

Inhibition of palmitoyltransferase ZDHHC12 sensitizes ovarian cancer cells to cisplatin through ROS-mediated mechanisms

Xining Zhang | Xingming Liao | Min Wang | Jiao Liu | Jiaxin Han | Dong An | Tiezheng Zheng | Xuefei Wang | Hailing Cheng  | Pixu Liu

Cancer Institute, Dalian Key Laboratory of Molecular Targeted Cancer Therapy, The Second Hospital of Dalian Medical University, Dalian Medical University, Dalian, China

Correspondence

Hailing Cheng and Pixu Liu, Cancer Institute, Dalian Key Laboratory of Molecular Targeted Cancer Therapy, The Second Hospital of Dalian Medical University, Dalian Medical University, No. 467 Zhongshan Road, Dalian, Liaoning 116023, China.
Email: hailingcheng_dmu@163.com and pixu_liu@163.com

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Abstract

Platinum-based therapies have revolutionized the treatment of high-grade serous ovarian cancer (HGSOC). However, high rates of disease recurrence and progression remain a major clinical concern. Impaired mitochondrial function and dysregulated reactive oxygen species (ROS), hallmarks of cancer, hold potential as therapeutic targets for selectively sensitizing cisplatin treatment. Here, we uncover an oncogenic role of the palmitoyltransferase ZDHHC12 in regulating mitochondrial function and ROS homeostasis in HGSOC cells. Analysis of The Cancer Genome Atlas (TCGA) ovarian cancer data revealed significantly elevated ZDHHC12 expression, demonstrating the strongest positive association with ROS pathways among all ZDHHC enzymes. Transcriptomic analysis of independent ovarian cancer datasets and the SNU119 cell model corroborated this association, highlighting a strong link between ZDHHC12 expression and signature pathways involving mitochondrial oxidative metabolism and ROS regulation. Knockdown of ZDHHC12 disrupted this association, leading to increased cellular complexity, ATP levels, mitochondrial activity, and both mitochondrial and cellular ROS. This dysregulation, achieved by the siRNA knockdown of ZDHHC12 or treatment with the general palmitoylation inhibitor 2BP or the fatty acid synthase inhibitor C75, significantly enhanced cisplatin cytotoxicity in 2D and 3D spheroid models of HGSOC through ROS-mediated mechanisms. Markedly, ZDHHC12 inhibition significantly augmented the anti-tumor activity of cisplatin in an ovarian cancer xenograft tumor model, as well as in an ascites-derived organoid line of platinum-resistant ovarian cancer. Our data suggest the potential of ZDHHC12 as a promising target to improve the outcome of HGSOCs in response to platinum-based chemotherapy.

KEY WORDS

cisplatin, HGSOC, mitochondrial ROS, protein palmitoylation, ZDHHC12

Abbreviations: 2BP, 2-bromopalmitate; 3D, three-dimensional; DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; ETC, electron transport chain; FASN, fatty acid synthase; H₂O₂, hydrogen peroxide; HGSOC, high-grade serous ovarian cancer; NAC, N-acetylcysteine; ROS, reactive oxygen species; TCGA, The Cancer Genome Atlas; ZDHHC12, zinc finger Asp-His-His-Cys-Type Containing 12.

The authors Xining Zhang and Xingming Liao contributed equally.

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1 | INTRODUCTION

High-grade serous ovarian cancer is the most common subtype of ovarian cancer, and patients with advanced diseases are associated with a poor prognosis.^{1–3} Platinum-based compounds, i.e., cisplatin or carboplatin, are first-line chemotherapeutic agents for solid tumors. While platinum-based therapies have significantly improved the survival of ovarian cancer patients, disease recurrence or development remains a major clinical concern.^{4–6} Therefore, there remains an unmet clinical need for the development of effective combinations to improve the response to platinum-based therapy in this disease.

Protein S-palmitoylation is one of the common posttranslational lipid modifications involving the addition of a 16-carbon fatty acid palmitate to proteins at cysteine residues via a thioester linkage mediated by protein S-acyltransferases.^{7,8} The family of protein S-acyltransferases have a conserved catalytic domain carrying a zinc finger, Asp-His-His-Cys (DHHC) motif and consists of 23 members (ZDHHC1 through ZDHHC24 without ZDHHC10).⁹ Protein palmitoylation is known to be involved in a variety of essential cellular processes by altering protein structures, translocation, stability, and function.^{10,11} Recent studies have suggested that several ZDHHC enzymes and their mediated protein palmitoylation are involved in tumorigenesis and influence treatment response.^{12–14}

Cisplatin exerts its cytotoxic effects primarily through inducing DNA damage. However, its efficacy is often hampered by the development of chemoresistance. Reactive oxygen species are emerging as critical players acting as a double-edged sword that can both enhance and impede cisplatin sensitivity.^{15,16} Cisplatin disrupts ROS homeostasis, leading to elevated intracellular ROS levels. Understanding how cancer cells deal with ROS stress may unlock specific vulnerabilities for targeted therapies.^{17,18} This could involve the development of therapeutic targets that selectively modulate ROS pathways in cancer cells while sparing healthy tissues. Conversely, studying the involvement of ROS in cisplatin-resistant cells can also reveal potential avenues for therapeutic intervention.^{19,20} Inhibiting antioxidant pathways or increasing ROS generation could effectively re-sensitize resistant tumors to cisplatin, thereby improving treatment efficacy. The present study reports that ZDHHC12 expression shows the strongest positive association with ROS pathways among all ZDHHC enzymes. Subsequent functional studies revealed an oncogenic role of ZDHHC12 in regulating mitochondrial function and ROS homeostasis. Importantly, our findings suggest that ZDHHC12 is a potential therapeutic target to enhance cisplatin efficacy via ROS-mediated mechanisms, providing a new direction to improve platinum-based chemotherapy in ovarian cancer.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

The HGSOC cell lines SNU119 and OVSAHO, purchased from Otwo Biotech, were maintained in culture medium supplemented with 10%

fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO₂. Cisplatin and C75 were purchased from MedChemExpress. 2-Bromopalmitate, H₂O₂ and N-acetylcysteine were purchased from Sigma. The siRNAs were custom synthesized from GenePharma. Lipofectamine 3000 was purchased from Invitrogen.

