Identification of LIV1, a Putative Zinc Transporter Gene Responsible for HDACi-Induced Apoptosis, Using a Functional Gene Screen Approach

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Abstract
Histone deacetylase inhibitors (HDACi) show promise as a novel class of antitumoral agents and have shown the ability to induce apoptosis of tumor cells. To gain a better understanding of the action of HDACi, we conducted a functional gene screen approach named suppression of mortality by antisense rescue technique to identify the key genes responsible for the tumor-selective killing trichostatin A. Over 20 genes associated with HDACi-induced mortality were identified. One of the confirmed positive hits is LIV1, a putative zinc transporter. LIV1 is significantly induced by treatment with HDACi in a number of tumor cells, but not in normal cells. Knockdown of LIV1 suppressed apoptosis induced by HDACi in tumor cells. Although HDACi induced a slight increase in the free intracellular zinc concentration, knockdown of LIV1 significantly enhanced the intracellular zinc level, which was associated with resistance to apoptosis. On the other hand, pretreatment of the cells with a specific zinc chelator TPEN reversed the apoptosis resistance conferred by knockdown of LIV1. However, the biological effects of TPEN were abolished by addition of physiologic concentrations of zinc. Taken together, the present study identifies LIV1 as a critical mediator responsible for HDACi-induced apoptosis. The effect of LIV1 is, at least in part, mediated by affecting intracellular zinc homeostasis, which may be related to alteration of the catalytic activity of the Caspase 3 and expression of some BCL-2 family genes. As such, these findings highlight a novel mechanism underlying the action of HDACi that could be potentially useful in the clinical setting.

Introduction
Histone deacetylase inhibitors (HDACi) are potent apoptosis inducers in a variety of tumor cells and have been promoted as a promising novel class of antitumoral agents in over 100 clinical trials (1, 2) to date. The clinical potential of HDACi has been well exemplified by the successful development of Vorinostat (suberoylanilide hydroxamic acid, SAHA), which has recently been approved by the U.S. Food and Drug Administration for treating cutaneous T-cell lymphoma (3). Initially, the mechanism of action for HDACi was believed to be mediated by inhibiting HDACs, increasing the acetylation state of the nucleosomal histones and thereby influencing gene transcription (4). Indeed, early studies using gene profiling techniques have revealed that up to 17% of all known genes are affected by HDACi at the transcriptional level. A large number of apoptosis (i.e., CD95, CD95 ligand, gelsolin, Apaf-1, Bak, Bax, caspase-9, Bim, Bcl-2, Bcl-XL, c-Flip, and survivin) and cell cycle regulatory genes (i.e., cyclin D, cyclin E, cyclin A, and p21Waf1/Cip1) have been independently identified by a number of groups as being activated or repressed by different HDACi, although the functional importance of most of these genes in mediating the biological effects of HDACi has not been addressed (5). In addition to HDACs, many nonhistone proteins are also important mediators of the action of HDACi including transcription factors (i.e., p53, GATA1, and RelA), nuclear import proteins (i.e., importin-a7), signal transduction molecules (i.e., β-catenin), cytoskeletal proteins (i.e., α-tubulin), and DNA repair enzymes (i.e., Ku70, WRN). Interestingly, in contrast to conventional chemotherapeutic agents, HDACi show strong tumor selectivity and cause less toxicity in normal tissues (5). These findings suggest that the mechanisms of HDACi activities are far more complex than previously thought. Despite the rapid clinical progress achieved, the mechanisms of action of HDACi are not yet well understood, which significantly limits the optimal application of this class of therapeutic drugs.

