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HDAC10 as a potential therapeutic target in ovarian cancer

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Abstract

Objective—We analyzed histone deacetylase 10 (HDAC10) for function in the context of the DNA damage response in *BRCA1*-null ovarian cancer cells as well as evaluated the potential of general HDAC inhibitors in primary ovarian carcinoma cells. HDAC10 had previously been shown to be highly stimulatory to the process of homology directed repair in HeLa cells, and in this study we investigated whether HDAC10 could impact in vitro the response to anticancer therapies. We hypothesized that the loss of HDAC10 would sensitize cells to platinum therapy.

Methods—We combined informatics analysis of large DNA sequencing datasets from ovarian cancer tumors with tissue culture based assays of primary and established cell lines to test for sensitivity to platinum therapy if HDAC10 activity was inhibited or depleted.

Results—Using The Cancer Genome Atlas (TCGA) dataset, we found that deep deletions in *HDAC10* occurred in 5–10% of ovarian cancer tumors. From the TCGA data we found that low *HDAC10* mRNA levels correlated with platinum sensitivity of the tumors. Cell proliferation and DNA damage assays in a *BRCA1*-null ovarian carcinoma cell line demonstrated reduced DNA repair capacity and sensitization of platinum therapy. Similarly, primary ovarian carcinoma cells demonstrated a sensitization to platinum therapies when treated with HDAC inhibitors.

Conclusions—From the results of this study, we suggest that the inhibition of HDAC10 may potentiate the effects of platinum therapies in ovarian tumors.

Introduction

Histone modifications have been central in the understanding of post-translational modifications and their effects on the regulation of gene expression [1]. Histone acetylation is a reversible process, governed by two classes of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs). The acetylation of lysine on histone proteins is generally stimulatory to mRNA synthesis [2]. One suggested mechanism is the neutralization of the positively charged lysine residue by acetylation loosens the interaction of the DNA with the underlying nucleosome, providing access to transcriptional machinery

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[3]. Alternatively, the transcriptional control could be modulated through direct protein interactions, or a combination of the above [4]. HDACs catalyze the reverse reactions, contributing to transcriptional repression by rendering the chromatin to a state that is functionally silenced [5]. In this study we focus specifically on one of these histone deacetylases, HDAC10.

Although originally classified as 'histone' deacetylases, these enzymes are not limited to histone substrates. Acetylated lysine on non-histone proteins have been shown to regulate multiple cellular functions, which can be reversed by HDACs [6]. Most findings about HDACs focus on these factors as repressors of transcription, but our understanding of the roles of HDACs continues to expand [7, 8]. As an example, HDAC1, HDAC2, and HDAC3 have all shown tumor-suppressive genomic stability function [9, 10]. HDAC9 and HDAC10 have been shown to stimulate homologous recombination in HeLa cells [11]. The DNA repair function of HDAC10 prompted us to explore a possible association with cancer.

Ovarian cancer is in many cases associated with defects in the repair of DNA double strand breaks (DSB). Mutation in either *BRCA1* or *BRCA2* is associated with ovarian cancer, and the proteins encoded by these genes are essential regulators of DSB repair [12, 13]. Tumor cells that are BRCA1 or BRCA2 deficient are sensitive to inhibitors of Poly-ADP-ribose Polymerase (PARP) [14], and PARP inhibitors are increasingly found to be effective in killing ovarian cancer cell lines and as a potential therapy for ovarian cancer [15]. Other proteins that stimulate the DSB repair pathway may also contribute to tumorigenesis when mutated and may provide targets for therapy.

In this study we find that HDAC10 is either expressed at low level or deleted in a subset of ovarian cancers. Additionally, we find a significant correlation with sensitivity to platinum-based therapy and low levels of HDAC10 mRNA within the same tumor samples. Based on our results from the in vitro studies, we suggest that inhibition of HDAC10 may potentiate the response to platinum-based therapy in ovarian cancer.

Materials and Methods

Cell Culture and Reagents

HeLa DR-13-9 cells utilized for homology directed repair have been previously described [16] and cultured using standard HeLa culturing protocols. UWB1.289 ovarian carcinoma cells were purchased from ATCC (Manassas, VA) and cultured according to manufacturer specifications. HDAC inhibitors trichostatin A (TSA) and suberanilohydroxamic acid (SAHA) were purchased from Sigma-Aldrich (St. Louis, MO). HDAC10 and control siRNAs were synthesized and purchased from Integrated DNA Technologies (Coralville, IA). Sequences for the siRNAs are listed in Table 1. MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and comet assay lysis buffer were purchased from Trevigen (Gaithersburg, MD). SYBR Green used in the comet assay was purchased from Bio-Rad (Hercules, CA).

Primary ovarian carcinoma cell population isolation

Primary ovarian carcinoma cell populations used in this study were isolated from patient ascites at the time of primary surgery or collected at the time of tumor recurrence from patients with advanced stage ovarian carcinoma. All patients signed consent forms, and the use of patient samples was approved under the Ohio State University Human Investigations Committee (IRB # 2004C0124). Cells were grown using MCDB/M199 media as previously described [17, 18].

Homology Directed Repair Assay (HDR)

The HDR assay we utilized has been previous described and characterized [16, 19, 20]. 300,000–400,000 HeLa cells were plated in each well of a 6-well tissue culture dish (~9.5 cm^2) on day 1. On Day 2, media in wells was replaced with media containing 0, 75, 150, or 300 nM of trichostatin A (TSA) or 0, 0.5, 1.0, or 2.0 μM of suberanilohydroxamic acid (SAHA). Cisplatin was added to each of the TSA and SAHA treatments at 0, 100, and 300 nM concentrations. On Day 3, in fresh media and in the absence of the drug, 3 μg of pCBASce1 plasmid containing I-Sce1 endonuclease was transfected using 3–5 μl of Lipofectamine 2000, first diluted in 125 μl Opti-MEM each. Four to six hours later, the culture media was replaced with fresh media containing 0, 75, 150, or 300 nM of trichostatin A (TSA) or 0, 0.5, 1.0, or 2.0 μM of SAHA, as appropriate. Cisplatin was added to each of the TSA and SAHA treatments at 0, 100, and 300 nM concentrations. Cells were then incubated for 72 hours. The cells in each treatment sample were then treated with trypsin and resuspended in 0.5–1 mL PBS. 10,000 cells were counted using a BD Biosciences FACSCalibur instrument available at the Ohio State University Comprehensive Cancer Center Analytical Flow Cytometry core laboratory. The 10,000 cells were gated using an area of the forward scatter-side scatter plot to optimize live cell counting. The number of cell expressing green fluorescent protein (GFP) was recorded for each sample and the GFP percentage was normalized against untreated samples. The experiment was repeated in quadruplicate.

