Cancer Therapy: Preclinical

Clinical Cancer Research

Overcoming Resistance to DNA-Targeted Agents by Epigenetic Activation of Schlafen 11 (*SLFN11*) Expression with Class I Histone Deacetylase Inhibitors

Sai-Wen Tang[1], Anish Thomas[1], Junko Murai[1], Jane B. Trepel[1], Susan E. Bates[1,2], Vinodh N. Rajapakse[1], and Yves Pommier[1]

Abstract

Purpose: Schlafen 11 (SLFN11), a putative DNA/RNA helicase is a dominant genomic determinant of response to DNA-damaging agents and is frequently not expressed in cancer cells. Whether histone deacetylase (HDAC) inhibitors can be used to release *SLFN11* and sensitize SLFN11-inactivated cancers to DNA-targeted agents is tested here.

Experimental Design: *SLFN11* expression was examined in The Cancer Genome Atlas (TCGA), in cancer cell line databases and in patients treated with romidepsin. Isogenic cells overexpressing or genetically inactivated for SLFN11 were used to investigate the effect of HDAC inhibitors on *SLFN11* expression and sensitivity to DNA-damaging agents.

Results: *SLFN11* expression is suppressed in a broad fraction of common cancers and cancer cell lines. In cancer cells not expressing *SLFN11*, transfection of *SLFN11* sensitized the cells to camptothecin, topotecan, hydroxyurea, and cisplatin but not to paclitaxel. *SLFN11* mRNA and protein levels were strongly induced by class I (romidepsin, entinostat), but not class II (roclinostat) HDAC inhibitors in a broad panel of cancer cells. *SLFN11* expression was also enhanced in peripheral blood mononuclear cells of patients with circulating cutaneous T-cell lymphoma treated with romidepsin. Consistent with the epigenetic regulation of *SLFN11*, camptothecin and class I HDAC inhibitors were synergistic in many of the cell lines tested.

Conclusions: This study reports the prevalent epigenetic regulation of *SLFN11* and the dominant stimulatory effect of HDAC inhibitors on *SLFN11* expression. Our results provide a rationale for combining class I HDAC inhibitors and DNA-damaging agents to overcome epigenetic inactivation of *SLFN11*-mediated resistance to DNA-targeted agents. *Clin Cancer Res; 24(8); 1944–53. ©2018 AACR.*

Introduction

Despite the promise of kinase inhibitors and immunotherapy, DNA-damaging agents remain widely used for a broad range of solid tumors and hematologic malignancies. DNA-damaging agents have a wide range of antitumor activity but are associated with toxicities related to their effects on normal tissue. Use of predictive biomarkers to maximize treatment efficacy and to minimize the risk of deleterious toxicities has long been recognized as an important goal in the clinical application of these drugs. However, currently, there are no reliable biomarkers of activity of DNA-targeted agents that have proven useful for patient stratification.

Recent studies have identified Schlafen 11 (SLFN11), a nuclear protein belonging to the Schlafen family of mammalian proteins, as a causal and dominant genomic determinant of response to topoisomerase (TOP) 1 inhibitors [camptothecin (CPT), topotecan, and irinotecan], TOP2 inhibitors (etoposide, doxorubicin, mitoxantrone), alkylating agents (cisplatin and carboplatin, nitrogen mustards, ifosfamide), DNA synthesis inhibitors (gemcitabine, cytarabine, hydroxyurea), and poly-(ADP)-ribose polymerase (PARP) inhibitors (olaparib, rucaparib, niraparib, and talazoparib; refs. 1–6). In addition to the role of SLFN11 in sensitizing cancer cells to DNA-damaging agents, Schlafen proteins play important roles in regulating biological functions including cellular proliferation, induction of immune response, and suppression of viral replication (7, 8). In patient tumors and cancer cell lines, *SLFN11* demonstrates a broad range of expression in a wide range of cancer types (Fig. 1; Supplementary Fig. S1), which suggests its use as a predictive biomarker of response to DNA-damaging agents. Indeed, *SLFN11* has been identified as a predictor of response to chemotherapy in ovarian cancer, small cell and non–small cell lung cancer, colorectal cancer, and Ewing sarcoma (5, 6, 9–13). For example, retrospective analyses show that high *SLFN11* expression is positively correlated with tumor-free survival in Ewing sarcoma and colorectal and ovarian cancer patients (1, 5, 12).

Although the expression of *SLFN11* is linked to sensitivity to DNA-targeted agents, lack of SLFN11 expression observed in approximately half of the 60 cancer cell lines in the U.S. National

[1]Developmental Therapeutics Branch and Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland. [2]Division of Hematology/Oncology, Columbia University, New York, New York.

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S.-W. Tang, A. Thomas, and J. Murai contributed equally to this article.

Corresponding Author: Yves Pommier, National Cancer Institute, 31 Center Drive, Building 37, Room 5068, Bethesda, MD 20892-4255. Phone: 240-760-6142; Fax: 240-541-4475; E-mail: pommier@nih.gov

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Translational Relevance

SLFN11 is a predictive biomarker of sensitivity and a determinant of response to a wide range of DNA-targeted therapies. Epigenetic inactivation of *SLFN11* confers resistance to DNA-damaging agents and is a frequent occurrence. We report here that class I HDAC inhibitors derepress *SLFN11* expression and are synergistic with DNA-damaging drugs. These findings provide a SLFN11-dependent epigenetic mechanism in the synergy between DNA-targeted agents and HDAC inhibitors, and present a rational therapeutic combination to overcome drug resistance.

Cancer Institute (NCI) Human Tumor Cell Line Screen (NCI-60) (Supplementary Fig. S1) is related to resistance to these agents (1, 9, 14, 15). DNA methylation analysis of the NCI-60 cell line panel identified CpG promoter island hypermethylation of *SLFN11* as a biomarker of resistance to platinum drugs as well as a broad range of DNA-targeted anticancer therapies (9, 15). A significant association was observed between *SLFN11* CpG promoter island hypermethylation and diminished *SLFN11* mRNA levels in cancer cells. The epigenetic silencing of *SLFN11* and the fact that promoter hypermethylation only accounts for a fraction of cancer cells that lack *SLFN11* expression (Fig. 1B; Supplementary Fig. S1A), and are therefore likely chemotherapy resistant, raises the question of whether epigenetic modifying drugs can derepress *SLFN11*, and sensitize *SLFN11*-inactivated cancer cells to chemotherapy. Consistent with this possibility, two recent studies showed that the DNA demethylating drug, 5-aza-2′-deoxycytidine (decitabine), and the EZH2 inhibitor, EPZ011989, induced SLFN11 expression both at the transcript and protein levels (9, 13). Moreover, treatment with the EZH2 inhibitor was shown to enhance the cytotoxicity and antitumor activity of topotecan in small cell lung cancer (SCLC) models (13).