Identification of the target genes essential for HDACi-induced apoptosis has proven to be very difficult. Previously,
a significant number of apoptosis and cell cycle regulatory genes have been identified by using gene expression profiling techniques as proposed effectors responsible for the tumor-selective action of HDACi. For example, the failure of HDACi to activate cell cycle checkpoints in tumor cells had been proposed as one of the mechanisms responsible for tumor selectivity of HDACi. In acute promyelocytic leukemia, however, preferential induction of tumor necrosis factor-related apoptosis-inducing ligand or FAS by HDACi was proposed to serve as the targets responsible for the tumor-selective action. Although these findings have greatly extended our understanding of the action of HDACi, it is difficult to conclude which genes/pathways are really universal, essential targets responsible for HDACi-mediated apoptosis. To address this, new analytic approaches are needed to identify the essential genes from the numerous proposed effectors of HDACi.

Suppression of mortality by antisense rescue technique (SMART) is a powerful technique for discerning functionally relevant genes involved in cell death induced by certain agents. In the first step of SMART, the global gene expression of tumor cells is randomly knocked out in an unbiased manner by transfection with the antisense cDNA expression library. The transfected tumor cells are then continuously exposed to treatment of certain agents. The assumption is that the tumor cells would survive the treatment of certain agents if key genes essential for certain agent-induced apoptosis are inactivated (6). A number of investigators have successfully applied the technique for isolation of death-associated genes involved in various agent-induced apoptosis. In the present study, we used SMART to identify the key genes responsible for the tumor-selective action of trichostatin A (TSA; ref.7), one of most extensively studied HDACi.

We identified 24 genes including Smad4, LIV1, or Ubiquitin B, which were essential for the TSA-induced apoptosis, of which LIV1, as a novel zinc transporter, was isolated by its remarkable ability to confer a resistance against HDACi-induced apoptosis when expressed in an antisense orientation. In addition, we found that the reduced cellular sensitivity to HDACi was a consequence of disrupted intra-cellular zinc homeostasis caused by downregulated expression of LIV1. Based on our findings, we propose a novel mechanism of HDACi activity and suggest that modulation of the expression of LIV1 represents an attractive and potential target for anticancer therapy.

Materials and Methods

Cells and Reagents

The human breast cancer cell line MCF-7 and T47D and human cervical cancer cell line HeLa were purchased from the American Type Culture Collection and cultured in DMEM containing 10% FCS. The breast epithelial cell line MCF-10A was obtained from American Type Culture Collection and cultured in DMEM/F12 supplemented with 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone, and 5% horse serum. The cisplatin-sensitive ovarian cancer cell line (OV2008) and its resistant variant (C13K) were gifts from Dr. Rakesh Goel from the Ottawa Regional Cancer Center, Ottawa, Canada, and were cultured as previously reported (8). Primary normal respiratory endothelial cells (REC) were isolated from freshly excised human nasal polyp tissue using the protease method (9). All cells were cultured at 37°C in a humidified 5% CO2 atmosphere. HDACis TSA, apicidin, and SAHA were purchased from Sigma and dissolved in DMSO.

Cell Viability Assays

Cell viability was determined by using a MTT assay. In brief, 5 × 104 cells were plated into each well of 96-well plates at 72 h after the indicated treatments, after which 5 mg/mL MTT was added and incubated at 37°C for 4 h. Media were then removed, and 1 mL of DMSO was added to solubilize the MTT-formazan product. The MTT absorbance was then measured at 570 nm on a Multiscan JX ver.1 (Thermo Labsystems). Results are expressed as a percentage of the viable cells in the DMSO-treated group. Each data point is the mean ± SEM of six replicates.

Apoptosis Assays

Cells were stained with Annexin V and propidium iodide and the percentage of apoptotic cells were determined by flow cytometry as described previously (10). CELL Quest software was used for data acquisition and analysis.