Comet Assay

The comet assay measures eukaryotic cell DNA damage using gel electrophoresis [21]. Damaged DNA is electrophoresed away from cells suspended in agarose, forming a comet tail shape that can be visualized and quantified. UWB1.289 cells were plated in 6-well tissue plates (~9.5 cm^2 per well). Once the cells reached a confluence of approximately 60%, they were transfected with control siRNA targeting luciferase (GL2), or siRNA targeting two different HDAC10 sequences (HDAC10-1 and HDAC10-2). 100 pmol of each siRNA was diluted in 250 μl of Opti-MEM, and 5 μl of Oligofectamine was similarly diluted in 250 μl of Opti-MEM and the transfection was carried out according to manufacturer specifications. 48 hours later, a second transfection was repeated with the same volumes and incubation times. Five days after the initial transfection, the cells were exposed to 4 Gy of x-ray ionizing radiation. The cells were then incubated for four hours, then detached from the plate with trypsin, mixed with culture media, pelleted at low speed, and resuspended in 1 mL of PBS. 5 μl of the suspension was diluted with 45 μl melted low melting point agarose and applied to a microscope slide. Slides were refrigerated to solidify the agarose, and then

incubated in comet assay lysis buffer at 4 °C for 45 minutes. The lysis buffer was then aspirated, replaced with electrophoresis buffer, and equilibrated in this buffer for 25 minutes. The cells are then placed in an electric field of 21 volts for 45 minutes. DNA is then precipitated using a precipitation buffer and incubated at room temperature for 25 minutes followed by aspiration and drying overnight. SYBR Green I solution (0.1 ml) was added to each slide for 30 minutes, and the solution was then gently removed and dried for 10–20 minutes. Slides were imaged with an Axiocamera instrument and analyzed with CometScore (TriTek, Wilmington, DE) software. Each treatment and slide was prepared in triplicate.

MTT Assay

UWB1.289 MTT assays follow similar cell culture and transfection procedures as the comet assay until day five. On day five, or 48 hours after the second transfection, 2000 cells of each treatment were counted and plated in a 96-well plate (~0.143 cm^2). Cells were cultured for 72 hours in a 96 well plate.

Primary ovarian cancer cells were counted and 1000 cells were plated per well of a 96-well tissue culture plate. 0, 5, 10, and 20 μM cisplatin and 0 and 75 nM TSA were applied to both platinum sensitive and platinum resistant primary ovarian cells.

Three days after the start of the MTT assay, 10 μl of 12 mM MTT stock solution (~10% of the well volume) was added to each well. The cells were incubated at 37 °C for four hours, after which cell membranes were disrupted with a 10% sodium dodecyl sulfate (SDS), 0.01 M hydrochloric acid (HCl) solution. After a final four-hour incubation at 37 °C, the plate is shaken to ensure mixing and the absorbance is measured at 600 nm. Each treatment was performed in triplicate.

Statistics

Error bars represent the standard error of mean. P-values represent two-sided student's t-tests unless otherwise indicated. Two-way ANOVA analysis was used to test for interaction between TSA and cisplatin treatments in chemotherapy sensitive and resistant primary ovarian tumor cells. ANOVA P-values are interaction P-values.

Results

HDAC10 is deleted in ovarian cancer and associated with sensitivity to cisplatin

We had found that HDAC9 and HDAC10 stimulate the repair of DNA double strand breaks (DSBs) by homologous recombination [11]. We utilized the Database of Genomic Variants (DGV) [22], to detect common deletions in the loci. The *HDAC10* gene is in the middle of a large multi-gene deletion that has been observed as heterozygous in 3 out of 443 normal individuals investigated [23] and in 34 cases in 6533 samples [24]. The HDAC10 locus on chromosome 22 is indicated with the deletions (Figure 1A). When looking at the incidence of mutations in the genes encoding these proteins in tumor samples, using The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/) and the web tool cBioPortal for visualization and analysis [25, 26], we found that *HDAC10* was deleted in a set of serous ovarian cancers (Figure 1B). We initially screened genetic changes to *HDAC10* across

multiple tumor types, including a large dataset for serous ovarian cancer. This ovarian dataset had two different gene copy analyses and indicated a high rate of *HDAC10* deletion. From a TCGA provisional dataset with 311 samples, 10% of the tumors had a deep deletion of the *HDAC10* gene. Deep deletion indicates that more than one allele is deleted, and if there are only two copies of the chromosome, then the locus would be homozygous deleted. A similar dataset analyzed in 2011 with 316 samples indicated about 5% of ovarian cancers with a deep deletion of *HDAC10*. Both gene copy analyses indicate ovarian tumors had the highest *HDAC10* deletion rates out of all the available cancer datasets. Certainly, the frequency of deletion of *HDAC10* was higher among ovarian cancers than observed in the general population using DGV. The dataset was also analyzed for loss of *BRCA1*. Although deep deletion of *BRCA1* was relatively rare, approximately 10% of the tumors had a nonsense *BRCA1* mutation. Two tumor samples had both an *HDAC10* deletion and *BRCA1* nonsense mutation.