In this study, we investigated the effect of histone deacetylase (HDAC) inhibitors on *SLFN11* expression and sensitivity to DNA-damaging agents across a panel of 10 cancer cell lines from various tissues of origin. Nine of them did not express *SLFN11* and did not exhibit CpG promoter methylation (two SCLC cell lines: H446 and H82; two ovarian cell lines: OVCAR3 and OVCAR4; two melanoma cell lines: SK-MEL28 and UACC-257; one fibrosarcoma cell line: HT1080; two leukemia cell lines: K562 and HH). We also tested one cell line with high promoter methylation (breast cancer MCF7; Fig. 1B; Supplementary Fig. S1A). Our rationale (see Fig. 1C) was to determine whether epigenetic activation by clinically used HDAC inhibitors could derepress *SLFN11* and sensitize cells to the prototypical TOP1 inhibitor CPT.

Materials and Methods

Cell lines, culture, and drugs

The K562, HCT116, OVCAR-3, SK-MEL-28, OVCAR-4, UACC-257, MCF7, and CCRF-CEM cell lines were obtained from the Division of Cancer Treatment and Diagnosis (DCTD), Developmental Therapeutics Program (DTP, NCI). HT1080 cells were kindly provided by Dr. Lee Helman (NCI/NIH). H82 (HTB-175), H446 (HTB-171), and HH (CRL-2105) cells were obtained from ATCC®. All cells were grown in RPMI medium with 10% FBS (Gibco-BRL) at 37°C in 5% CO_2. CPT, topotecan, cisplatin, hydroxyurea, romidepsin, entinostat, and rocilinostat were obtained from the DCTD. Actinomycin D (A9415) was obtained from Sigma-Aldrich.

Generation of SLFN11-expressing cells

SLFN11 cDNA was amplified using the forward primer (5′-ATCGGATCC GCGGCCAACATGGAGGCAAATCAGTGC-3′) and the reverse primer with the sequence for the Flag tag (5′-ATTGTC-GACGCGGCCCTACTTATCGT CGTCAT CCTTGTAATCATGGC-CACCCCACGGAA-3′) and cloned into the pCDH-EF1-MCS-(PGK-copGFP) lentiviral expression vector (System Biosciences) using the In-Fusion HD Cloning Kit (Clontech). The lentiviral *SLFN11*-expressing vector and the pPACKH1 lentivector packaging plasmids were cotransfected into 293TN cells (System Biosciences) and the viral particles were collected to infect K562 cells with TransduxTM (System Biosciences). The *SLFN11*-expressing cells with GFP signal were sorted using a FACS.

Cell viability assays

Cells were treated or untreated with different concentrations of HDAC inhibitors for 16 hours prior to adding CPT. After the pretreatment, cells were collected, washed, and seeded in 96-well white plates (#6005680 Perkin Elmer Life Sciences) in 100 μL of medium per well with the following cell number; 30,000 cells for K562, 3,000 cells for HT1080 and HCT116, and 5,000 cells for H446, H82 and HH cells. Cells were continuously exposed to the indicated drug concentrations for 48 to 72 hours in triplicate. Cellular viability was determined using ATPlite 1-step kits (PerkinElmer). The ATP level in untreated cells was defined as 100%. Viability of treated cells was defined as: (ATP in treated cells)/(ATP in untreated cells) × 100.

Quantitative real-time PCR

The RNAs of drug-treated or -untreated cells were extracted using RNeasy Mini RNA Kit (Qiagen) and the complementary DNA was generated by SuperScript II Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions. The primers specific for *SLFN11*, *FLI-1*, and *GAPDH* and materials for quantitative real-time PCR were described in our previous study (5). The melting curve was measured to show the amplification specificity. The expression levels of *GAPDH* was considered as the reference gene. The relative expression of *SLFN11* and *FLI-1* to reference gene *GAPDH* was determined using the $2(-\Delta\Delta C_t)$ method.

To measure the half-life of mRNA for *SLFN11*, *GAPDH*, *PUMA*, and *Cyclin E*, CCRF-CEM cells were treated or untreated with actinomycin D (1 μmol/L) for 6 hours, and total RNA was extracted. The expression level of 18S ribosomal RNA was considered as the reference gene. Primers for each gene was designed by PrimerBank (https://pga.mgh.harvard.edu/primerbank/).

Western blotting

Cells were treated with the indicated concentrations of HDAC inhibitors for 16 hours and lysed in 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 0.1% SDS supplemented with protease inhibitor cocktail (Roche). The lysates were separated in 8% to 15% of SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). The antibodies specific to SLFN11 (sc-374339 or sc-515071; Santa Cruz Biotechnology), acetyl histone H3 (ab4441; Abcam), FLI1 (sc-356; Santa Cruz Biotechnology), actin (MAB1501; Chemicon