2.2 | RNA sequencing analysis

Total RNA was isolated from SNU119 cells transfected with siZDHHC12 or siNC using TRIzol Reagent (Invitrogen). RNA-Seq was performed by the Novogene Corporation. The sequencing libraries were created using the NEB Next® Ultra™ RNA Library Prep Kit for Illumina® (NEB) according to the manufacturer's instructions. Gene set enrichment analysis (GSEA) was performed to identify the molecular pathways correlated to ZDHHC12 knockdown in SNU119 cells using the *clusterProfiler* R package. In total, 1000 permutations were conducted, and gene sets with a false discovery rate (FDR) ≤0.05 and nominal *p*-values ≤0.01 were considered significantly enriched.

2.3 | Data analysis

The relative expression levels of ZDHHC12 (mRNA) across the pan-cancer were analyzed using TCGA datasets obtained from UCSC Xena (<https://xena.ucsc.edu>). The tumor-normal comparison was performed by comparing the tumor tissue data from TCGA datasets with the normal tissue data from the GTEx datasets. The high-throughput transcriptome data of TCGA ovarian cancer (OV) were downloaded using the R package TCGAbiolinks.²¹ GSE74357 and GSE26193 are published ovarian cancer data obtained from the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). HALLMARK gene sets were downloaded from the molecular signatures database (MSigDB), and signature scores were calculated using the R package GSVA.²² To perform GSEA,²³ Gene Ontology annotations and HALLMARK pathways were tested for (FDR <0.05) using the “gseGO” function and the “GSEA” function in the *clusterProfiler* R package, respectively. The correlation between gene expression levels and signature scores was calculated using the Pearson correlation coefficient. In the dot plot graphs, each dot indicates an individual sample.

Gene expression profiling data of cisplatin-sensitive and resistant OVCAR8 ovarian cancer spheroids were obtained from the GEO database (accession number GSE4553²⁴). Normalized values of gene expression levels were analyzed for differentially expressed genes using the LIMMA R package. Log₂FC>0.5 and FDR <0.05 were considered differentially expressed genes.

2.4 | Quantitative reverse transcription PCR

Total RNA was isolated using NucleoZOL (Macherey-Nagel) according to the manufacturer's instructions. Reverse transcription

reaction was performed using the Prime Script RT reagent kit (TaKaRa), and gene expression levels were analyzed using qPCR and a SYBR Green master mix on a QuantStudio 5 Real-Time PCR System (Applied Biosystems).

2.5 | Flow cytometric analysis

Mitochondria ROS levels were measured using the MitoSOX™ Red Mitochondrial Superoxide Indicator (Invitrogen) according to the manufacturer's protocol. Cellular ROS levels were measured using ROS Detection Reagents (Thermo Fisher) according to the manufacturer's protocol. Apoptosis assay was performed with an Annexin V-FITC Apoptosis Detection Kit (Dojindo) according to the manufacturer's instructions. Stained cells were analyzed on a NovoCyte Advanteon flow cytometer (Agilent).

2.6 | Measurement of mitochondrial mass

Mitochondrial mass was assessed with MitoTracker™ Red CM-H2Xros (Invitrogen) according to the manufacturer's protocol. Briefly, cells were incubated with MitoTracker for 30 min at 37°C followed by fluorescence microscopy (Leica) or analysis using a NovoCyte Advanteon flow cytometer (Agilent).

2.7 | Determination of relative ATP levels

Cells seeded in 96-well plates were counted and assessed using Cell Titer-Glo Reagent (Promega). The luminescence signal is proportional to the amount of ATP present in the cells. Results were analyzed by dividing the relative luminescence units (RLUs) by the number of cells.

2.8 | Clonogenic survival assays

Clonogenic survival assays were conducted as previously described.²⁵ At the endpoint, cells were fixed, stained with 0.5% crystal violet solution, and dissolved in 50% acetic acid. The optical density was measured at 570 nm using an xMark Microplate spectrophotometer (Bio-Rad).

2.9 | Live-cell confocal imaging of 3D spheroids

A 3D tumor spheroid culture assay was carried out as previously described.²⁶ Tumor spheroid structures were imaged using an inverted phase-contrast microscope (Leica) and scored for 3D structure integrity. Tumor spheroids stained with Hoechst 33342 and DCFDA or Mitotracker Red at 37°C were imaged using confocal microscopy (Leica TCS SP8).

2.10 | In vivo mouse xenograft study

In total, 5×10^6 SNU119 cells mixed with Matrigel were inoculated subcutaneously into 8-week-old female Balb/c nude mice. When tumor volumes reached approximately 90 mm^3 , mice were randomized and subjected to drug treatment. Cisplatin (3 mg/kg in PBS) was injected intraperitoneally every other day. siNC or siZDHHC12 (40 µg per tumor) was injected intratumorally every 3 days. The tumor volumes were measured with calipers and calculated according to the following formula: tumor volume = (length × width²) / 2.

2.11 | Organoid culture

An organoid line was established from malignant ascites of a moderately differentiated serous ovarian cancer patient who had developed resistance to treatment with carboplatin, cisplatin, and paclitaxel. Organoids were cultured as previously described.²⁷ Organoid cell viability was determined using CellTiter-Glo® 3D Reagent according to the manufacturer's instructions (Promega). Luminescence signals were measured using a SpectraMax microplate reader (Molecular Devices).

2.12 | Statistical analysis

The data obtained from in vitro and in vivo studies were analyzed statistically using an two-tailed unpaired Student's t-test, and one-way and two-way ANOVA with multiple comparisons tests using GraphPad Prism software. A p-value of <0.05 was considered statistically significant. n.s. not significant. *p<0.05, **p<0.01, ***p<0.001.

3 | RESULTS

3.1 | Correlation of ZDHHC12 expression with redox regulation and mitochondrial metabolism

Recent research highlights the critical role of ROS in cisplatin-mediated cytotoxic effects.¹⁶ By analyzing TCGA ovarian cancer data using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, we identified ZDHHC12 as the ZDHHC enzyme most significantly associated with ROS-related pathways (Figure 1A), suggesting its potential regulatory role in these processes. Notably, ZDHHC12 expression is significantly elevated in 12 cancer types, including ovarian cancer (Figure 1B). Further analysis using HALLMARK and Gene Ontology (GO) signature pathways revealed strong correlations between ZDHHC12 expression and pathways related to ROS regulation, oxidative phosphorylation, and mitochondrial metabolism in ovarian cancer (Figure 1C,D). Strikingly, these correlation patterns were consistent in two independent ovarian

cancer datasets (GSE74357²⁸ and GSE26193²⁹) (Figure 1E,F). Taken together, these findings implicate ZDHHC12 as a potential therapeutic target for modulating ROS and mitochondrial processes that may influence cisplatin sensitivity in ovarian cancer.