The uncontrolled cell division of cancers makes DNA a prime target for disrupting the multiple processes needed to sustain the proliferation. Cisplatin is an interstrand DNA crosslinker, interfering with mitosis as well as initiating the apoptosis response of the DNA damage response pathway [27]. Since HDAC10 has been shown to be involved in DNA repair [11], the first characteristic we evaluated was platinum sensitivity. We hypothesized that patients who were deficient in HDAC10 would be more sensitive to platinum therapy. Sensitivity to platinum was known for a subset of ovarian cancers in the TCGA dataset. As shown in Figure 1C, all cancers that had deep deletions of *HDAC10* were sensitive to platinum therapy. 66.2% of shallow deletions and 63.6% of diploid or amplified *HDAC10* tumors were sensitive to platinum therapy. These results indicated the possibility that the loss of HDAC10 in tumors with deep deletions helps sensitize cells to platinum therapy, and we suggest that when HDAC10 is diploid or amplified other factors influence platinum sensitivity. However the sample size of the deep deletion patients was too small to evaluate statistical significance.

Data regarding *HDAC10* DNA copy numbers in cisplatin sensitive tumors were complemented by transcriptional analysis. The *HDAC10* mRNA levels correlated with platinum sensitivity in the patients. In the subset of tumors in which platinum status was available, 62 patients were resistant to platinum therapy, while 128 patients were sensitive to platinum therapy. The mean of *HDAC10* mRNA levels in the resistant samples was significantly higher than the sensitive samples (Figure 1D). Tumor data suggested that *HDAC10* copy loss or low expression were correlated with maintaining sensitivity to cisplatin. This result initiated our investigation of HDAC10 and HDAC inhibitor effects in a number of cell culture assays with an emphasis on ovarian cancer cells and platinum therapy.

HDAC inhibitors enhance the cytotoxicity of primary tumor cells to cisplatin

Since decreased activity of HDAC10 enhanced the sensitivity of cells to cisplatin, we tested whether generally functioning HDAC inhibition enhances cisplatin sensitivity in primary ovarian cancer cells. The primary cells were derived from ascites of patients with ovarian tumors. These cells can only be grown for three passages, making it impossible to use the siRNA transfection; instead, we were able to culture these cells in the presence of HDAC

inhibitors and cisplatin. We performed MTT proliferation assays in primary tissues cells, treating cells with various concentrations of cisplatin (0, 5, 10, and 20 μM) and the HDAC inhibitor, Trichostatin A [TSA; 0 nM (red), 75 nM (blue), and 150 nM (green)]. We analyzed three cell lines derived from cisplatin sensitive ovarian tumors, A-195, A2780CA, and OVCAR8 (Figure 2, left), and two cell lines derived from cisplatin resistant ovarian tumors, TR-127 and TR-182 (Figure 2, right); each analysis was done in triplicate. As shown in Figure 2, TSA enhanced the effect of cisplatin on cell proliferation in both sensitive and resistant primary cells. In the case of the TR-182 cells, the effect of the HDAC inhibitor was strongest at low concentration of cisplatin. The results in Figure 2 are consistent with a similar analysis of SAHA and paclitaxel in ovarian cancer cell lines [28].

HDAC inhibitors in combination with cisplatin inhibit DNA repair by homologous recombination

Cisplatin has multiple mechanisms of action through which it modulates its cytotoxic effect, including DNA crosslinking, and we tested whether cisplatin inhibited DNA repair by homology directed repair (HDR) and whether HDAC inhibition would enhance the effect cisplatin had on HDR. HDR activity was measured in HeLa cells, and we infer that effects of HDAC inhibitors in this assay would relate to ubiquitous function and not be specific to ovarian cells. We focused on inhibitors that are effective with HDAC10. Trichostatin A (TSA) and suberanilohydroxamic acid (SAHA) both inhibit HDAC10, though SAHA inhibits HDAC10 to a lesser degree than the other HDACs. There is currently no HDAC10 specific inhibitor [29]. Cisplatin, alone, inhibited homologous recombination in our HDR assay at concentrations as low as 100 nM cisplatin. The effect of cisplatin inhibition of HDR had been observed before [30]. Interestingly, the inhibition of HDR was at concentrations lower than those at which cisplatin is cytotoxic. Importantly, HDAC inhibition with TSA concentrations of 150 nM or 300 nM further reduced the HDR activity of these cells, even when already inhibited by cisplatin (Figure 3A). Similarly, when SAHA was included in the medium at 1.0 μM or 2.0 μM, the HDR activity was also reduced when in the presence of cisplatin (Figure 3B).

We have previously established that HDAC10 stimulates HDR in HeLa cells, consistent with the results of the effects of cisplatin and HDAC inhibition in the HDR assay. In the following experiment we tested whether HDAC10 was involved in DNA repair with ovarian cancer cells. To study this, we utilized a comet assay in an ovarian carcinoma cell line, UWB1.289. We exposed cells treated with HDAC10 siRNA to 4 Gy ionizing radiation, allowed four hours for recovery, and embedded treated cells in agarose with neutral pH buffer. When exposed to an electric field, DNA with double strand breaks (DSBs) electrophorese out and form a comet tail, which can be quantified for the level of unrepaired DNA. The tail moments detected in irradiated cells that had been transfected with either of two different non-overlapping siRNAs targeting HDAC10 were significantly larger than the tail moments from cells transfected with the control siRNA. The larger tail moment indicated more unrepaired DNA damage four hours post irradiation than in control transfected cells (Figure 4). The UWB1.289 cells are null for the *BRCA1* gene, and both BRCA1 and HDAC10 function in the repair of DSBs by homology directed repair [11]. Since both factors participate in the same pathway, it might be expected that depletion of

HDAC10 would be epistatic with the *BRCA1* deletion, but instead the DSB repair defect was worsened when HDAC10 was depleted. It is possible that this ovarian cancer cell line has partially compensated for the deficiency in BRCA1 by activating a bypass pathway, and HDAC10 inhibition caused the compensation to fail. UWB1.289 cells did not show significant DNA damage after ionizing radiation under control conditions despite being BRCA1 negative, while other cell lines that have BRCA1 deficiency exhibit significant DNA damage in a comet assay [31].