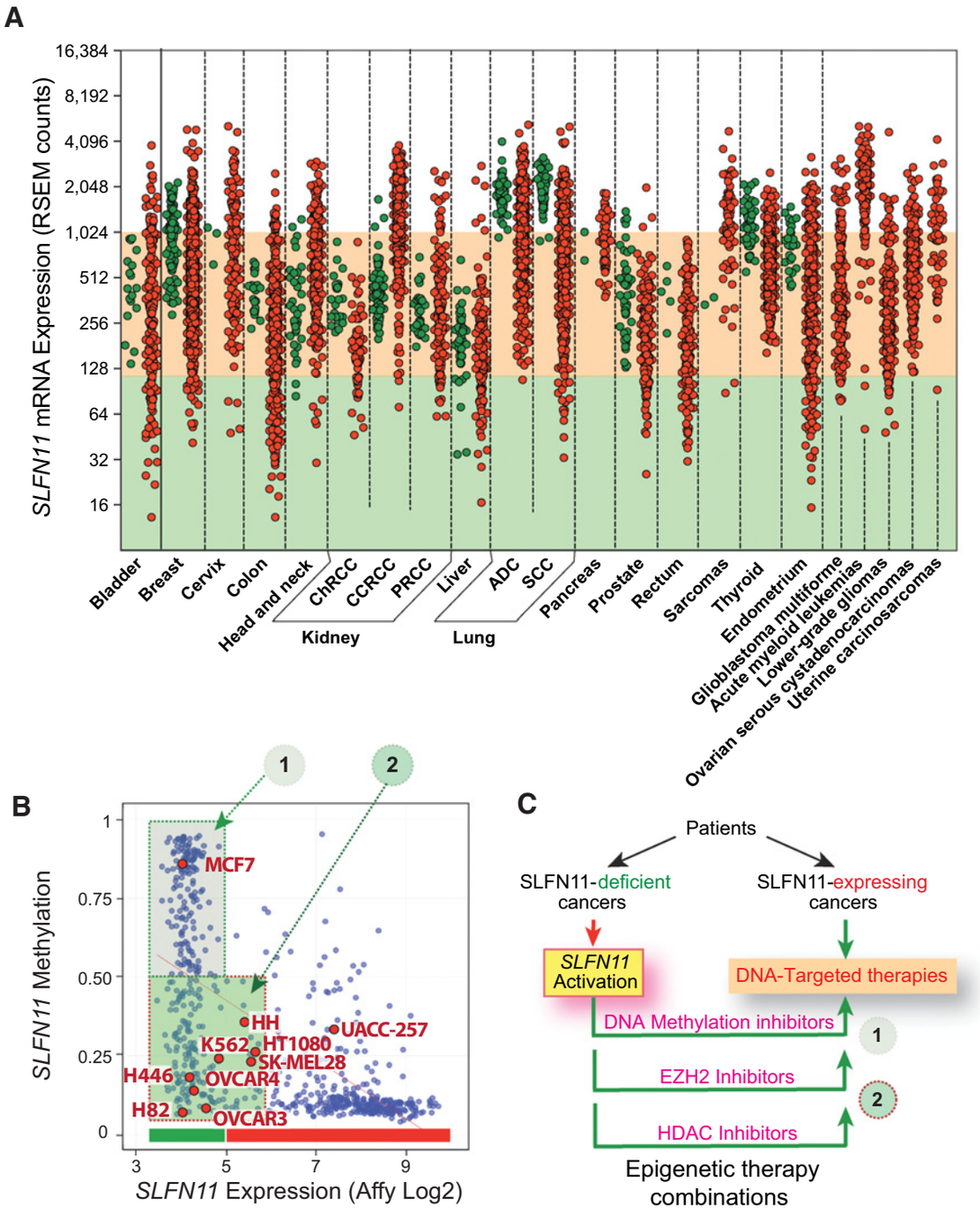

Figure. 1.

SLFN11 is both repressed and overexpressed across the various cancer types of the TCGA database and is epigenetically regulated in the 1,000 cancer cell line databases. **A,** When available, for each tissue type, normal samples are shown in green and cancer samples in red. *SLFN11* transcript expression was determined by RNASeq and is represented as the $\log_2$ of the RSEM-quantified transcript abundance level. The salmon color–shaded area corresponds to average normal transcript levels. ChRCC, chromophobe renal cell carcinoma; CCRCC, clear cell renal cell carcinoma; PRCC, papillary renal cell carcinoma; ADC, adenocarcinoma; SCC, squamous cell carcinoma. **B,** *SLFN11* expression in the cancer cell lines of the CCLE collection (http://www.broadinstitute.org/ccle/) matched with promoter methylation determined from the data from the GDSC database (http://www.cancerrxgene.org/) for the individual common cell lines across the two databases (blue dots) obtained with CellMiner (http://discover.nci.nih.gov/cellminercdb/). Cell lines in group 1 do not express *SLFN11* and have promoter methylation. Cell lines in group 2 do not express SLFN11, presumably because of chromatin epigenetic drivers such as histone deacetylation. Cell lines used in this study are marked in red (see also Supplementary Fig. S1). **C,** Scheme for the rationale of the study. Recent studies have shown that *SLFN11* can be derepressed by inhibiting DNA methylation and by EZH2 inhibitors (9, 13). This study explores the relevance of epigenetic acetylation by using HDAC inhibitors.

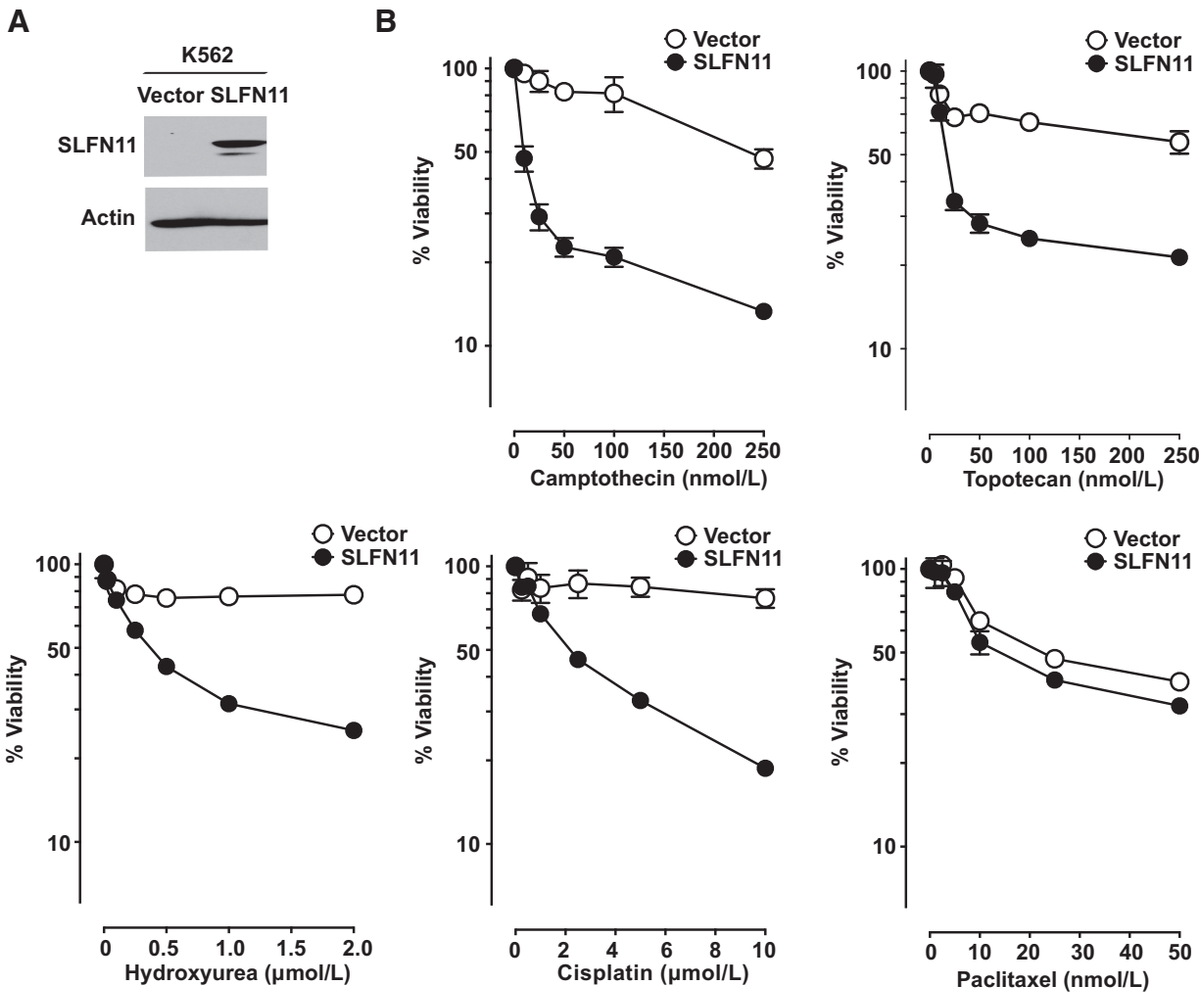

Figure 2.