3.2 | Inhibition of ZDHHC12 expression sensitizes HGSOC cells to cisplatin treatment

Our investigation of the effect of ZDHHC12 on cisplatin sensitivity revealed that, among six nuclear-localized palmitoyltransferases (including ZDHHC5, ZDHHC8, ZDHHC12, ZDHHC15, ZDHHC16 and ZDHHC23^{30–32}), only knockdown of ZDHHC12 significantly enhanced cisplatin sensitivity in HGSOC cells (SNU119 and OVSAHO) (Figure 2A–C). Furthermore, the knockdown of ZDHHC12 in combination with cisplatin proved to be more effective than either alone, as evidenced using clonogenic survival assays and increased apoptosis (Figure 2D,E). Combined treatment with cisplatin and siZDHHC12 also induced massive disintegration of the tumor spheroids of SNU119 and OVSAHO cells (Figure 2F). These results demonstrate the therapeutic potential of ZDHHC12 inhibition in potentiating the cytotoxic effects of cisplatin in HGSOC.

To explore the clinical relevance of our findings, we investigated whether inhibition of ZDHHC12 could overcome cisplatin resistance. Gene expression analysis revealed significantly higher levels of ZDHHC12 in cisplatin-resistant OVCAR8 spheroids (Figure 2G), suggesting an association with cisplatin resistance. Furthermore, we evaluated the effect of ZDHHC12 on cisplatin response using an ascites-derived organoid line of cisplatin-resistant ovarian cancer as a preclinical model (Figure 2H). The combined use of siZDHHC12 and cisplatin, but not either alone, significantly reduced tumor organoid growth (Figure 2I). These data suggest that ZDHHC12 is a promising therapeutic target for overcoming cisplatin resistance.

3.3 | ZDHHC12 is involved in the regulation of mitochondrial metabolism and redox homeostasis in ovarian cancer cells

We further asked whether ZDHHC12 is involved in the modulation of mitochondrial activity in ovarian cancer. We found that the knockdown of ZDHHC12 resulted in pronouncedly increased internal complexity of SNU119 and OVASHO cells using flow cytometry analysis

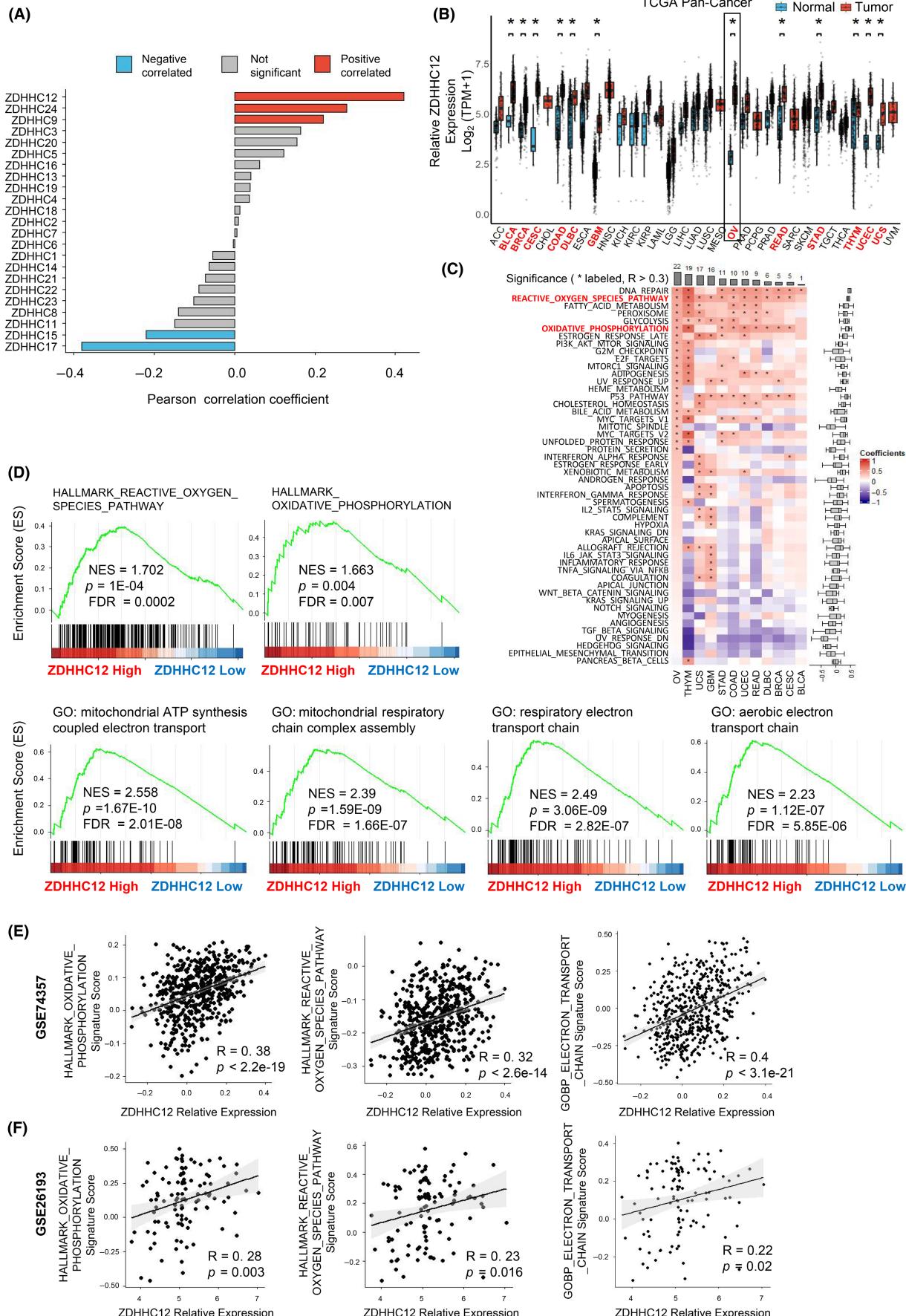
(Figure 3A), indicating increased organelles and metabolic functions within the cytoplasm. As the knockdown of ZDHHC12 also resulted in increased ATP levels (Figure 3B), we speculated that ZDHHC12 may be involved in the regulation of mitochondrial function for ATP production. Indeed, inhibition of ZDHHC12 resulted in increased MitoTracker staining as determined using immunofluorescence and flow cytometric analysis, indicating increased mitochondrial activity (Figure 3C,D). Confocal microscopy analysis revealed higher intensity MitoTracker staining in 3D spheroids of siZDHHC12-transfected cells than in those of control cells, further supporting a potential functional link between ZDHHC12 and the regulation of mitochondrial function (Figure 3E). Taken together, these data indicate that inhibition of ZDHHC12 may increase mitochondrial mass and promote mitochondrial function for ATP synthesis.

