysis of HDAC6 in human cancer remains scarce, HDAC6 expression has been shown to be up-regulated in primary oral squamous cell carcinoma tissue and cell lines. Elevated levels of HDAC6 were also documented in primary acute myeloid leukemia blasts, several myeloblastic cell lines, and some human breast cancers (38–41). Interestingly, in breast cancer cell lines, HDAC6 was identified as an estrogen-regulated gene. HDAC6 was proposed to play a role in estrogen-induced breast tumor cell motility and invasiveness (38, 40). In fact, HDAC6 is required for estrogen-induced proliferation in MCF7 cells (42). The potential importance of HDAC6 in estrogen receptor–dependent tumor phenotype is supported by a clinical study of breast cancer patients, which revealed that HDAC6 levels positively correlate with a favorable response to anti-estrogen tamoxifen treatment (40). These findings are consistent with our conclusion that HDAC6 plays an important role in maintaining tumor growth and suggest a potential utility for targeting HDAC6 in breast cancer.

HDAC6 likely modulates tumor formation by several different mechanisms. We found that HDAC6 is induced in Ras oncogene-transformed cells in vitro (Fig. 1). The significance of this finding is underlined by the observation that HDAC6-deficient cells and mice are more resistant to oncogenic Ras-induced oncogenesis. Indeed, in HDAC6-null mice, the Ras-dependent signaling is impaired (Fig. 6). Taken together, HDAC6 likely contributes to tumorigenesis, at least in part, by facilitating the activation of Ras and downstream PI3K and MAPK pathways. The exact molecular targets mediating such a tumor-promoting effect of HDAC6 remains to be established. To date, at least four HDAC6 substrates have been identified: α-tubulin (17), Hsp90 (21), cortactin (13), and β-catenin (43). α-Tubulin is the first HDAC6 substrate identified. While the exact function of microtubule acetylation remains uncertain, it has been correlated with a moderate enhancement in microtubule stability and altered focal adhesion turnover (18, 44). In this regard, the involvement of HDAC6 in anoikis caused by a loss of proper adhesion is of particular interest. Our results showed that HDAC6 activity is required for cancer cells to efficiently escape anoikis and grow in soft agar. Transformed cells often acquire resistance to anoikis so they can invade and metastasize to distant organs (45).

Figure 4. HDAC6 is critical for tumorigenic growth of human cancer cells in vivo. A, top, representative subcutaneous flank tumor in mice and resected tumors 28 d after SKOV3 cells expressing the described construct were injected in mice; bottom, tumor volume (mm³) and SD versus time (d) of SKOV3 cells stably expressing a scramble control sequence (scram), HDAC6 shRNA (HD6KD), HDAC6 shRNA in the presence of vector (HD6KD + V), HDAC6 shRNA in the presence of siRNA-resistant wild-type HDAC6 (HD6KD + HD6WT), HDAC6 shRNA in the presence of siRNA-resistant enzymatically inactive HDAC6 (HD6KD + HD6CD) injected into the flanks of immunocompromised mice. B, re-expression of HDAC6 in HDAC6 knockdown cells in xenograft. Detection of HDAC6, acetylated tubulin, and tubulin by immunoblot in SKOV3 human ovarian cancer cells stably expressing either a scramble control sequence or HDAC6 shRNA and the same cells recultured from xenograft tumors. Tubulin serves as a loading control. C, representative histologic sections with Ki-67 staining of the aforementioned tumors from mice injected with the indicated cells. Scale bar, 50 μm. The graph shows the average number and SD of Ki-67–positive cells from 10 different fields of two different tumor sections. D, cellular growth in monolayer culture of SKOV3 cells stably expressing a scramble control sequence or HDAC6 shRNA.
Whether HDAC6 is required for efficient tumor metastasis is being investigated.

HDAC6 also regulate acetylation of Hsp90, cortactin, and β-catenin (13, 14, 21, 43), which all play important roles in oncogenesis. The molecular chaperone Hsp90 is critical for the structural maturation and activity of many oncogenic proteins (46). As Hsp90 requires HDAC6 for its full activity (21), inactivation of HDAC6 in cancer cells could affect oncogenic signaling, thereby inhibiting tumor growth. Indeed, hyperacetylation of Hsp90 has been associated with a defect in oncogenic kinase stability and signaling (12). The amplification of cortactin gene, also termed EMS1, has been shown to correlate with lymph node metastasis and unfavorable clinical outcome (47). Interestingly, similar to the effect of HDAC6 shRNA, knockdown of cortactin in esophageal cancer cells also leads to susceptibility to anoikis and impairment of malignant growth in soft agar and in mice (48). In HDAC6-deficient cells, hyperacetylated cortactin showed reduced activity in stimulating actin polymerization, suggesting that HDAC6 might be required for cortactin to promote tumor formation as well (13).