Forcing *SLFN11* expression sensitizes K562 cells to DNA-damaging drugs. **A,** Protein levels of SLFN11 in K562/Vector and K562/SLFN11 cells determined by Western blotting using antibodies against SLFN11 (98 kDa). Actin (42 kDa) was used as loading control. **B,** K562/Vector (empty circles) and K562/SLFN11 (solid circles) cells were treated with CPT, topotecan, hydroxyurea, cisplatin, or paclitaxel for 2 days before cell viability was assessed. Representative results in triplicate from three independent experiments are shown as mean ± SD.

International), or GAPDH (2118S; Cell Signaling Technology) were applied to the membranes 4°C overnight followed by horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Life Sciences). The signals were developed by ECL Western Blotting Substrate (Thermo Scientific). Actin was used as the loading control.

Microarray and RNA-Seq data

Peripheral blood mononuclear cells from patients with circulating tumor cells were obtained before infusion (pre), and at 4 hours after start of the infusion of the first cycle of treatment (16). The normalized gene expression from microarray analysis (16) were downloaded from the Gene Expression Omnibus website (GSE45405, http://www.ncbi.nlm.nih.gov/geo; ref. 17). The RNA-Seq data for SLFN11 gene expression in the datasets of various cancers and normal tissues were retrieved from TCGA portal (http://cancergenome.nih.gov).

Generation of SLFN11-deleted cells

To disrupt the *SLFN11* gene, we designed a guide RNA targeting just downstream of the start codon in the 4th exon using the CRISPR design tool (http://crispr.mit.edu; ref. 18). A human codon-optimized SpCas9 and chimeric guide RNA expression plasmid (pX330: pX330-U6-chimeric_BB-CBh-hSpCas9) was purchased from Addgene. Each guide RNA was inserted into the pX330 plasmid (pX330-SLFN11). The gene-targeting constructs harboring homology arms and a puromycin-resistance gene were prepared. Briefly, ~1 kb genomic sequences just upstream and downstream of the Cas9 cleavage sites were amplified by PCR methods from genomic DNA. The PCR products of the upstream site (left homology arm) and downstream site (right homology arm) were subcloned into pCR2.1-TOPO vector (Invitrogen) at the TA cloning site and *Apa*I/*Xho*I restriction endonuclease site, respectively in the desired direction. Puromycin resistance gene was finally

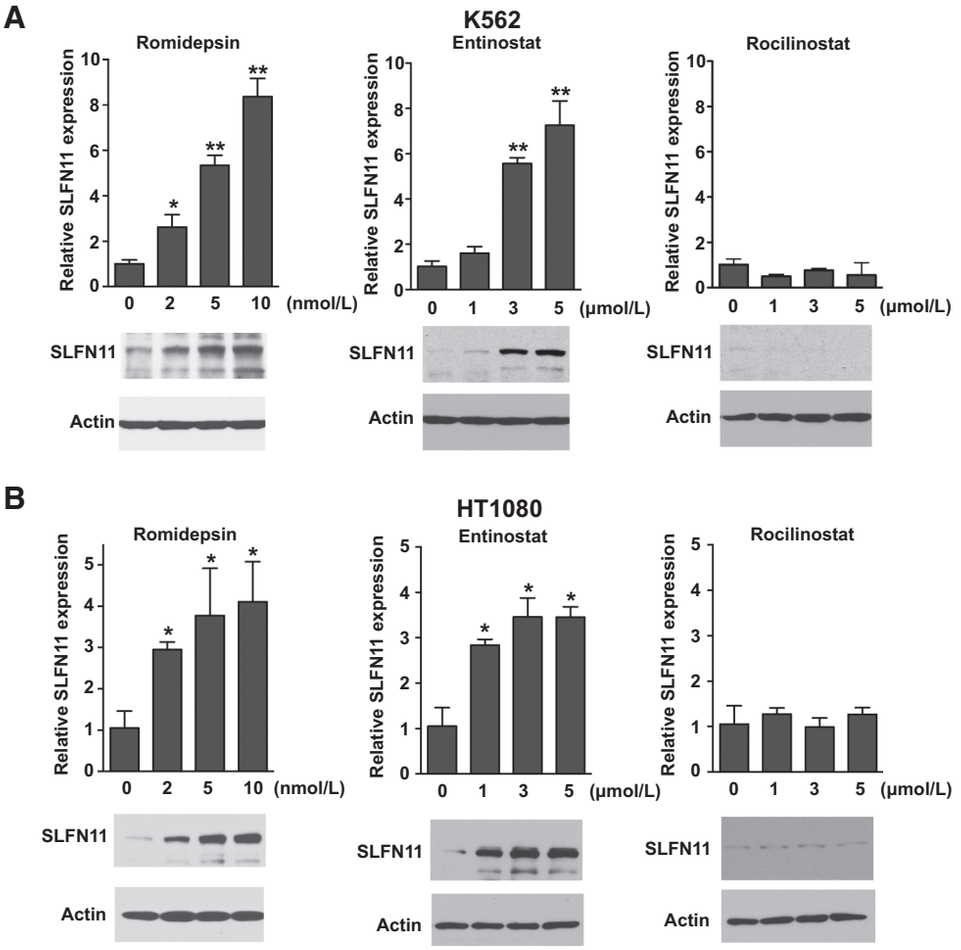

Figure 3.
Class I HDAC inhibitors induce SLFN11 expression. K562 (**A**) and HT1080 (**B**) cells were treated with romidepsin, entinostat, or rocilinostat for 16 hours. Top: *SLFN11* mRNA levels were measured by quantitative real-time PCR. The *y*-axis represents relative *SLFN11* expression normalized to untreated cells (0). Representative results in triplicate from three independent experiments are shown as mean ± SD. *, $P < 0.05$; **, $P < 0.001$ by *t* test. Bottom: SLFN11 protein levels were determined by Western blotting using antibodies against SLFN11 (98 kDa). Actin (42 kDa) was used as loading control.

subcloned between the homology arms at the *Not*I restriction endonuclease site. The targeting construct and pX330-SLFN11 were cotransfected into K562 cells by electroporation. After transfection, cells were released into drug-free medium for 48 hours followed by puromycin selection until single colonies were formed. Single clones were expanded, and gene-deletion was confirmed by Western blotting. PCR primers and guide RNA sequences will be provided on request.