To gain insight into the mechanism underlying the high expression levels of ZDHHC12 in ovarian cancer, we performed RNA sequencing analysis to assess the effect of siRNA-mediated ZDHHC12 knockdown on specific transcriptional changes in SNU119 cells. Significant transcriptional changes were observed compared with the control cells (Figure 4A). GSEA identified a significant association of ZDHHC12 expression with pathways involved in oxidative phosphorylation and mitochondrial function (Figure 4B), mirroring findings from independent ovarian cancer datasets (Figure 1D–F). Consistent with these pathways relying on mitochondrial activity for ROS generation, ZDHHC12 knockdown significantly increased basal mitochondrial and cellular ROS levels in SNU119 and OVSAHO cells as determined using flow cytometry (Figure 4C,D). Notably, ZDHHC12 knockdown further exacerbated H₂O₂-induced ROS elevation, which was reversed with the ROS scavenger NAC. These findings suggest that ZDHHC12 plays a role in redox regulation in ovarian cancer, highlighting its potential as a therapeutic target to regulate redox balance and combat the disease.

3.4 | Inhibition of ZDHHC12 enhanced cisplatin sensitivity through ROS-mediated mechanisms

Given the association of ZDHHC12 with mitochondrial function, we hypothesized that its inhibition could potentially enhance cisplatin sensitivity through ROS regulation. Combination treatment with siZDHHC12 and cisplatin significantly increased ROS levels, while subsequent treatment with the ROS scavenger NAC reversed these effects on ROS production (Figure 5A), cell viability (Figure 5B), and apoptosis

FIGURE 1 Correlation analysis of ZDHHC12 expression with signature pathways. (A) Correlation analysis between the expression levels of all ZDHHCs and the HALLMARK ROS pathway in ovarian cancer from TCGA (TCGA-OV). Pearson correlation coefficient >0.2 and p -value <0.01 is considered a significant positive correlation (as shown in red). Pearson correlation coefficient <-0.2 and p -value <0.01 is considered a significant negative correlation (as shown in blue). (B) Patterns of ZDHHC12 expression levels between tumor and normal tissues. (C) Correlation heatmap showing the correlation between ZDHHC12 and HALLMARK signature scores across 12 tumor types with ZDHHC12 expression levels significantly higher than normal tissues. Correlation values (R) and p -values are shown. (D) Enrichment plots for HALLMARK or GO signature pathways associated with ZDHHC12 expression using ovarian cancer datasets from TCGA ($n=374$). NES (normalized enrichment score), p -value and false discovery rate (FDR) value of the correlation are shown. (E,F) Correlation analysis (Pearson's test) between ZDHHC12 and HALLMARK or GO signature scores using ovarian cancer datasets from GSE 74357 (E, $n=529$) and GSE 26193 (F, $n=107$). Each dot represents an individual sample of human ovarian cancer.



in SNU119 and OVASHO cells (Figure 5C), indicating a ROS-dependent mechanism. Confocal microscopy in 3D spheroids further corroborated these findings, revealing a much stronger ROS signal upon combined treatment compared with single-agent exposures (Figure 5D). These data provide compelling evidence that inhibition of ZDHHC12 enhances the cytotoxic effects of cisplatin in HGSOC cells by potentiating cisplatin's cytotoxicity through ROS-mediated mechanisms.

3.5 | Inhibition of protein palmitoylation sensitized HGSOC cells to cisplatin treatment

Next, we validated the role of ZDHHC12-mediated protein palmitoylation in cisplatin response. The broad-spectrum protein palmitoylation inhibitor 2BP has been widely used to validate the biochemical and biological effects of ZDHHC protein inhibition in preclinical studies.^{11,33} In addition, FASN is known to be required for de novo palmitate synthesis, and thus the inhibition of FASN would block de novo protein palmitoylation.^{34,35} We used 2BP and C75 to potentially mimic ZDHHC12 inhibition. Indeed, treatment with 2BP, and to a greater extent with C75, significantly enhanced the cytotoxic effect of cisplatin in the 2D culture and 3D spheroid model of HGSOC cells (SNU119 and OVSAHO) (Figure 6A,B). Notably, the enhanced cytotoxicity induced by the combination treatment was correlated with a significant increase in apoptosis (Figure 6C). Furthermore, while the combined use of 2BP or C75 with cisplatin resulted in a marked ROS accumulation (Figure 6D), treatment with the ROS scavenger NAC significantly reduced ROS levels and abrogated the sensitivity of HGSOC cells to the drug combination, as evidenced using restored cell viability and decreased apoptosis (Figure 6E,F). Taken together, these results suggest that inhibition of protein palmitoylation or blockade of de novo palmitate synthesis sensitizes HGSOC cells to cisplatin through ROS-mediated mechanisms.

3.6 | Inhibition of ZDHHC12 enhanced cisplatin response in vivo

To validate the therapeutic potential of ZDHHC12 inhibition in vivo, we used a xenograft mouse model of SNU119 ovarian cancer cells