Suppression of Mortality by Antisense Rescue Technique

Construction of an antisense cDNA library and subsequent analysis using SMART were done following protocols described elsewhere with some modification (11). Initially, MCF-7 cells were treated with TSA at 250 nmol/L for various lengths of time. At 0, 8, 12, 24, 36, and 48 h posttreatment with TSA, MCF-7 cells were collected, and total mRNA was extracted for subsequent construction of an antisense cDNA library by random insertion of cDNAs into the mammalian replication permissive plasmid PCEP-4. A total of 40 μm of library DNA was electroporated into 1 × 107 HeLa cells. The cDNA library–transfected HeLa cells were then selected by incubation with 150 nmol/L of TSA in the presence of 200 μg/mL of hygromycin-B for 4 wk. Parallel selection was done similarly in MCF-7 cells transfected with the plasmid control PCEP4-CAT. Culture medium was changed, and TSA were readded daily for the first week and then every other day for the rest of weeks. At the end of 4 wk of selection, the surviving colonies were pooled and amplified in the presence of hygromycin (200 μg/mL) for isolation of plasmid using the Hirt DNA extraction method (12). The isolated DNA was digested with Dpn I and electroporated into Escherichia coli DH10B. The resultant colonies were picked and screened by enzyme digestion to detect the presence of inserts. The individual plasmid with inserted antisense cDNA was subjected to a second round of selection to confirm resistance to TSA treatment. Ten micrograms of individual episomes mixed with 30 μg of salmon sperm DNA were electroporated into HeLa or MCF-7 cells. The cells were then selected with TSA (150 nmol/L) and hygromycin B (200 mg/mL) for 20 d. Episomes that were confirmed to be with TSA resistance were chosen for DNA sequencing and further characterization.
Real-time PCR

Quantitative PCR was done in ABI Prism 7000 using the SYBR Green PCR Master Mix (Sigma) with the following set of primers: LIV1: 5’- GGT GAT GCC CTG AAC CAT TTC-3’ and 5’-TCA GGC ATG ATC GCC GCC TTA TGT ATG-3’; 18s RNA: 5’-AGT CCC TGC CCT TTG ACA CA-3’ and 5’-GAT CCC AGG GCC TCA CTA AAC-3’. 18s RNA was used as an internal control. All primers were designed with the Primer3 software (13). A melting curve assay was done to determine the purity of the amplified product. Contamination with genomic DNA was not detected in any of the analyzed samples. Each sample was assayed in triplicate, analysis of relative gene expression data used the 2^-ΔΔCT method (14), and the results were expressed as fold induction compared with the untreated group.

Western Blot

Preparation of protein samples and Western blotting were done as described previously (15). Antibodies against LIV1 were kindly supplied by Dr. Kathryn M. Taylor (Tensovus Cancer Research Centre, Welsh School of Pharmacy, Cardiff University, U.K.). Antibodies against Bcl-2, Bcl-XL, Bax, and caspase 3 were purchased from Cell Signaling technology. Antibodies against β-actin were purchased from Santa Cruz Biotechnology.

Clonal Forming Assays

MCF-7 and HeLa cells were stably transfected with PCEP4-CAT and AS-LIV1 (PCEP4 carrying antisense LIV1 cDNA) and cultured for 24 h. Cells were then treated with 500 nmol/L TSA or 500 nmol/L apicidin or 2.5 μmol/L SAHA for 24 h and plated in triplicate in 24-wells plate at 50 cells per well. Plates were subsequently incubated for 14 d in a humified incubator at 37°C, and colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet and counted using a dissecating microscope (×50 magnification). Three random fields were counted for each triplicate of samples, and average values were presented as the means ± SD.

RNA Interference

Annealed, purified, and desalted double-stranded siRNA LIV1 (SiRNA1: AUU AUG ACC AUC CAG GCC AAA GUG G; SiRNA2: AUA AAG GAC AGC CUG CUU AAC GGU C; SiRNA3: AUU AUG GUC AGA CUG AUG CUC GUG G) were ordered from Invitrogen Corporation. Then 1.5 × 105 cells were plated in a six-well dish on day 0. On day 1, cells were transfected with 200 nmol/L siRNA in Opti-MEM medium (Invitrogen) without fetal bovine serum using Lipofectamine 2000 reagent (Invitrogen).