We hypothesized that the inhibition of HDAC10 would sensitize the UWB1.289 cells to the DNA damage and cytotoxic effects of platinum therapy. To test this hypothesis, we performed an MTT assay. MTT is a measure of metabolically active cells, which we use to measure proliferative activity. We transfected the cells with two rounds of siRNA specific for HDAC10 or control, and 48 hours after the second transfection, we incubated the cells in the presence of different concentrations of cisplatin. After three days of treatment with cisplatin at a range of concentrations from 0 to 80 μM, we performed the MTT assay. When treating UWB1.289 with cisplatin, cells transfected with HDAC10 siRNA had significantly lower proliferation as compared to cells transfected with control siRNA (Figure 5A) consistent with a decrease in abundance of the HDAC10 protein analyzed in these cells (Figure 5B). This result demonstrated the potential enhancement the inhibition of HDAC10 can have on cisplatin in BRCA1 deficient ovarian tumors.

Discussion

The standard front-line adjuvant therapy for ovarian cancer includes platinum-based chemotherapy [32]. Initial response is often promising, where 80% of patients have a response to platinum. Unfortunately, most patients relapse and go on to die of chemotherapy resistant disease [33]. As long as ovarian tumors are sensitive to cisplatin, progression of the cancer is arrested; thus any mechanism for maintaining cisplatin sensitivity of tumors is desirable. In this study we discovered: 1) serous ovarian cancers more commonly have *HDAC10* deletions than the general population; 2) in ovarian cancers homozygous deletion of *HDAC10* correlated with tumor sensitivity to cisplatin; 3) HDAC inhibitors potentiated the cytotoxicity of cisplatin in primary ovarian cancer cell lines derived from tumor ascites; 4) HDAC inhibitors potentiated the inhibition by cisplatin of DNA repair by homologous recombination; and 5) HDAC10 was required for DNA repair and survival in cells damaged by ionizing radiation or cisplatin treatment.

Our results indicated that HDAC inhibition of HDR could enhance the first line platinum therapy and thus could improve survival in patients with ovarian cancer. Several clinical trials are already underway of HDAC inhibitors (including SAHA) in combination with platinum-based chemotherapy in ovarian cancer [34]. Preclinical studies corroborate our finding of platinum sensitization in ovarian cancer cells using other ovarian cells lines when treated with SAHA [28]. We propose, based on our findings, that HDAC10 inhibition enhances the platinum sensitization of ovarian carcinoma cells. Strikingly, even in the ovarian cell line with *BRCA1* deleted, HDAC10 stimulated DSB repair.

A major issue with platinum therapy and ovarian cancer is relapse as a result of development of platinum resistance. One strategy to potentiate the effectiveness of platinum therapy is to use a combination therapy including HDAC inhibitors along with the platinum; however, such combination therapy is associated with significant drug toxicities [35, 36]. Since most HDAC inhibitors do not inhibit, or have only low levels of inhibition of HDAC10 [29], results from this study suggest that an HDAC10 isoform-specific inhibitor could improve the platinum sensitivity in ovarian carcinoma tumors and possibly with lower toxicity.

HDAC inhibitors have become an important class of drugs in the search for new therapies against cancer among other diseases. Several current clinical trials analyze HDAC inhibitors as anti-tumor agents [29]. Most of these inhibitors are broad spectrum inhibitors, affecting both class I and class II HDACs. Although many HDAC inhibitor studies are in progress, most of these are based on empirical data, and the exact mechanisms through which these agents are producing their cytotoxic effects are currently not well understood.

Valproic acid (VPA) has been used to treat neurological disease such as epilepsy for more than 30 years. However, VPA is specific to class I HDACs, and has no effect on the class II HDAC10 [29]. VPA had no effect in the HDR assay, supporting the concept that HDAC10 is the HDAC protein associated with the DNA repair activity [11]. VPA is currently in phase I and phase II clinical trials for leukemia and cervical cancer [37]. However, VPA does have neurological toxicity side effects, which limits its therapeutic window.

Another currently FDA approved HDAC inhibitor is SAHA, also known as vorinostat. Approved for the treatment of CTCL, SAHA is currently being studied in multiple phase I and phase II studies [29, 34]. SAHA is a broad spectrum HDAC inhibitor, affecting both class I and II HDACs. Unlike the other FDA approved HDAC inhibitors, it does inhibit HDAC10, albeit to a reduced effect compared to other HDACs [29]. Out of the current clinical trials, SAHA has potential to sensitize cancer cells to platinum therapy mediated by inhibition of HDAC10 [38, 39].

Although our analysis was limited to ovarian cancer cells, the DNA repair mechanisms through which HDAC10 potentially modulates cisplatin sensitivity could be present in other cell lines. A recent clinical trial demonstrated SAHA enhanced the efficacy of platinum therapy in patients with non-small-cell lung cancer [38, 39]. Similarly, HDAC inhibitor potentiation of platinum sensitivity is not mediated solely by HDAC10. This is evidenced by the multiple trials where HDAC inhibition is in combination with platinum therapy as well as preclinical studies demonstrating other HDACs mediating platinum sensitivity [37, 40].

HDAC inhibition has tremendous potential in the search for pharmacological solutions against cancer. HDAC inhibitors affect a large number of pathways and have already been demonstrated to improve patient outcomes in certain cancers. Although there is an ever-growing library of work that is analyzing the mechanisms through which HDAC and their inhibition modulate their effects, many of their functions are still unknown [37]. HDAC10 in particular is poorly understood, only recently being discovered to be involved in DNA repair [11]. Similarly, few of the current pharmacological solutions affect HDAC10 as strongly as other HDACs [29]. HDAC inhibitors are associated with significant side effects, but if an

inhibitor were developed that could target specifically HDAC10 and not HDAC1 or any of the ten other HDACs, then perhaps these significant side effects could be avoided. Our results provide evidence to explore HDAC10 specific inhibition as an adjuvant therapy to platinum therapies, especially for BRCA1-deficient ovarian carcinoma patients.