Statistical and bioinformatics analyses

Level 3 RSEM-quantified RNASeq gene expression data (platform code IlluminaHiSeq_RNASeqV2) was downloaded from the TCGA data portal for tumor and normal samples of the presented tissue types. IC_{50} was calculated as the treated cell counts relative to untreated control cells by nonlinear regression using GraphPad Prism5. The differences between two experimental groups was determined by the two-tailed independent-samples *t* test for the results from quantitative real-time PCR and microarray analysis. Synergism was assessed using the CompuSyn Software. Combination index between 0.7 and 0.3, combination index between 0.3 and 0.1, and combination index less than 0.1 indicate synergy, strong synergy, and very strong synergy, respectively.

Results

SLFN11 expression is suppressed in many human cancers and cancer cell lines

SLFN11 expression has previously been shown to vary broadly across cancer cell lines with some lines expressing no or very low *SLFN11* whereas others such as sarcomas and leukemias express very high levels of *SLFN11* transcripts (1, 2, 5). Analyzing the TCGA database, we found that *SLFN11* exhibits a broad dynamic range of expression within and across the collection of available tissues (Fig. 1A). Notably some normal tissues such as lung and thyroid, and to a lesser extent some breast and endometrium samples exhibit high *SLFN11* transcripts. Also, acute myeloid leukemias, sarcomas and clear cell renal cell carcinomas (CCRCC) show high *SLFN11* expression in the majority of patients. By contrast, a large fraction of the bladder, colon, liver, prostate and endometrial carcinomas show tumor samples with notably low *SLFN11* expression (Fig. 1; shaded green area below the shaded salmon color area corresponding to the range of expression in normal tissues).

SLFN11 expression was also widely distributed across the cell lines of the cancer cell line databases from the Broad Institute (CCLE; http://www.broadinstitute.org/ccle/), the Welcome Trust Sanger Institute-Massachusetts General Hospital Cancer Center

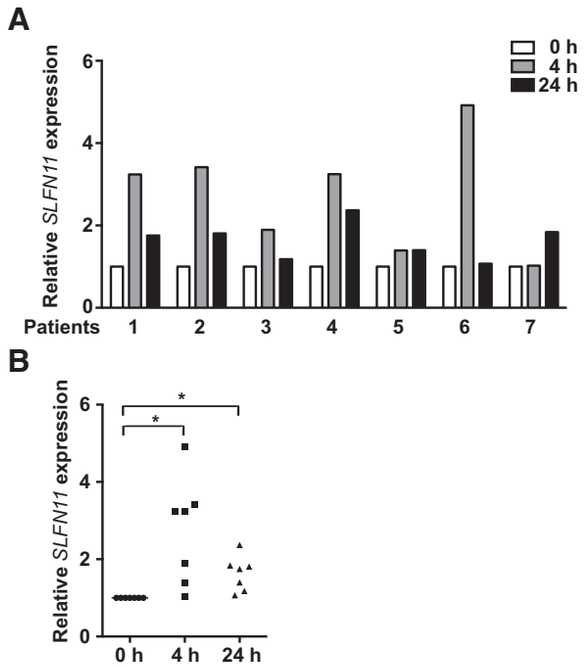

Figure 4.

SLFN11 expression is induced in response to romidepsin in patient samples. Microarray analysis of total RNA isolated from PBMCs of seven patients with significant CTCL blood involvement. **A** and **B,** Samples were obtained before romidepsin infusion and at 4 and 24 hours after the initiation of romidepsin therapy (at romidepsin infusion end). The *y*-axis represents relative *SLFN11* expression normalized to the samples before romidepsin infusion (open bars). *, $P < 0.05$.

(GDSC; http://www.cancerrxgene.org/), and the NCI-60 (http://discover.nci.nih.gov/cellminercdb). Figure 1B and Supplementary Fig. S1A show that approximately 45%–50% of the cell lines repress *SLFN11* (green-shaded areas), and that among them a significant fraction could not be linked to promoter hypermethylation (dark green-shading). Whole exome sequencing (19) and copy number analyses (20) of the NCI-60 (http://discover.nci.nih.gov/) showed that the SLFN11-non-expressing cells had no detectable copy loss or deleterious mutation. This led us to test the role of epigenetic acetylation (Fig. 1C).

Enhancing *SLFN11* expression in cancer cells increases sensitivity to DNA-damaging agents

To investigate whether forced *SLFN11* expression sensitizes cancer cells to DNA-damaging agents, *SLFN11* was exogenously expressed in leukemia K562 cells, which express a very low level of SLFN11 (Figs. 2A and 1B; Supplementary Fig. S1A; ref. 1; http://discover.nci.nih.gov/cellminer/). As shown in Fig. 2B, overexpression of *SLFN11* strongly sensitized K562 cells to DNA-damaging agents including CPT (IC_{50} = 272.5 nmol/L vs. 11.5 nmol/L in vector control cells and *SLFN11*-expressing cells, respectively), topotecan (IC_{50} = 192.6 nmol/L vs. 23.9 nmol/L), hydroxyurea (IC_{50} = 6.4 μmol/L vs. 0.4 μmol/L), and cisplatin (IC_{50} = 15.6 μmol/L vs. 2.2 μmol/L), but not to the antimitotic agent paclitaxel (IC_{50} = 27.5 nmol/L vs. 18.1 nmol/L). A similar phenomenon was observed in the colorectal cancer cell line HCT116 with *SLFN11* overexpression (Supplementary Fig. S2). These results demonstrate that enhancing *SLFN11* expression in cancer cells increases sensitivity to DNA-damaging agents.

Class I HDAC inhibitors induce *SLFN11* expression both *in vitro* and *in vivo*

HDAC inhibitors have been shown to regulate gene expression. To date, the US Food and Drug Administration has approved four HDAC inhibitors—vorinostat, romidepsin, belinostat, and panobinostat—for cutaneous/peripheral T-cell lymphoma and multiple myeloma. HDAC inhibitors as monotherapy and in combination with other agents are in different stages of clinical development for the treatment of hematologic malignancies and solid tumors (21–23). To examine the effect of HDAC inhibitors on *SLFN11* expression, K562 cells were treated with two class I HDAC inhibitors, romidepsin and entinostat, and one class IIb HDAC inhibitor rocilinostat. Figure 3A shows that *SLFN11* mRNA and protein levels were strongly enhanced by romidepsin and entinostat, but not with rocilinostat. We also observed the induction of *SLFN11* expression both at the RNA and protein levels in the fibrosarcoma cell line HT1080 by the class I HDAC inhibitors, romidepsin and entinostat but not by the class IIb HDAC inhibitor rocilinostat (Fig. 3B).