Aside from oncogenic signaling, HDAC6 also plays a critical role in the disposition and clearance of toxic misfolded protein aggregates via the aggresome-autophagy pathway (10, 49, 50). Although toxic misfolded proteins have been primarily studied in neurodegenerative disease, tumor cells are likely prone to produce excessive amounts of misfolded proteins due to their high rates of metabolism (51), protein synthesis, and production of mutated proteins (52). This might be particularly true for tumors with secretory function, such as multiple myeloma, as secretory proteins are highly susceptible to misfolding. Taken together, we suggest that loss of HDAC6 inhibits cancer cell growth by causing accumulation of toxic misfolded proteins.

A tumor-promoting function for HDAC6 by clearing toxic proteins is strikingly similar to that of heat shock factor-1 (HSF-1), a transcription factor that induces heat shock protein expression in response to the accumulation of misfolded proteins caused by heat shock or proteasome inhibition (53). Both HDAC6- and HSF-1-null MEFs showed resistance to DMBA-TPA two-stage carcinogen-induced skin tumor formation and oncogenic Ras-driven anchorage-independent growth. Both HDAC6 and HSF-1 are required for maintenance of transformed phenotypes of established cancer cells and oncogenic MAPK signaling. The similar requirement of HDAC6 and HSF-1 in oncogenic transformation is consistent with the finding that HDAC6 is required for full activation of HSF-1 when proteasome functions are compromised (54) and strongly argues that the management of misfolded protein stress is critical for maintaining robust tumor phenotypes. Supporting this view, combined use of proteasome inhibitors and

Figure 5. HDAC6-null mice are resistant to carcinogen-induced skin tumors. A, reduction of spontaneous tumors in HDAC6-null mice. Representative mice of the indicated genotype at 20 wk; arrowhead, tumor. Percentage of wild-type and HDAC6-null mice with tumors versus time after initial application of DMBA (wk). B, reduction in the number of tumors in HDAC6-null mice. Mean number of tumors per wild-type and HDAC6-null mouse versus time after initial application of DMBA (wk). *, P < 0.05. C, reduction in tumor volume in HDAC6-null mice. Mean tumor volume per wild-type and HDAC6-null mouse versus time after initial application of DMBA (wk). *, P < 0.05.
Histone acetylation: mechanism and implications for cancer.

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HDAC6-selective inhibitors has synergistic cytotoxic effects on cancer cell lines (10, 55).

Regardless of the mechanism, our analysis clearly shows that HDAC6 is required for efficient oncogenic transformation and tumor formation. Importantly, as HDAC6 is not an essential gene and HDAC6-null mice are grossly normal (14, 56), our study further suggests that HDAC6 might be an ideal target for the design of chemotherapeutic agents for its low toxicity. Given that the deacetylase activity of HDAC6 is required to support robust tumorigenic growth (Figs. 2–5), these data suggest that pharmacologic inhibition of HDAC6 activity might be an effective strategy in cancer therapeutics.

For decades, protein phosphorylation/dephosphorylation controlled by oncogenic kinases has been the primary target for therapeutic manipulation in cancer treatment. The efficacy of HDACIs in model systems and clinical trials points to the potential importance of reversible protein acetylation and HDAC in oncogenic signaling. The antitumor activity of HDACIs has been mostly attributed to their effects on gene expression and chromatin dynamics. The identification of nonnuclear HDAC members and large number of nonnuclear acetylated proteins (57), however, has raised a critical question as to whether nongenomic mechanism might also play an important role underlying the therapeutic effect of HDACI. Our report now provides evidence that HDAC6, a cytosolic HDAC family member, is required for full and autonomous tumorigenic growth. This finding highlights the potential importance of nongenomic targets in the antitumor activity of HDACIs. These conclusions also strongly indicate that, similar to protein phosphorylation, reversible protein acetylation occurring outside the nucleus could play an important role in cancer biology.

**Disclosure of Potential Conflicts of Interest**

F. Ordentlich: ownership interest, Syndax Pharmaceuticals. T-P. Yao: consultant, Syndax Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

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