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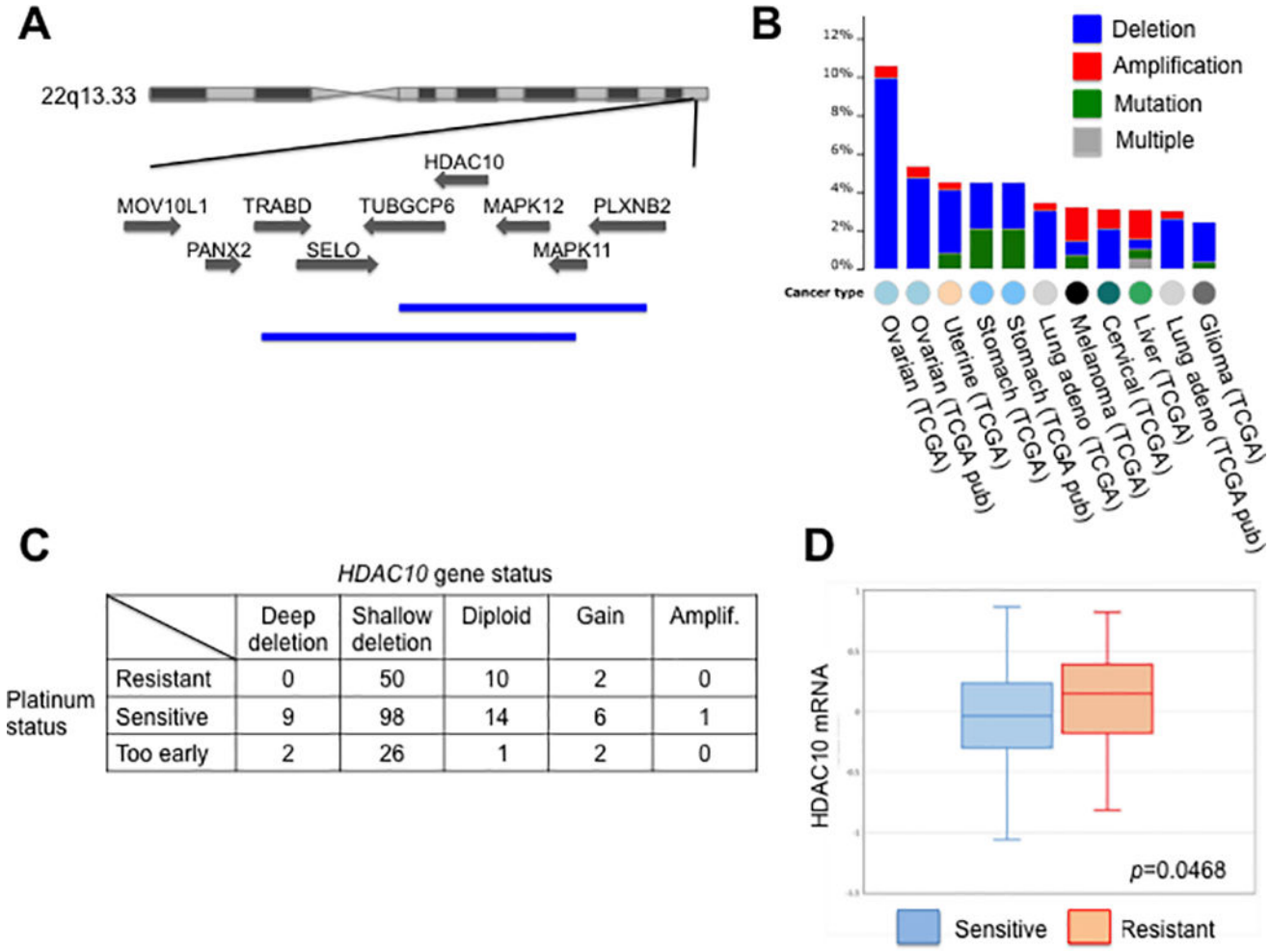

Figure 1. HDAC10 is deleted in many ovarian tumors, and loss of HDAC10 correlated with sensitivity to cisplatin

A. The chromosome 22 locus containing the *HDAC10* gene is shown, and deletions found as a common variant were shown in blue at the bottom. **B.** Frequency of HDAC10 alteration in tumor types is indicated. Data were taken from the TCGA database using software from CBioPortal. **C.** Some of the tumors in the TCGA ovarian cancer dataset were linked with information about cisplatin sensitivity of the tumor. The status of the *HDAC10* gene was indicated in columns. **D.** *HDAC10* mRNA abundance in tumor samples from cisplatin-sensitive tumors (blue) was compared to mRNA abundance in cisplatin-resistant tumors (red). The statistical test used was an unpaired student's t-test.