The increase in the *SLFN11* mRNA level was observed as early as 3 hours posttreatment, peaked at 24 hours, and returned to low levels 24 hours after romidepsin removal (Supplementary Fig. S3A and S3C). This transient upregulation is at least in part due to the relatively rapid turnover of SLFN11 transcripts. Using actinomycin D to block RNA synthesis and comparing *SLFN11* with transcripts known to have short half-life (*CCNE* and *PUMA*) and long half-life (*GADPH*), we determined the half-life of *SLFN11* mRNA to be relatively short (approximately 4 hours; Supplementary Fig. S3D) in CCRF-CEM cells that express high SLFN11 (1, 24).

Our recent publication showed *FLI1*, which is a member of ETS transcriptional factor family, to be a cellular regulator of *SLFN11* expression in cancer cells from various tissues of origin (5). However, we did not observe the induction of *FLI1* expression by romidepsin in K562 cells (Supplementary Fig. S3B) under conditions where SLFN11 was strongly induced (see Fig. 3A, left). Therefore, the derepression of *SLFN11* by the HDAC inhibitors is not due to *FLI1* activation.

We also examined whether romidepsin could activate *SLFN11* expression *in vivo* by assessing gene expression microarray data of PBMCs obtained before and after romidepsin from patients with cutaneous T-cell lymphoma (CTCL) and circulating malignant T cells (25). Four out of seven cases exhibited more than three-fold induction of *SLFN11* expression in response to romidepsin therapy. The induction of SLFN11 expression tended to be higher at 4 than 24 hours after romidepsin (Fig. 4), which is likely related to the rapid elimination of romidepsin (half-life around 3 hours) and to the short half-life of *SLFN11* transcripts (Supplementary Fig. S3D). No obvious romidepsin-induced changes in *SLFN11* expression were observed in two cases (Fig. 4). These results indicate that class I HDAC inhibitors activate *SLFN11* expression both in cancer cell line models and patients.

Class I HDAC inhibitors are synergistic with CPT

Because *SLFN11* expression determines the sensitivity of cancer cells to different classes of DNA-damaging agents (see Fig. 2; refs. 1, 6, 24), we examined whether class I HDAC inhibitors, which activate *SLFN11* expression show synergy with CPT, a

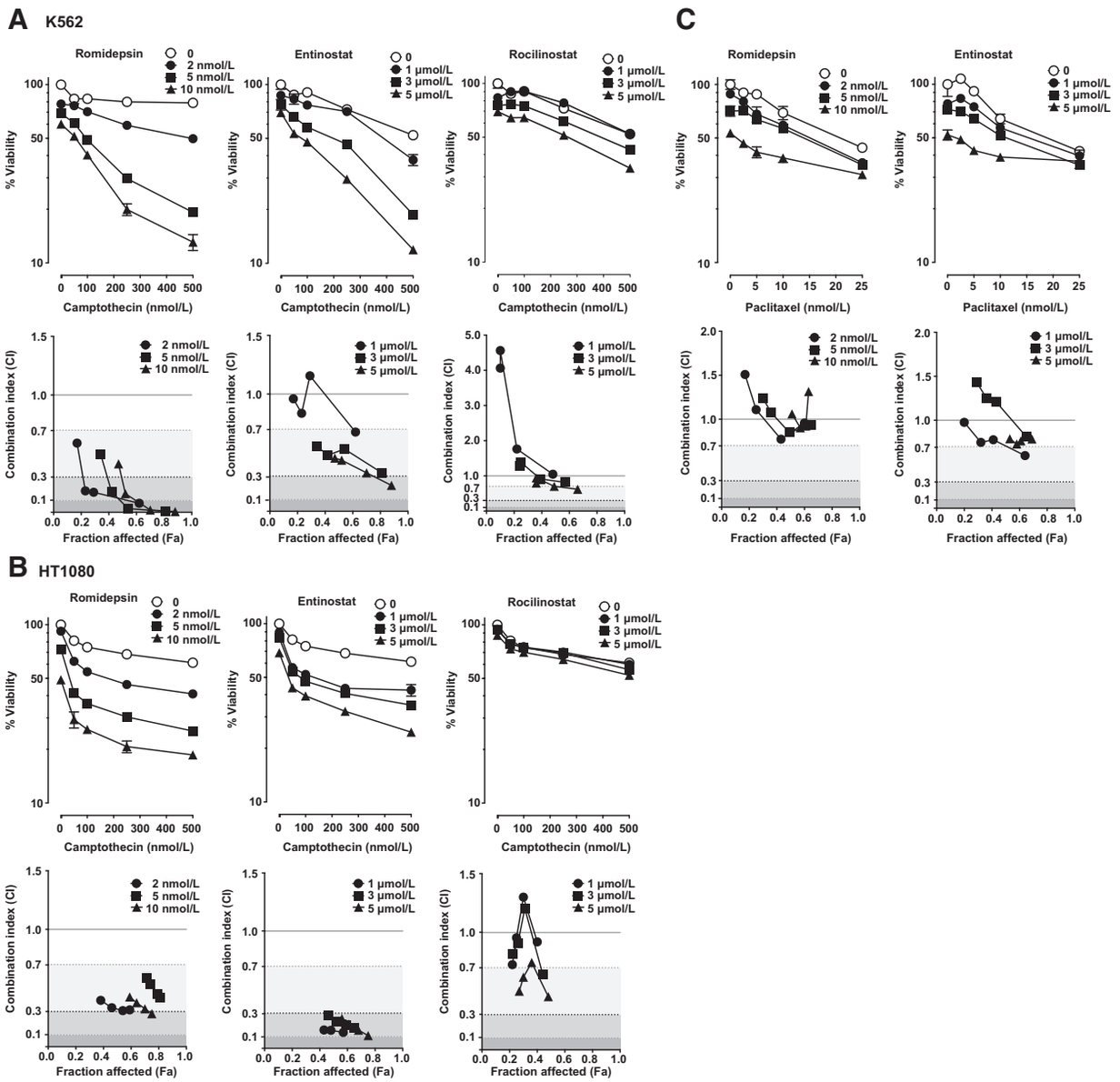

Figure 5.