Measurement of Intracellular Free Zn2+

The concentration of intracellular free zinc was assessed using the ion-specific fluorescent probe, FluoZin-3-AM (FluoZin-3; Molecular Probes). At each time point, ∼5 × 10^5 cells per sample were removed from treatment medium and washed thrice with HBSS without calcium, magnesium, or phenol red. Cells were resuspended in 100 μL HBSS containing 10 μmol/L FluoZin-3. The samples were incubated at 37°C for 30 min in the dark. After incubation, cells were washed twice with HBSS and resuspended in a final volume of 500 μL, and the samples were analyzed by flow cytometry within 1 h. The fluorescence excitation/emission maximum for FluoZin-3 was 494/516 nm. As controls, cells were cultured with 20 μmol/L pyrithione (Sigma) and 50 μmol/L zinc acetate or with 20 μmol/L N,N,N′,N′-tetrakis-(2-pyridylmethyl)-thiленедiamine (TPEN; Sigma) for 15 min, and they were then processed and analyzed as above.

Results

Selective Induction of Cell Death by TSA

HDACis have been shown to selectively induce tumor cell death when compared with normal cells (16). To determine whether TSA would selectively induce apoptosis, we treated MCF-7 cells, as well as the nonmalignant cell line of the same tissue origin, MCF-10A, with a TSA dose range between 250 and 1,000 nmol/L and measured cell viability using the MTT assay. As shown in Fig. 1A, TSA caused profound inhibition of cell growth in MCF-7 in a dose-dependent fashion, whereas TSA caused only slight growth inhibition in the corresponding normal cell MCF-10A (Fig. 1B). We then chose the lowest concentration of TSA (250 nmol/L) to compare the induction of apoptosis in MCF-7 versus MCF-10A. Exposure of MCF-7 or MCF-10A to TSA displayed similar kinetics of apoptosis, which was detectable at 24 hours and peaked at 72 hours. At every time point examined, TSA exhibited preferential induction of apoptosis in MCF-7 (Fig. 1C). To test whether TSA at a dose of 250 nmol/L also preferentially induced apoptosis in other types of tumor cells, tumor (HeLa, C13K, OV2008) and primary normal RECs were treated with TSA for 72 hours and subjected to apoptosis analysis. Again, 250 nmol/L TSA preferentially induced apoptosis in tumor (HeLa, C13K or OV2008), but not in normal (REC) cells (Fig. 1D). Thus, these data show that 250 nmol/L TSA can selectively induce apoptosis of tumors.

SMART Identifies the Genes Associated with TSA-Induced Apoptosis

To identify the genes essential for TSA-induced apoptosis, we performed the SMART approach. In the first step, MCF-7 cells were treated with TSA at a dose of 250 nmol/L, at which TSA would preferentially induce apoptosis in tumor, but not normal, cells. At 0, 8, 12, 24, 36, and 48 hours post-treatment with TSA, MCF-7 cells were then collected and extracted for total mRNA for construction of an antisense cDNA library. Over this time range, TSA-induced apoptosis became evident but did not reach a maximum, and hence it is supposed to be in a stage at which early apoptosis events are substantially activated. An antisense cDNA library was then constructed by random insertion of cDNA in an antisense orientation in a mammalian replication-permissive plasmid PCEP-4, which could replicate in the manner of an extra chromosome in human cells. Next, a total of 40 μg of library DNA was electroporated into 1 × 10^7 HeLa cells. The library-transfected HeLa cells were then selected by incubation with 150 nmol/L of TSA in the presence of 200 μg/mL of hygromycin-B for 4 weeks. At the end of the experiment, HeLa cells transfected with PCEP4-CAT yielded no surviving cell colonies. On the other hand, HeLa
cells transfected with antisense cDNA library yielded several TSA-resistant colonies. The TSA-resistant colonies were pooled for identification of cDNA inserts. Twenty-four inserted cDNA samples were then identified from the TSA-resistant colonies. Some of the positive hits were chosen for second-round screening to confirm resistance to TSA in HeLa or MCF-7 cells. Of the positive hits examined, the plasmid containing the antisense cDNA of \textit{LIV1} displayed the obvious resistance to TSA treatment in HeLa or MCF-7 cells (Fig. 2A). Under the same conditions, only a few colonies transfected with the episomal vector \textsc{pCEP}4-CAT survived 4 weeks of TSA/hygromycin-B screen. To rule out the possible interference of hygromycin-B, HeLa or MCF-7 cells were stably transfected with the \textit{AS-LIV1} antisense plasmid \textsc{(AS-LIV1)} or \textsc{pCEP}4-CAT followed by 5-day treatment with TSA, and they were then examined for growth inhibition. \textsc{AS-LIV1} was confirmed to significantly knockdown the basal and TSA-induced level of \textit{LIV1} expression (Fig. 2B), and transfection of \textsc{AS-LIV1} again displayed resistance to TSA treatment (Fig. 2C). Furthermore, stable transfection of \textsc{AS-LIV1} also made HeLa and MCF-7 cells resistant to treatment of apicidine, a structurally diverse HDACi (Fig. 2D). Therefore, \textit{LIV1} seems to be essential for TSA-induced cell death in tumor cells.