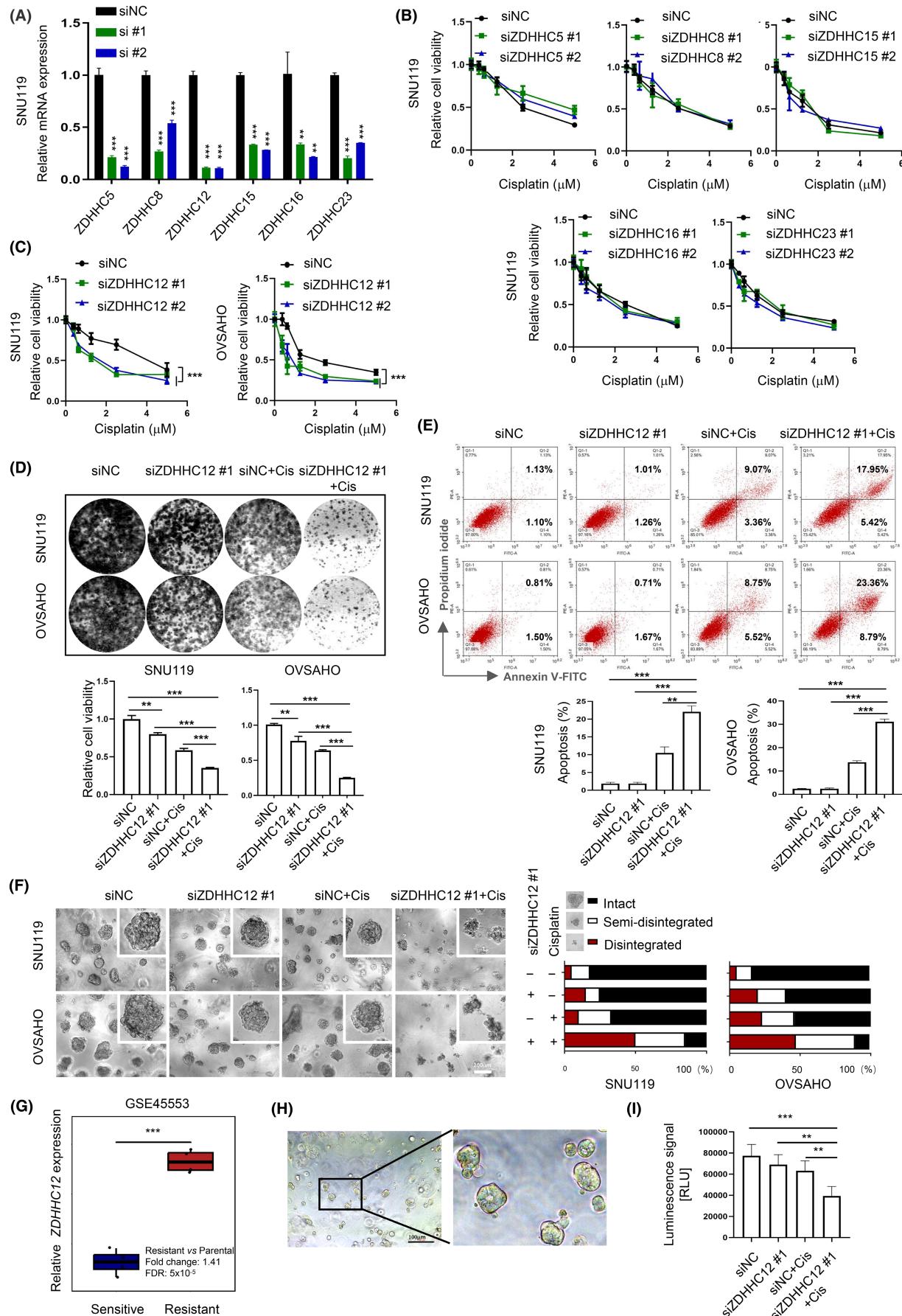
for preclinical evaluation of cisplatin response. Strikingly, while single-agent treatment with siZDHHC12 or cisplatin showed limited tumor growth reduction, the combination treatment significantly potentiated the efficacy of cisplatin (Figure 7A,B). ZDHHC12 knockdown was confirmed in siZDHHC12-treated tumors (Figure 7C). Consistent with the cytotoxic effect, the combination treatment resulted in prominent induction of cleaved poly-ADP-ribose polymerase (PARP) and γH2AX in tumors, consistent with oxidative stress-induced cytotoxicity (Figure 7D). These data suggest the potential of inhibition of ZDHHC12 as a therapeutic strategy to significantly enhance the cytotoxic effects of cisplatin *in vivo*.

4 | DISCUSSION

Our study reveals ZDHHC12 as a novel regulator of mitochondrial function and ROS homeostasis, playing an uncharacterized oncogenic role in HGSOC cells. Importantly, we used established HGSOC cell line models, SNU119 and OVASHO, chosen for their genomic profiling closely resembling clinical ovarian tumors.³⁶ Markedly, inhibition of ZDHHC12 sensitized ovarian cancer cells to cisplatin through ROS-mediated mechanisms, offering a promising therapeutic vulnerability. These findings pave the way for further investigation of ZDHHC12 as a therapeutic target for enhancing cisplatin response and improving clinical outcomes in ovarian cancer.

Recent studies have reported that several ZDHHC enzymes are critical for mitochondrial oxidative phosphorylation and ROS homeostasis.^{37–39} S-Palmitoylation of substrate proteins mediated by ZDHHC13 has been implicated in liver mitochondrial function and metabolism.³⁷ ZDHHC3-mediated palmitoylation regulates cellular oxidative stress and senescence in breast cancer.³⁸ ZDHHC21 regulates oxidative phosphorylation hyperactivity to induce differentiation block and stemness in acute myeloid leukemia cells.³⁹ It has been documented that high expression of ZDHHC12 is associated with poor prognosis in glioma and promotes malignant cell growth,⁴⁰ and that ZDHHC12-mediated S-palmitoylation of Claudin-3 promotes ovarian cancer progression.⁴¹ While previous studies have identified an oncogenic role for ZDHHC12, its potential involvement in the regulation of mitochondrial activity has not been reported until the present study. Our work uncovers ZDHHC12 as a regulator

FIGURE 2 Knockdown of ZDHHC12 increased the cytotoxic effects of cisplatin in HGSOC cells. (A) Quantitative RT-PCR analysis of mRNA expression levels in SNU119 cells transfected with specific siRNAs as indicated. (B) SNU119 cells transfected with specific siRNA as indicated were treated with cisplatin for 5 days and subjected to cell viability assay. (C) siZDHHC12 or siNC-transfected HGSOC cells were treated with or without cisplatin for 5 days, followed by cell viability assay. mean ± SD for three independent experiments is shown (two-way ANOVA with Tukey's multiple comparisons). (D) Clonogenic survival was measured in the indicated cells treated with or without 2 μM cisplatin. (E) Apoptosis levels were determined in the indicated cells. (F) 3D spheroid assay of the indicated cells treated with or without cisplatin. Representative images and quantification of scored structures (intact, semi-disintegrated and disintegrated) are shown. (A,D,E) Data are shown as mean ± SD for three independent experiments (Student's *t*-test). (G) The expression level of ZDHHC12 in cisplatin-sensitive and cisplatin-resistant 3D spheroids of OVCAR8. Data were obtained from GEO with accession number GSE45553.²⁴ Each point represents a biological repeat (*n*=4). Data are shown as the mean ± SD, *** *p*<0.0001 (Student's *t*-test). (H) Representative phase-contrast image of an ascites-derived cisplatin-resistant organoid line of ovarian cancer. (I) ATP levels (luminescence signal) were determined for organoids treated as indicated. Data are shown as mean ± SD (one-way ANOVA with multiple comparisons test).