Class I HDAC inhibitors show synergistic effects with CPT but not with paclitaxel. K562 (**A**) and HT1080 (**B**) cells were pretreated with romidepsin (left), entinostat (middle), or rocilinostat (right) for 16 hours; washed; and then treated with the indicated concentrations of CPT for another 2 days before cell viability was assessed (top). Representative results in triplicate from three independent experiments are shown as mean ± SD. Bottom: Graphs of combination index versus Fa (fraction affected) for data points of romidepsin (left), entinostat (middle), or rocilinostat (right) in combination with CPT. **C,** K562 cells were pretreated with romidepsin (left) or entinostat (right) for 16 hours, washed, and then treated with CPT for another 2 days before cell viability was assessed (top). Representative results in triplicate from three independent experiments are shown as mean ± SD. Bottom: Graphs of combination index versus Fa (fraction affected) for data points of romidepsin (left) or entinostat (right) in combination with CPT. Shading represents the levels of synergism. Combination index between 0.7 and 0.3, combination index between 0.3 and 0.1, and combination index less than 0.1 indicate synergy, strong synergy, and very strong synergy, respectively.

specific TOP1 inhibitor (26) representative of the DNA-damaging drugs whose activity is under the control of *SLFN11* expression level (1, 2). K562 and HT1080 cells were pretreated with HDAC inhibitors for 16 hours, washed out, and then incubated with various doses of CPT. Pretreatment with romidepsin or entinostat, but not rocilinostat, sensitized K562 and HT1080 cells to CPT in a dose-dependent manner (Fig. 5A and B). Combination index values were calculated to evaluate synergy using the CompuSyn software (bottom panels of Supplementary Fig. S5A and S5B). Romidepsin (2–10 nmol/L) and entinostat (1–5 μmol/L) showed high synergistic values with CPT in K562 and HT1080 cells (except 1 μmol/L entinostat in K562 cells). As expected, based on the fact that SLFN11 does not determine response to tubulin inhibitors (see Fig. 2; refs. 1, 9, 15), the combination of class I HDAC

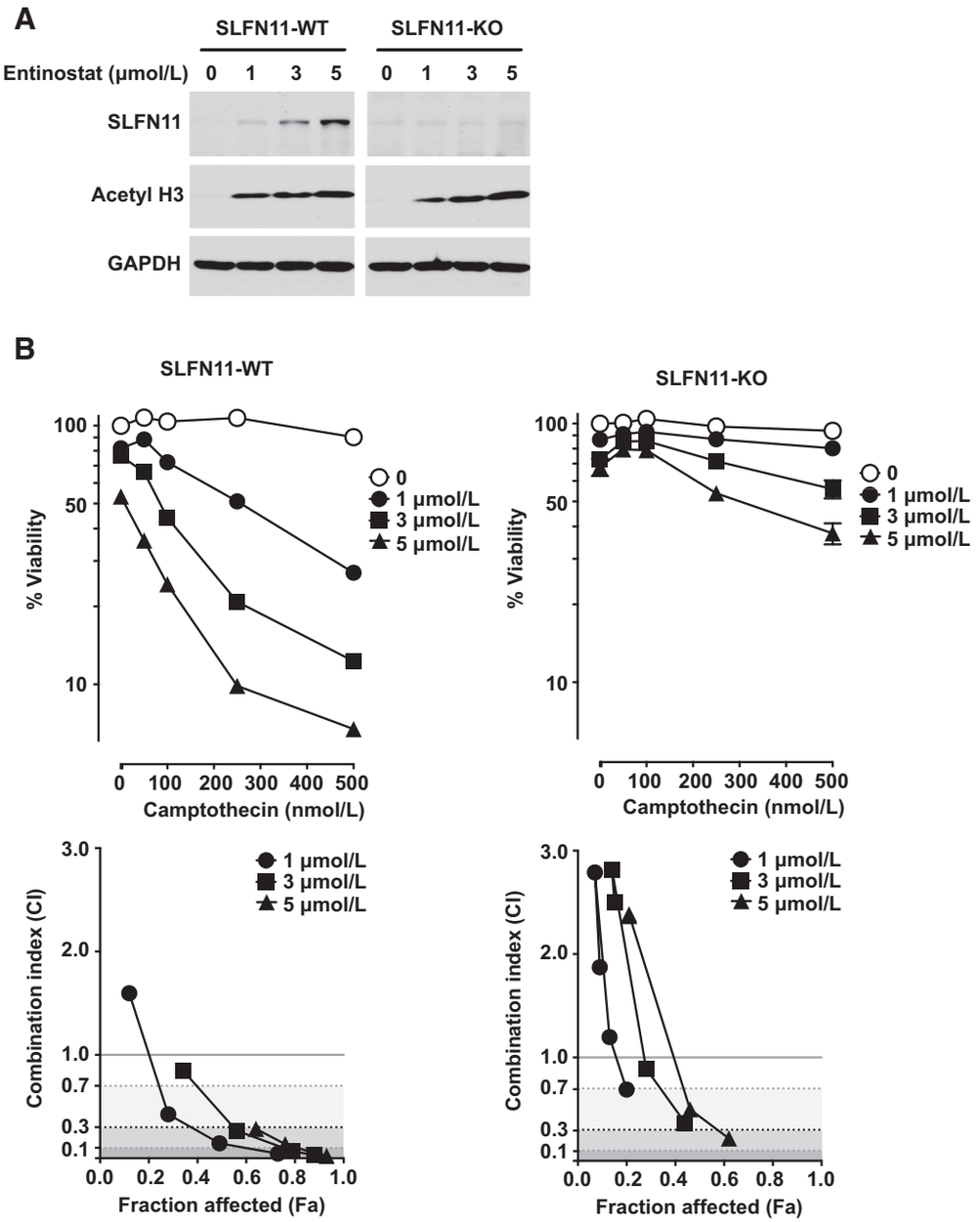

Figure 6.

SLFN11 induction is crucial for the synergy of class I HDAC inhibitors with CPT. **A,** Protein levels of SLFN11 and acetyl histone H3 (K9) in K562 WT and K562/SLFN11-knockout cells determined by Western blotting using antibodies against SLFN11 (98 kDa) and acetyl histone H3 (17 kDa). Entinostat treatments were for 16 hours. Actin (42 kDa) was used as loading control. **B,** K562 WT (left) and K562/SLFN11-knockout (right) cells were pretreated with entinostat for 16 hours, washed, and then treated with the indicated concentrations of CPT for another 2 days before cell viability was assessed (top). Representative results in triplicate from three independent experiments are shown as mean ± SD. Bottom: Graphs of combination index versus fraction affected (Fa) for data points of entinostat in combination with CPT.

inhibitors with paclitaxel did not show synergy under similar conditions (Fig. 5C).

We extended these experiments to three additional cancer cell lines: two SCLC cell lines, H82 and H446 that were used recently to demonstrate the epigenetic regulation of SLFN11 by EZH2 (13), and the HH cutaneous T-cell lymphoma cell line (Supplementary Fig. S4). The results show that entinostat derepressed *SLFN11* in all three cell lines and produced synergy with CPT in H82 and to a lesser extent in HH cells. Entinostat produced only additional cytotoxicity in H446 cells in spite of *SLFN11* induction. Together these results demonstrate the synergy of class I HDAC inhibitors with CPT, which is consistent with the epigenetic derepression of *SLFN11* by HDAC inhibitors.