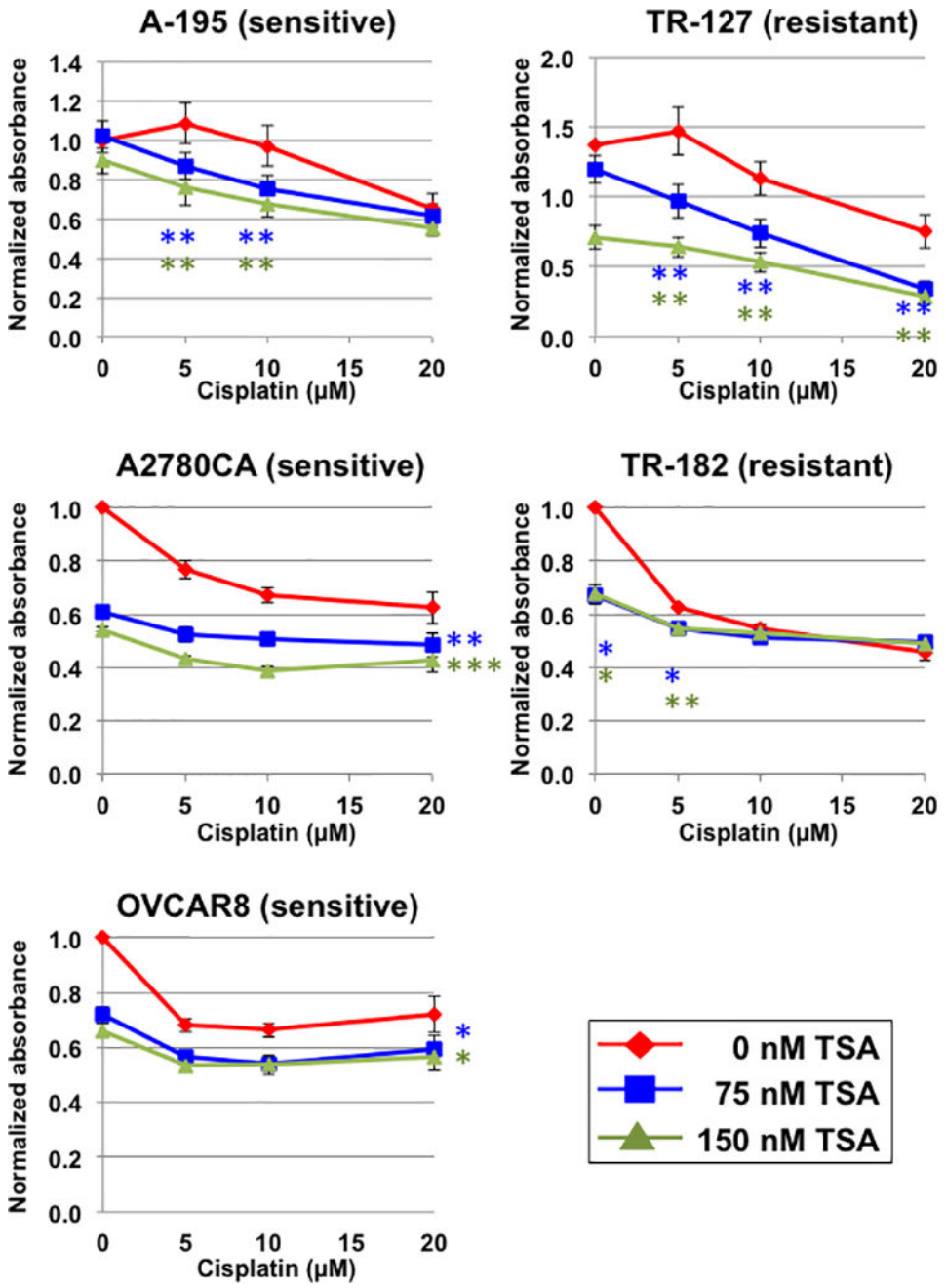

Figure 2. HDAC inhibitor increases the sensitivity to cisplatin of cells derived from primary ovarian tumors

Five primary cell lines derived from the ascites of ovarian tumors were cultured in the presence of cisplatin and the HDAC inhibitor TSA. A-195, A2780CA, and OVCAR8 cells were derived from cisplatin sensitive tumors, and TR-127 and TR-182 were derived from cisplatin resistant tumors. After 3 days, cells were analyzed for proliferation using the MTT assay. For each condition, analyzed in triplicate, cells were normalized to the MTT value of the cells without added cisplatin or TSA. For statistical analysis the unpaired student's t-test compared the effect of TSA at a given concentration of cisplatin; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. In the cases of A2780CA and OVCAR8, the statistical analysis was

similar for each concentration of cisplatin tested, and the asterisks are indicated to the right of the line.