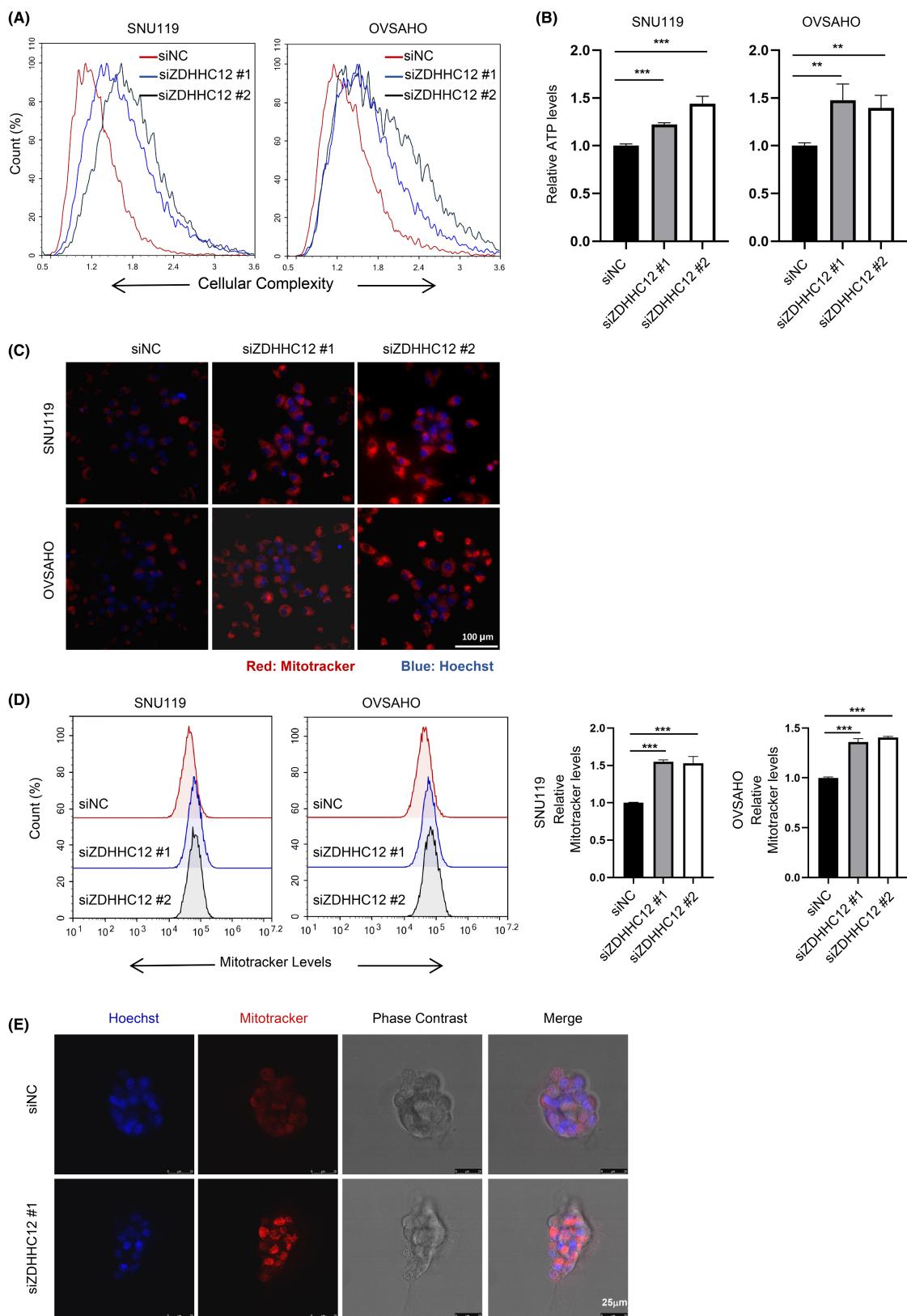


FIGURE 3 The expression of ZDHHC12 is correlated with mitochondrial and cellular ROS levels. (A) Representative flow cytometry histogram displaying cellular complexity on the x axis for the indicated cells. (B) Relative ATP levels in the indicated cells. (C) Fluorescence imaging of mitochondrial mass in the indicated cells. (D) Flow cytometry histograms showing comparison of MitoTracker Red fluorescence of the indicated cells. (E) 3D spheroids of the indicated cells were stained with MitoTracker (red) for mitochondria and Hoechst 33342 (blue) for nuclei. Images were captured using confocal microscopy. (B,D) Data are shown as the mean \pm SD for three independent experiments (Student's t-test).