\textbf{Induction of LIV1 Expression by TSA in Tumor Cells}

To test whether expression of \textit{LIV1} is induced by HDACi, we investigated the effects of HDACi on mRNA and protein expression of \textit{LIV1} in several tumor and normal cells. Tumor (MCF-7, HeLa, or T47D) or normal (MCF-10A and REC) cells were treated with 500 nmol/L TSA or 500 nmol/L apicidine for various lengths of time. As shown in Fig. 3A and B, transcription of \textit{LIV1} was highly induced in tumor, but not normal cells. At 12 hours posttreatment with TSA or apicidine, transcriptional induction reached a maximal level (5.25 - 0.08-fold for HeLa; 2.65 - 0.18-fold for T47D; \textit{P} < 0.01, compared with the basal transcriptional level). Interestingly, the basal levels of \textit{LIV1} protein were lower in tumor cells compared with normal cells. However, treatment of HDACi led to a significant increase in \textit{LIV1} protein levels in tumor, but not normal cells (Fig. 3C). Taken together, treatments with HDACi selectively induced \textit{LIV1} expression in cancer cell lines, which supports the finding that \textit{LIV1} is a target gene of HDACi in tumor cells.

\textbf{Knockdown of LIV1 Suppresses Cell Death Induced by HDACi in Tumor Cells}

To further confirm whether knockdown of \textit{LIV1} suppressed HDACi-induced cell death in tumor cells, HeLa and MCF-7 cells stably transfected with \textsc{AS-LIV1} or \textsc{pCEP}4-CAT were exposed to TSA or apicidine in a dose range between 300 nmol/L and 700 nmol/L for 72 hours and subjected to MTT assay for the determination of cell viability. As shown in Fig. 4A and B, knockdown of \textit{LIV1} decreased TSA- or apicidine-induced killing efficiency in both HeLa and MCF-7 cells at every dose, with a maximum effect seen at 500 nmol/L concentration, where the rate of viable cells was increased over 35% in HeLa and 25% in MCF-7. Furthermore, knockdown of \textit{LIV1} suppressed HDACi-induced killing and gave rise to more survival colonies (Fig. 4C and D). Accordingly, knockdown of \textit{LIV1} made HeLa and MCF-7 cells resistant to TSA-induced apoptosis (Fig. 4E and F). HeLa or MCF-7 cells stably transfected with \textsc{AS-LIV1} were much less sensitive to TSA-induced apoptosis than cells stably transfected with \textsc{pCEP}4-CAT (HeLa, 18.36 ± 2.6% versus 37.55 ± 3.8%, \textit{P} < 0.01; MCF-7, 19.48 ± 1.3% versus 46.96 ± 4.5%, \textit{P} < 0.01). In addition, \textit{LIV1} RNAi was also verified to significantly reduce TSA-induced apoptosis (Supplementary Fig. S1). Collectively, the data indicate that downregulation of \textit{LIV1} is capable of conferring a significant resistance to HDACi-induced cell death in tumor cells.
Knockdown of LIV1 Protects Cells from HDACi-Induced Apoptosis by Affecting Intracellular Zinc Homeostasis