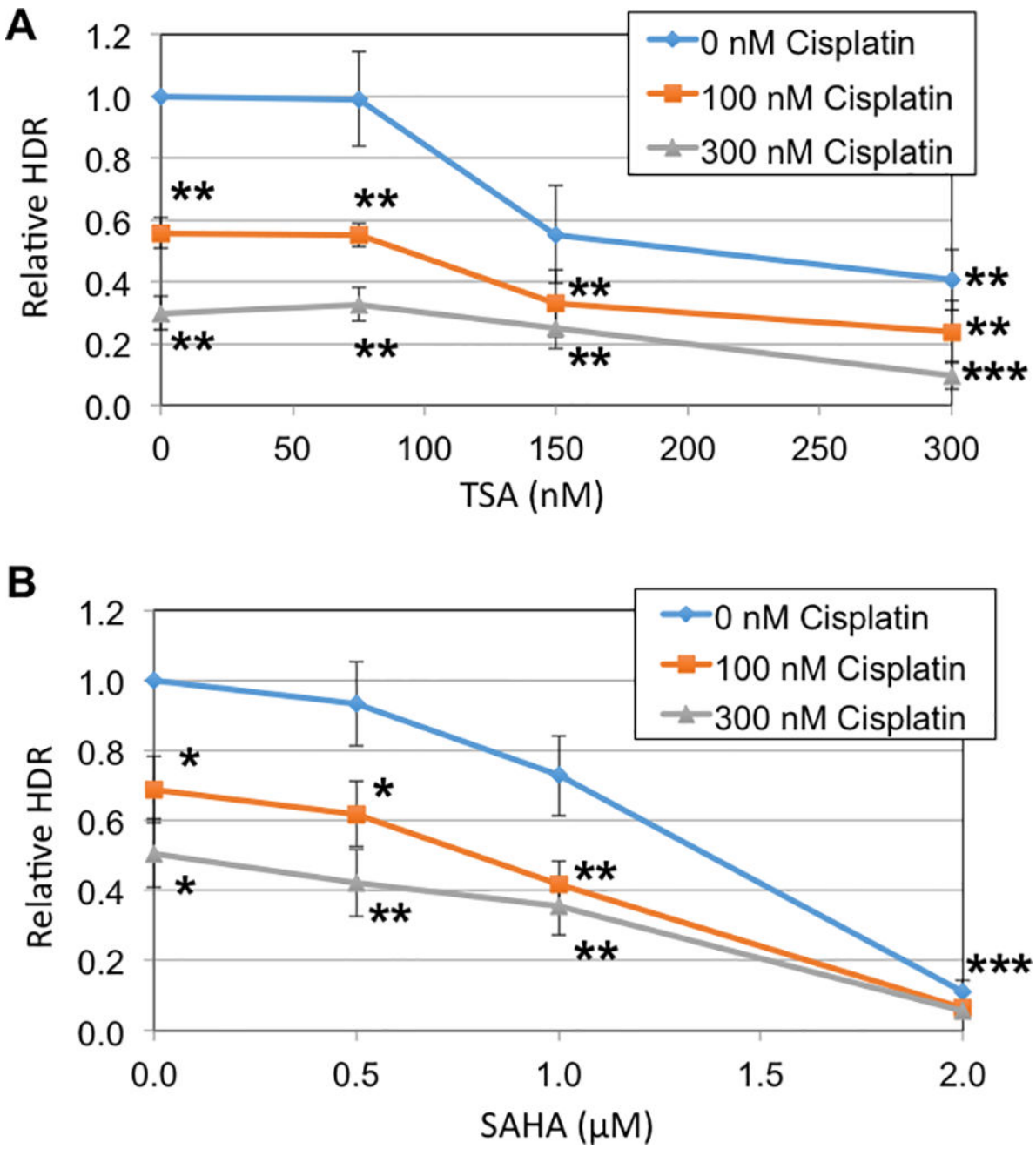

Figure 3. HDAC inhibitors, in combination with cisplatin, potently inhibit the homology directed repair of DNA double strand breaks

A. HeLa-DR cells, which have stably integrated a DNA for measuring HDR activity, were treated with cisplatin and TSA at the indicated concentrations. HDR activity was measured as the percentage of cells that convert to GFP positive, and results were normalized relative to the sample without drug added. Statistical analysis compared each data point with the indicated drug to the results in the absence of added drug; the student's two-tailed t-test was used, and asterisks represent *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **B.** HeLa-DR cells were tested as in panel A using the HDAC inhibitor SAHA. Statistical tests were done as in Panel A; for the results in the presence of 2.0 μM SAHA, all three curves had $p < 0.001$.

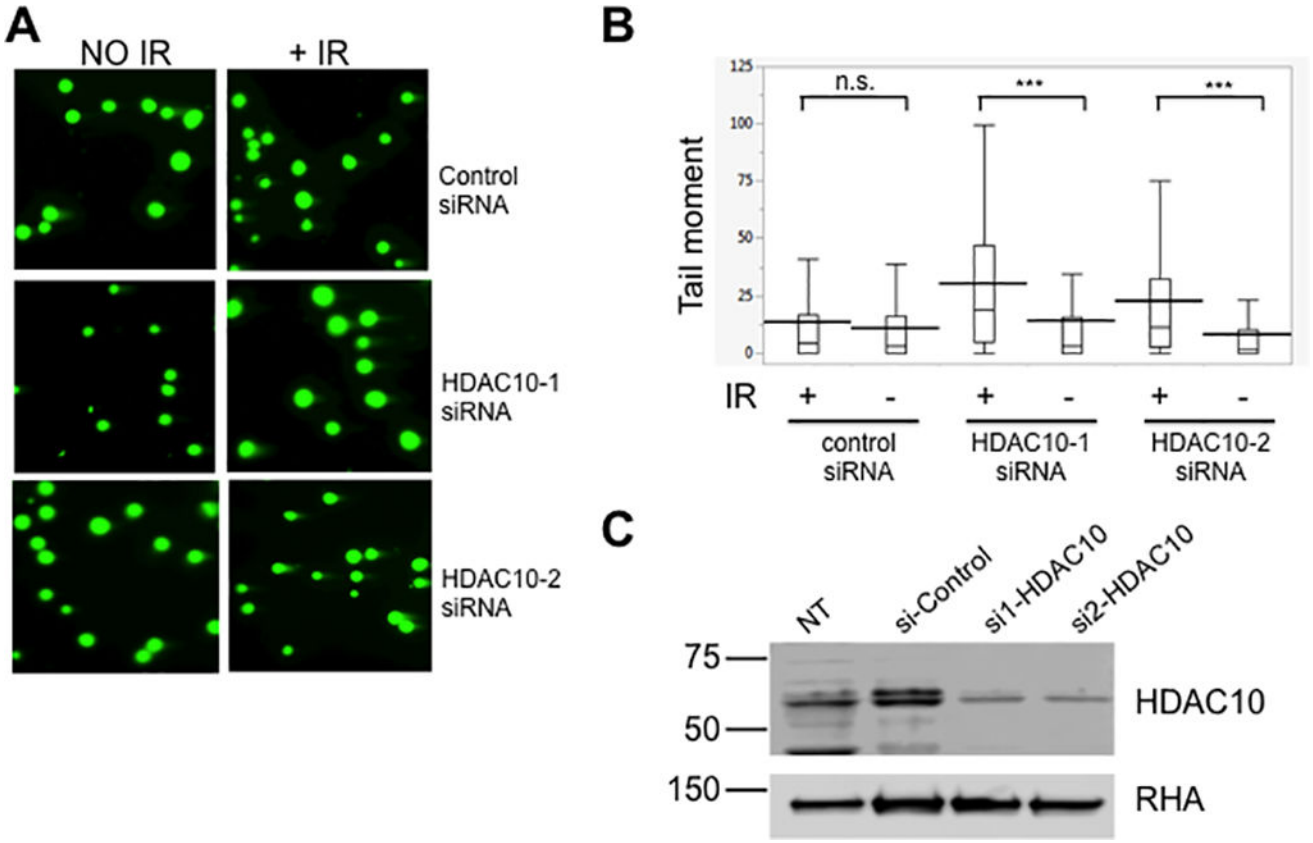

Figure 4. Depletion of HDAC10 from the UWB1.289 ovarian cancer cell line resulted in delay in repair of DSBs

A. UWB1.289 cells were transfected with two rounds of the indicated siRNA, and 48 h post transfection irradiate with 4 Gy of x-ray. After 4 h of recovery, cells were subjected to a comet assay in the presence of neutral agar. **B.** The amount of unrepaired DSBs in each sample from panel A was quantified by measuring the tail moment for each cell. Statistical analysis used the unpaired student's t-test; *** indicates $p < 0.0001$. **C.** Immunoblot analysis of the depletions of HDAC10 in samples used in panel A (top) and analysis of RNA Helicase A (RHA; bottom) to evaluate equal loading of the samples. Samples were whole cell extracts from non-transfected (NT), control siRNA (si-Control), HDAC10 specific siRNA-1 (si1-HDAC10), and HDAC10 specific siRNA-2 (si2-HDAC10) cells.