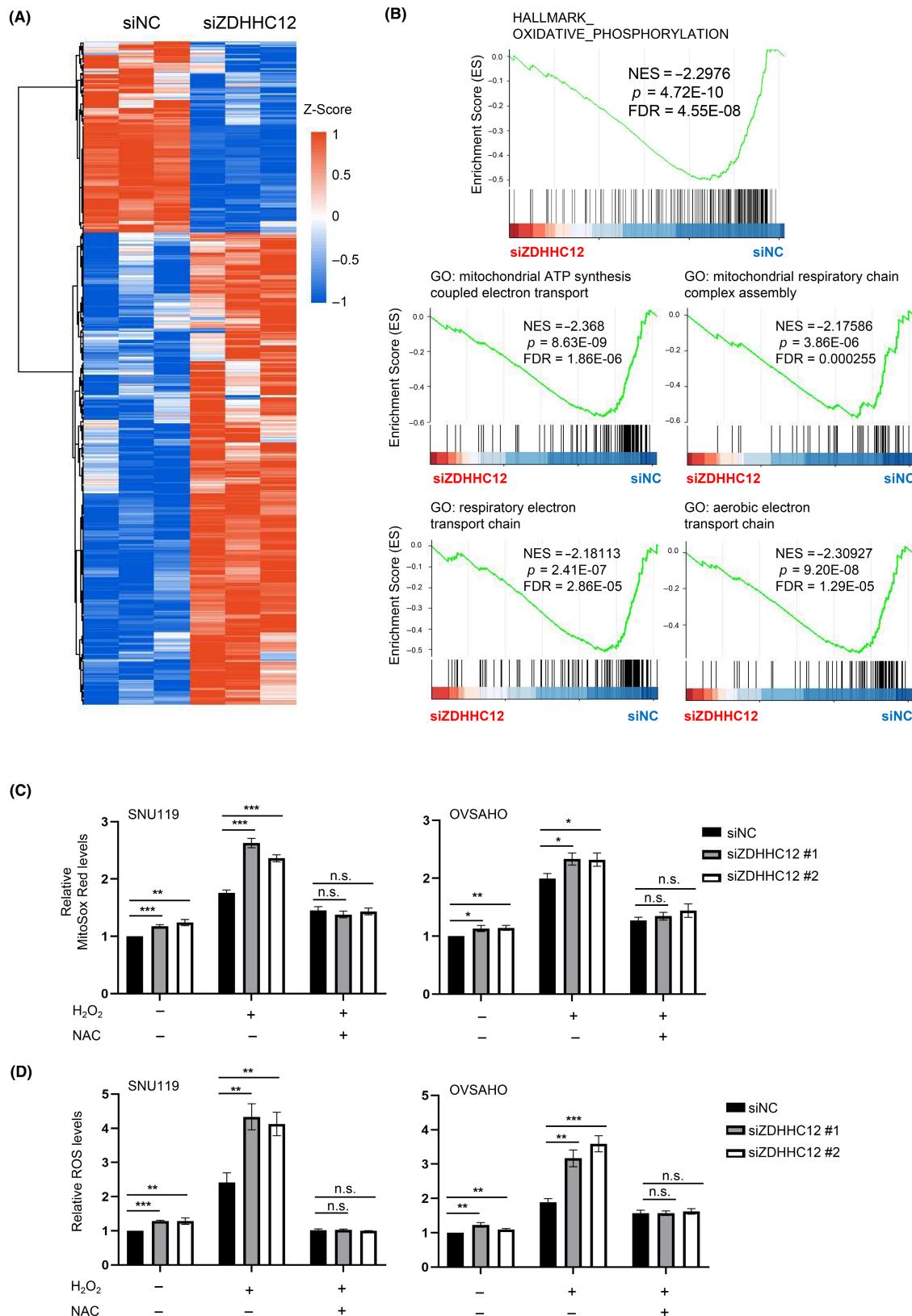


FIGURE 4 Transcriptome sequencing analysis in ZDHHC12 knockdown SNU119 cells. (A) Changes in the top 200 differentially expressed genes in SNU119 cells transfected with siZDHHC12 or siNC for 72 h. Gene upregulation is in red and gene downregulation is in blue. (B) Enrichment plots for HALLMARK or GO signature pathways associated with ZDHHC12 expression in SNU119 cells. (C) SNU119 and OVSAGO cells transfected with siZDHHC12 or siNC were pretreated with or without NAC followed by incubation with H_2O_2 . MitoSOX Red was used to assay for mitochondrial ROS levels by FACS. (D) DCFDA was used to assay for cellular ROS levels by FACS. (C,D) Data are shown as the mean \pm SD for three independent experiments (Student's t-test).