In light of the clues that LIV1 is a zinc transporter, which plays an important role in maintaining intracellular zinc homeostasis (17, 18), we wonder whether the protective effects of AS-LIV1 in the presence of HDACi is the consequence of alteration of intracellular free zinc. To test this, we treated HeLa cells stably transfected with AS-LIV1 and PCEP4-CAT with TSA and then stained them with a zinc-specific fluorescent probe, FluoZin-3, to monitor free intracellular zinc concentration (19). Figure 5A shows the representative data from these experiments. Obviously, the cells stably transfected with PCEP4-CAT or AS-LIV1 displayed similar level of FluoZin-3 green fluorescence before TSA treatment. However, the cells transfected with AS-LIV1 showed a much higher cellular fluorescence than the cells transfected with PCEP4-CAT after TSA treatment (0 hours, 30.52 ± 0.42 versus 30.09 ± 0.31, P > 0.05; 12 hours, 117.96 ± 3.25 versus 81.46 ± 1.38, P < 0.01; 24 hours, 196.25 ± 3.87 versus 62.23 ± 2.15, P < 0.01; 48 hours, 155.14 ± 3.12 versus 53.78 ± 0.96, P < 0.01). Moreover, knockdown of LIV1 with LIV1 RNAi again induced a significantly higher concentration of intracellular zinc compared with control RNAi after TSA treatment (Supplementary Fig. S2). Thus, knockdown of LIV1 seems to enhance the TSA-induced increase of free intracellular zinc concentration. Furthermore, expression level of LIV1 was high in MCF-10A and low in MCF-7 cells, and accordingly, zinc level was lower in MCF-10A cells compared with in MCF-7 cells (Supplementary Fig. S3). The results further support the putative role of LIV1 as a zinc transporter gene. To address the effects of LIV1 overexpression on intracellular zinc homeostasis and cell death, MCF-10A and MCF-7 cells were transfected with LIV1 expression plasmid FL-LIV1 or empty vector (pcDNA4/TO/Myc-His B, Invitrogen) and selected by antibiotic for stably transfected cell lines. Significant apoptosis response was triggered upon transfection of FL-LIV1 into MCF-7 cells, and consequently, stably transfected cell line could not be developed. The result showed the high toxicity of wild-type LIV1 to MCF-7 D,
cells. In contrast, stably transfected cell lines of FL-LIV1 could be developed from nonmalignant MCF-10A cells. Interestingly, overexpression of LIV1 had no obvious effects on intracellular zinc homeostasis in the presence of TSA and consequently did not modulate the TSA-induced apoptosis in MCF-10A cells (Supplementary Fig. S4). Next, we examined whether manipulating zinc concentration could modulate the antiapoptotic efficacy of knockdown of LIV1. First, we used TPEN, a cell-permeable, specific zinc chelator, N,N,N′,N′-tetrakis-(2-pyridylmethyl)-ethylenediamine, and physiologic amounts of zinc for zinc deficiency or supplementation without affecting cell growth (Supplementary Fig. S5). Second, cells stably transfected with PCEP4-CAT showed relatively smooth change of intracellular free zinc after addition of 1 μmol/L TPEN or 10 μmol/L zinc under TSA treatment. In contrast, free intracellular zinc levels in cells stably transfected with AS-LIV1 fluctuated sharply, indicating the failure to maintain intracellular zinc homeostasis (Fig. 5B). Third, HeLa cells stably transfected with AS-LIV1 were much less sensitive to TSA-induced apoptosis compared with cells stably transfected with PCEP4-CAT. Interestingly, pretreatment with TPEN of HeLa cells stably transfected with AS-LIV1 resensitized tumor cells to TSA-induced apoptosis (18.36 ± 2.6% versus 31.57 ± 2.9%, P < 0.05). The sensitized effects of TPEN could be reversed by addition of 10 μmol/L zinc (31.57 ± 2.9% versus 11.79 ± 1.2%, P < 0.01). On the other hand, neither zinc deficiency nor supplementation could affect TSA-induced apoptosis in cells stably transfected with PCEP4-CAT (Fig. 5C). Collectively, these data indicated that the disrupted intracellular zinc homeostasis by knockdown of LIV1 seems to, at least in part, correlate with the antiapoptotic efficacy of AS-LIV1.