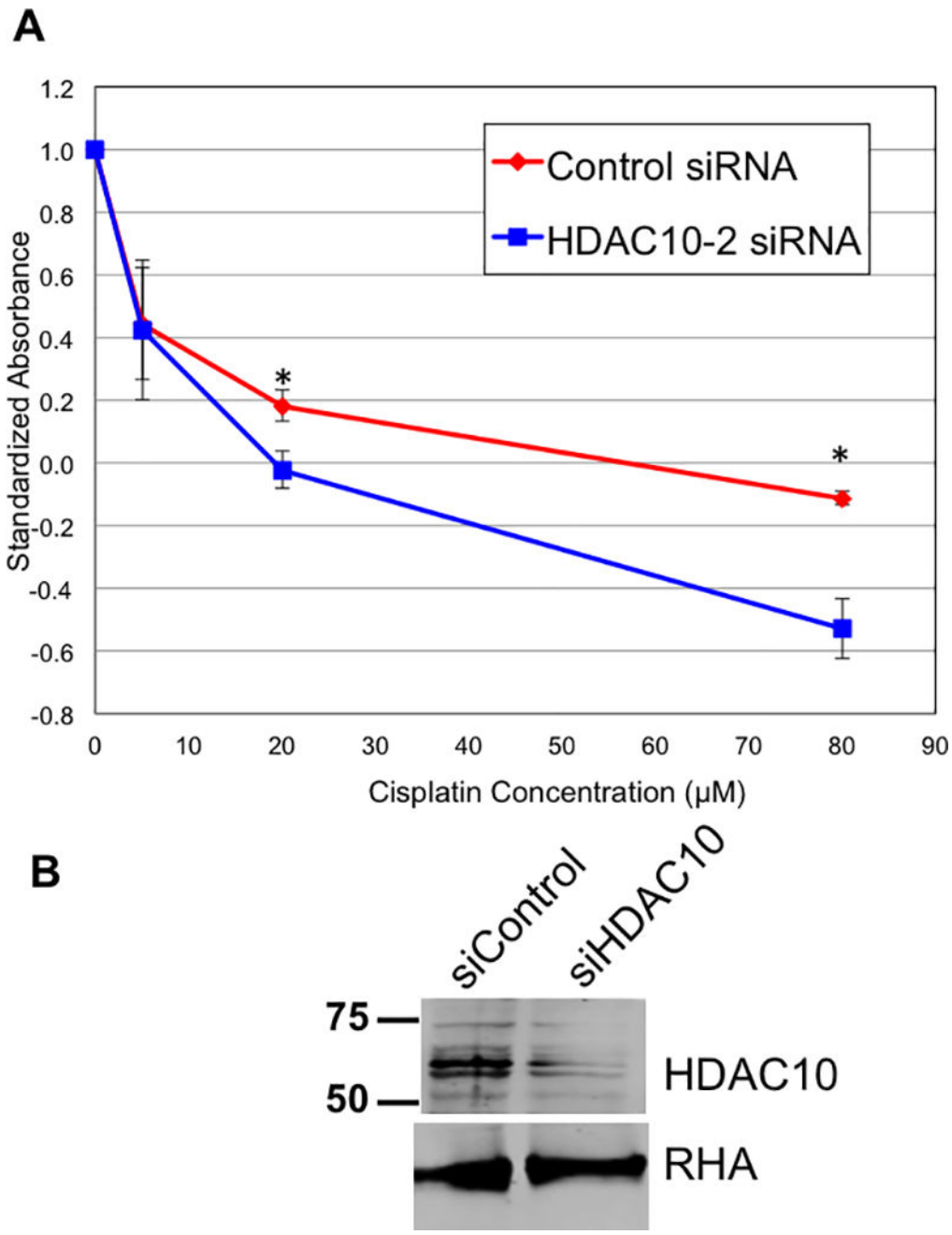

Figure 5. Depletion of HDAC10 potentiates cytotoxicity of cisplatin on UWB1.289 cells

A. UWB1.289 cells were subjected to two rounds of siRNA transfection with control siRNA (red) or HDAC10 specific siRNA (blue) and analyzed for the effect of cisplatin on proliferation using the MTT assay. Statistically different datapoints were determined by student's t-test and indicated by * ($p < 0.05$). **B.** Immunoblot analysis of the depletions of HDAC10 in samples used in panel A (top) and analysis of RNA Helicase A (RHA; bottom) to evaluate equal loading of the samples.

Table 1

siRNA sequences for HDAC10 study

Name	Sequence
GL2 sense	5’- CGU ACG CGG AAU ACU UCG ATT - 3’
GL2 antisense	5’- UCG AAG UAU UCC GCG UAC GTT - 3’
HDAC10-1 sense	5’ - CGG AGU CAG UGU GCA UGA CAG UAC A - 3’
HDAC10-1 antisense	5’- UGU ACU GUC AUG CAC ACU GAC UCC G - 3’
HDAC10-2 sense	5’- UCA CUG CAC UUG GGA AGC UCC UGU A - 3’
HDAC10-2 antisense	5’ - UAC AGG AGC UUC CCA AGU GCA GUG A - 3’

Sequences of siRNAs in study. dTdT are present on the 3’ end of every oligo.