Induction of *SLFN11* expression is a dominant factor for the synergy of class I HDAC inhibitors with CPT

To investigate the causal contribution of *SLFN11* to the synergistic effect of class I HDAC inhibitors with DNA-damaging agents, we generated *SLFN11*-knockout (*SLFN11*-KO) K562 cells using CRISPR/Cas9. As expected, entinostat induced acetylation of histone H3 but did not increase SLFN11 expression in *SLFN11*-KO cells (Fig. 6A). We further examined the synergistic effect of entinostat and CPT in *SLFN11*-WT and –KO K562 cells. Combination of entinostat and CPT showed much weaker synergy in *SLFN11*-KO cells than in *SLFN11*-WT cells (Fig. 6B), indicating that enforced *SLFN11* expression by class I HDAC inhibitors dominantly drives the synergy of class I HDAC inhibitors with CPT.

Discussion

Availability of robust biomarkers is an unmet need to optimize the efficacy of DNA-damaging agents and maximize their impact on cancer treatment. Yet, despite their broad utility in multiple cancers, DNA-damaging agents lack clinically useful predictive biomarkers of response and resistance (14).

We initially discovered expression of *SLFN11* as a genetic determinant of response to a broad range of DNA-damaging agents (1), a finding that has since been confirmed in multiple independent studies (2–5, 9, 13, 24). Recently, we demonstrated that epigenetic inactivation of *SLFN11* expression by promoter hypermethylation confers resistance to DNA-damaging therapies (9, 15). Based on these considerations, in this study we investigated the effect of epigenetic modifiers on *SLFN11* expression and sensitivity of cancer cells to DNA-damaging agents. Our experiments show that class I HDAC inhibitors induce *SLFN11* expression in epigenetically *SLFN11*-inactivated cells both *in vitro* and *in vivo*. We further demonstrate that pretreatment with class I HDAC inhibitors enhance cell killing by DNA-damaging agents via a *SLFN11*-dependent mechanism. Our study reports the effect of HDAC inhibitors on *SLFN11* expression and provides a proof of concept for novel combination therapy based on genomic determination of *SLFN11* status in tumors. This concept is further supported by recent findings by Gardner and colleagues (13) that SLFN11 expression is suppressed in resistant tumors after exposure to chemotherapy and that EZH2 inhibitor activates SLFN11 expression to re-sensitize SCLC cells and PDX murine models to topotecan. In Fig. 1C, we summarize the rationale and outline the strategy for combining class I HDAC inhibitors and DNA-targeted therapies to overcome the resistance of cells lacking both *SLFN11* expression and *SLFN11* promoter methylation. Notably, a recent publication by Zeng and colleagues (27) suggested the rationale for combining HDAC and BRD4 inhibitors. Further studies are warranted to determine whether the combination of HDAC inhibitors with other epigenetic therapies (EZH2 and/or BRD4 inhibitors) will improve the activity of DNA-targeted therapies for tumors that do not express *SLFN11* and do not show promoter hypermethylation.

Synergistic effects of HDAC inhibitors and DNA-damaging agents with respect to growth inhibition and apoptosis are well-established in preclinical studies (28). This synergistic effect is thought to be due to HDAC inhibition-mediated increase in chromatin accessibility which allows access of DNA-damaging agents to their targets. Alternately, HDAC inhibition may result in down-regulation of the DNA damage response. Genes involved in nonhomologous end-joining (NHEJ) and homologous recombination (HR) including *Ku70*, *Ku86*, *DNA-PKc*, *RAD51*, *BRCA1*, and *BRCA2* are down-regulated by HDAC inhibition (29, 30). Our study for the first time implicates HDAC inhibition-mediated direct activation of *SLFN11* in sensitization of cancer cells to DNA-targeted therapies. That this effect is specific for class I HDAC inhibitors may be a function of the distinct cellular and subcellular expression patterns, signaling pathways and substrates associated with class I and class II HDACs.

It is critical to generate and validate reliable techniques to assess *SLFN11* expression in patient tumors to translate these findings to a clinical setting. *SLFN11* promoter methylation has been found to explain *SLFN11* gene silencing in cancer cell lines (9). In earlier studies, we have found significant association between *SLFN11* CpG island methylation and diminished *SLFN11* RNA levels as well as *SLFN11* RNA levels and protein expression (9, 15, 24). However, based on the cancer cell line data (see Fig. 1B; Supplementary Fig. S1A), promoter methylation is clearly insufficient to determine lack of *SLFN11* expression. Quantitative assessment of *SLFN11* transcripts and SLFN11 protein expression by immunohistochemistry should provide clinically translatable assays to measure SLFN11 transcripts and protein expression in formalin-fixed paraffin-embedded tumor samples.

The precise mechanism by which SLFN11 sensitizes cancer cells to DNA-targeted agents is not fully established. Studies to date have provided seemingly contradictory data in this regard. Mu and colleagues found defective checkpoint maintenance and HR as the likely cause of SLFN11-dependent cell death (31). In contrast, our recent study showed that SLFN11-induced cell death is mediated by HR-independent irreversible and prolonged S-phase arrest (24, 32). In our model, SLFN11 inhibits replication and forces cell-cycle arrest at mid S-phase in response to treatment with DNA-damaging agents. Ongoing efforts are focused on elucidating the molecular mechanisms of SLFN11-dependent replication arrest and cancer cell death (32).

In conclusion, our study reveals the epigenetic modulation of *SLFN11* expression and its relevance to sensitivity to DNA-damaging agents. These promising results warrant future clinical investigations. Developing assays for assessing *SLFN11* expression status as a predictive marker for tumor response to DNA-damaging agents and clarifying the molecular mechanisms underlying *SLFN11* biology are important future considerations.

Disclosure of Potential Conflicts of Interest

J.B. Trepel reports receiving commercial research grants from Syndax Pharmaceuticals. S.E. Bates holds ownership interest (including patents) in and is a consultant/advisory board member for Celgene. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S.-W. Tang, J.B. Trepel, Y. Pommier
Development of methodology: S.-W. Tang, Y. Pommier
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-W. Tang, A. Thomas, J. Murai, Y. Pommier
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.-W. Tang, A. Thomas, J. Murai, J.B. Trepel, S.E. Bates, V.N. Rajapakse, Y. Pommier
Writing, review, and/or revision of the manuscript: S.-W. Tang, A. Thomas, J.B. Trepel, S.E. Bates, Y. Pommier

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.-W. Tang, Y. Pommier
Study supervision: Y. Pommier

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