To further assess the role of zinc homeostasis in LIV-1 knockdown–associated apoptosis, HeLa cells stably transfected with AS-LIV1 or PCEP4-CAT were treated with SAHA and subjected to analysis of zinc homeostasis and apoptosis. Again, knockdown of LIV-1 disrupted intracellular zinc homeostasis, which correlated with the resistance of HeLa cells to SAHA-induced apoptosis (Supplementary Fig. S6). In addition, as shown in Fig. 5D, TSA induced apoptosis by decreasing endogenous levels of Bcl-2/Bcl-XL, increasing Bax and enhancing cleavage of procaspase-3, which was not influenced by either of zinc deficiency or supplementation in the absence of LIV-1 knockdown. In contrast, the TSA-induced alteration mentioned above could be significantly reversed by either zinc deficiency or supplementation in the presence of LIV-1 knockdown, indicating that intracellular zinc homeostasis plays a critical role rather than a by-phenomenon in LIV-1 knockdown–associated apoptosis.

Discussion

For the time being, a significant number of apoptosis and cell cycle regulatory genes have been independently identified by different groups as the potential mediators of action of HDACi. However, the functional importance of most of these genes in mediating the biological effects of HDACi has not been addressed (20, 21). Given the large number of genes that can be affected in response to HDACi, the molecular basis for HDACi seems to be far more complex than previously thought. Identification of key mediators essential
to mediate the action of HDACi has proven to be a difficult task using the comparison of global gene expression profiling. To identify the essential genes responsible for the action of HDACi, we therefore used SMART in the current study, which permits the isolation of apoptosis-associated genes by functional inactivation (6).

Twenty-four candidate genes had been identified by virtue of resistance to TSA-induced apoptosis, of which LIV1 was selected for second-round screening and was confirmed as an authentic mediator in resisting TSA-induced apoptosis. Our data presented here show that LIV1 levels are specifically upregulated in a time-dependent manner by HDACi treatment in a variety of tumor cells. Inactivation of LIV1 alone blocked HDACi-induced apoptosis in various tumor cells. LIV1 belongs to a new subfamily of Zrt-, Irt-like proteins (ZIP) zinc transporters, now termed the LIV1 sub-family of ZIP zinc transporters (LZT; ref. 18). Based on its amino acid sequence and its cellular location on the plasma membrane, it has been proposed as a putative zinc transporter involved in maintaining intracellular zinc homeostasis (22). Previous investigations declare that LIV1 expression associates with small estrogen receptor–positive tumors of which 92% show lymph node involvement, and its expression may be both a suitable prognostic marker for lymph node involvement and metastatic spread in steroid hormone receptor–positive disease (17). In breast cancer, high LIV1 protein expression is associated with a better clinical outcome in patients with breast cancer (23). In zebrafish gastrula organizer, LIV1 controls epithelial-mesenchymal transition (24). Nevertheless, the biological function of the LIV1 gene is still not well understood. Our findings presented here highlight an essential role for LIV1 in HDACi-activated “death programs,” which might extend our understanding of LIV1 in the regulation of apoptosis in cancer.

Our data imply that the inhibition of HDACi-induced apoptosis by knockdown of LIV1 might be associated with its ability to disrupt intracellular zinc homeostasis rather than zinc level. Intracellular zinc level is not affected by either treatment of zinc depletion or supplementation providing the LIV1 is not knocked down. Treatment of tumor cells with HDACi just slightly increased the intracellular free zinc level. On the other hand, it seems that LIV1 modulates the killing efficacy of HDACi by drastically upregulating the intracellular free zinc level during treatment with HDACi. These findings are consistent with previous reports in which zinc was shown as a critical regulator of cell growth.

Figure 4. Knockdown of LIV1 suppresses cell death induced by HDACi in tumor cells. A and B, HeLa (A) or MCF-7 (B) cells were stably transfected with AS-LIV1 and PCEP4-CAT and were then exposed to TSA and apicidinc at the indicated concentrations for 72 h. Cell growth was assessed by MTT assay. Results show viable cells in the TSA- and apicidine-treated group and are expressed as a percentage of the viable cells in the DMSO-treated group. Points, mean of six replicates; bars, SEM. C and D, HeLa (C) or MCF-7 (D) cells were treated with 500 nmol/L TSA or 500 nmol/L apicidine or 2.5 μmol/L SAHA for 24 h, and subjected to clonal forming assay. *, P < 0.01. E and F, HeLa (E) or MCF-7 (F) cells stably transfected with AS-LIV1 and PCEP4-CAT were exposed to 1 μmol/L TSA for 48 h and subjected to apoptosis analysis using flow cytometry, where each data point represents the mean ± SEM of three replicates. The representative flow cytometric results depicted similar data as observed in the apoptosis analysis.
Knockdown of LIV1 protects cells from HDACi-induced apoptosis by affecting intracellular zinc homeostasis. A, HeLa cells stably transfected with AS-LIV1 and PCEP4-CAT were treated with 1 μmol/L TSA for 48 h. At each indicated time point, the cells were stained with Fluozin-3 and measured for intracellular free zinc using flow cytometry. The panel represents a typical result of Fluozin-3 fluorescence. Results are expressed as the average fluorescence intensity, where each data point represents the mean ± SEM of three replicates, *, P < 0.01; NC, negative control (cells were not stained with Fluozin-3). B, HeLa cells stably transfected with PCEP4-CAT and AS-LIV1 were treated with DMSO or 1 μmol/L TSA for 48 h, or pretreated with 1 μmol/L TPEN and then exposed to 1 μmol/L TSA for 48 h, or pretreated with 1 μmol/L TPEN and 10 μmol/L zinc acetate and then exposed to 1 μmol/L TSA for 48 h. Cells were stained with Fluozin-3 and measured for intracellular free zinc using flow cytometry. The panel represents a typical result of Fluozin-3 fluorescence. Results are expressed as the average fluorescence intensity, where each data point represents the mean ± SEM of three replicates, *, P < 0.01. Fluorescence-activated cell sorting analysis was used to detect apoptosis. The panel represents a typical result of cellular apoptosis. Each data point represents the mean ± SEM of three replicates, *, P < 0.01 (C). Western blot analysis was used to detect the expression of Bcl-2, Bcl-XL, Bax, and cleaved caspase 3 (D).
and death (25, 26). For example, in some tumor cells, increased levels of intracellular zinc or decreased zinc dependence can be a major and decisive factor in their relative resistance to apoptosis and accelerated growth (27–29). Furthermore, our data are also consistent with the previous findings that the mechanistic actions of zinc is shown through change of the caspase enzymes activities, as well as the direct alteration of apoptotic regulators expression, especially the Bcl-2 family (29). In addition, abnormal expression of genes involved in zinc homeostasis has been described in breast carcinomas and tumors of other organs (30–32). Taken together, our findings have identified LIV1 as a novel target responsible for the action of HDACi. The novel mechanism proposed here might have important clinical potential. Besides its role in maintaining intracellular zinc homeostasis and consequently stabiling the catalytic activity of the caspase 3 and other regulators of apoptosis, e.g., Bcl-2 family, LIV1 is also found to be associated with many other important functions. For example, LIV1 has been recently linked to signal transducers and activators of transcription 3 and Snail (24). Given that LIV1 is a novel identified gene with undefined functions and the prediction of clinical responses to HDACi have currently not been realized, further characterization of LIV1 and resistance to HDACi would help to develop more effective protocols and identify potentially susceptible patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest.

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