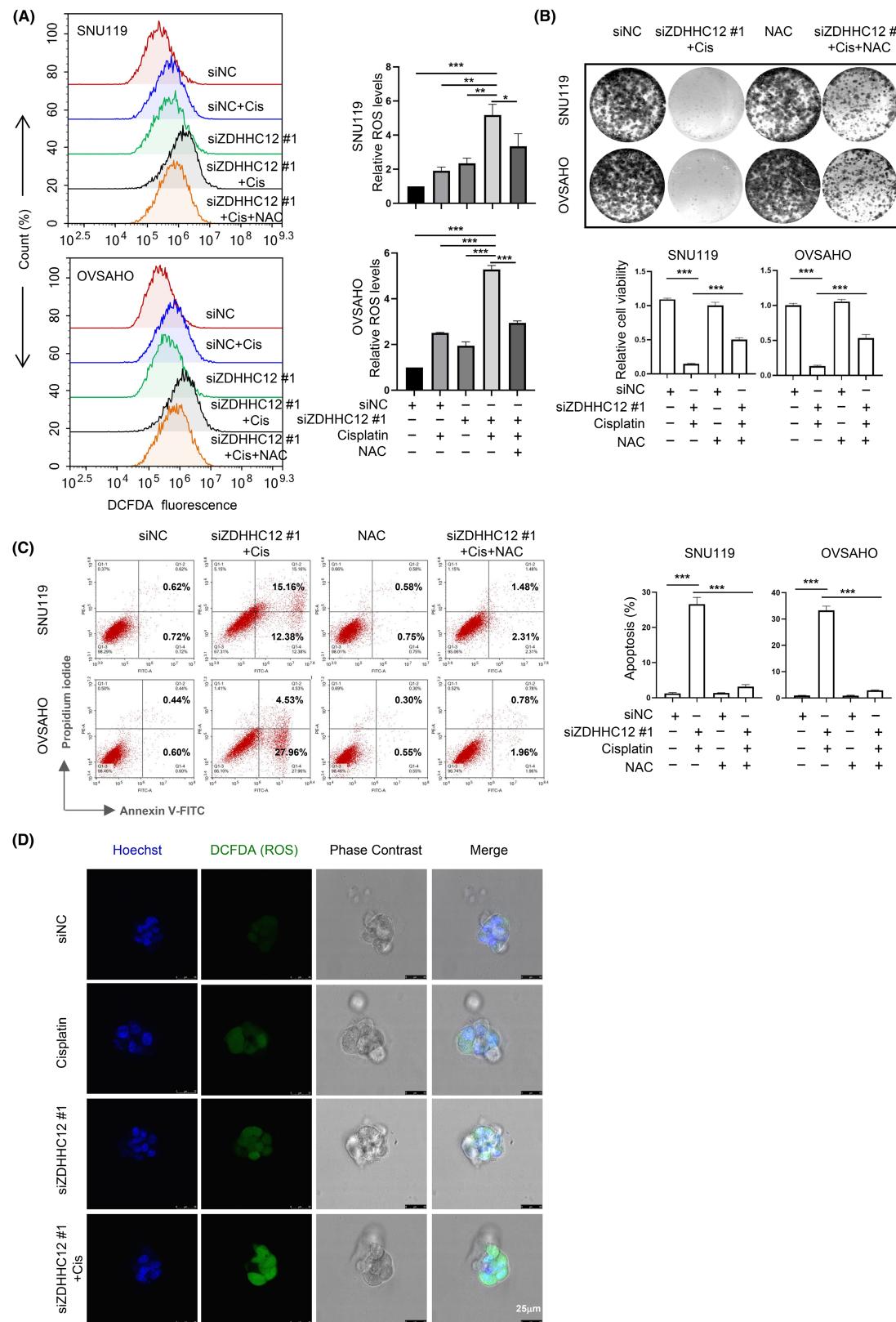


FIGURE 5 Treatment with N-acetylcysteine reversed the cytotoxic effects of siZDHHC12 on cisplatin sensitivity in HGSOC cells. (A) Flow cytometric analysis of ROS levels in SNU119 and OVSAHO cells treated as indicated for 72 h. Cisplatin, 5 μ M cisplatin; NAC, 1.25 mM NAC. (B) Clonogenic survival was measured using crystal violet assay in the indicated cells. (C) Apoptosis levels were determined in the indicated cells. (A–C) Data are shown as mean \pm SD for three independent experiments (Student's *t*-test). (D) Confocal images of 3D spheroids of SNU119 cells treated as indicated for 3 days. ROS was measured by staining with DCFDA (green) and nuclei were stained with Hoechst 33342 (blue).

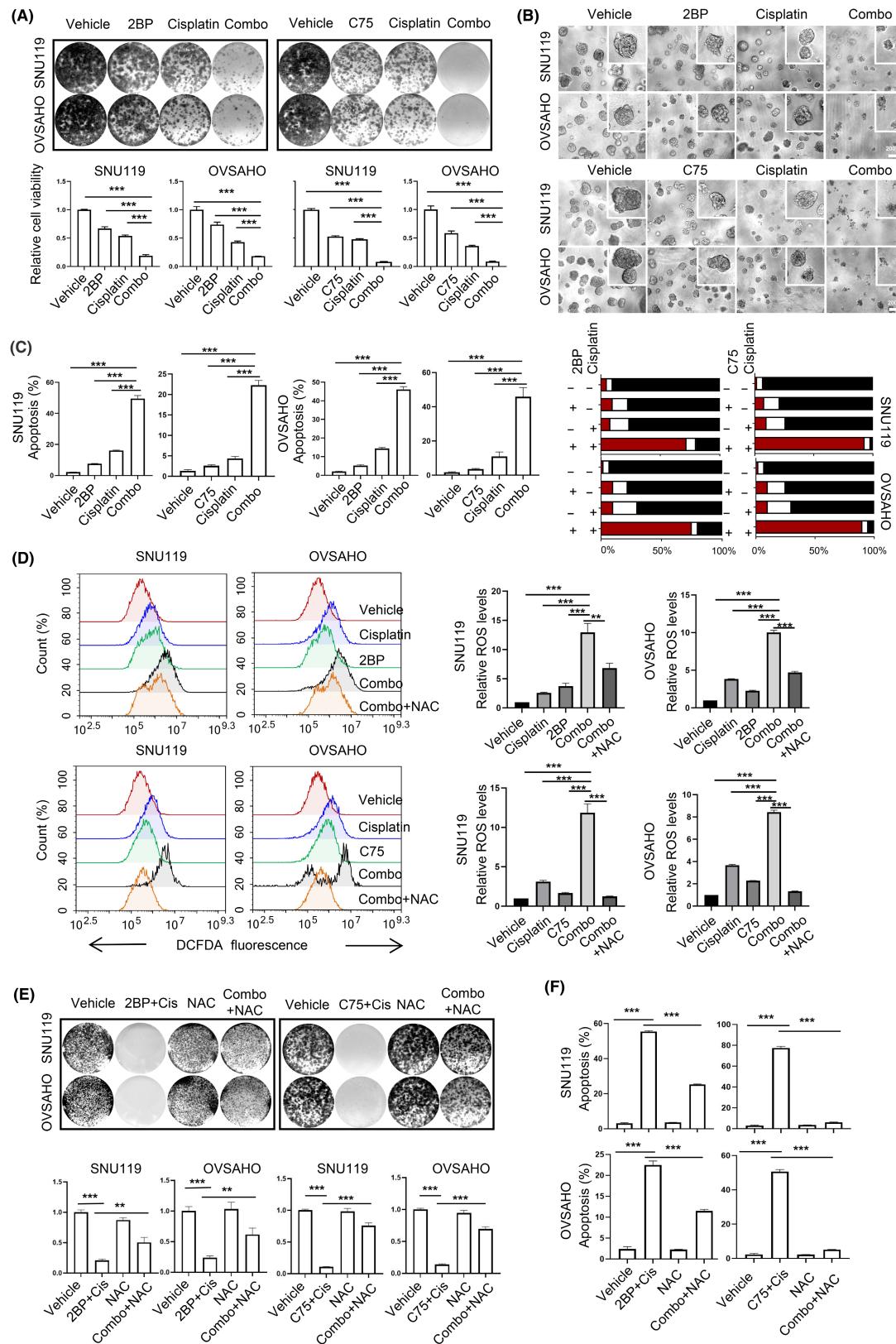


FIGURE 6 Treatment with 2BP or C75 increased the cytotoxic effects of cisplatin through ROS-mediated mechanisms. (A) Clonogenic survival was measured using crystal violet assay in SNU119 and OVSAHO cells treated as indicated. 2BP, 30 μ M; C75, 30 μ M; cisplatin, 3 μ M. (B) SNU119 and OVSAHO cells cultured in 3D Matrigel were treated as indicated. Apoptosis levels (C) and ROS levels (D) were determined in SNU119 and OVSAHO cells treated as indicated for 72 h. (E) Clonogenic survival was measured using crystal violet assay in SNU119 and OVSAHO cells treated as indicated. (F) Apoptosis levels were determined in SNU119 and OVSAHO cells treated as indicated for 72 h. (A–F) Data are shown as mean \pm SD for three independent experiments (Student's *t*-test).

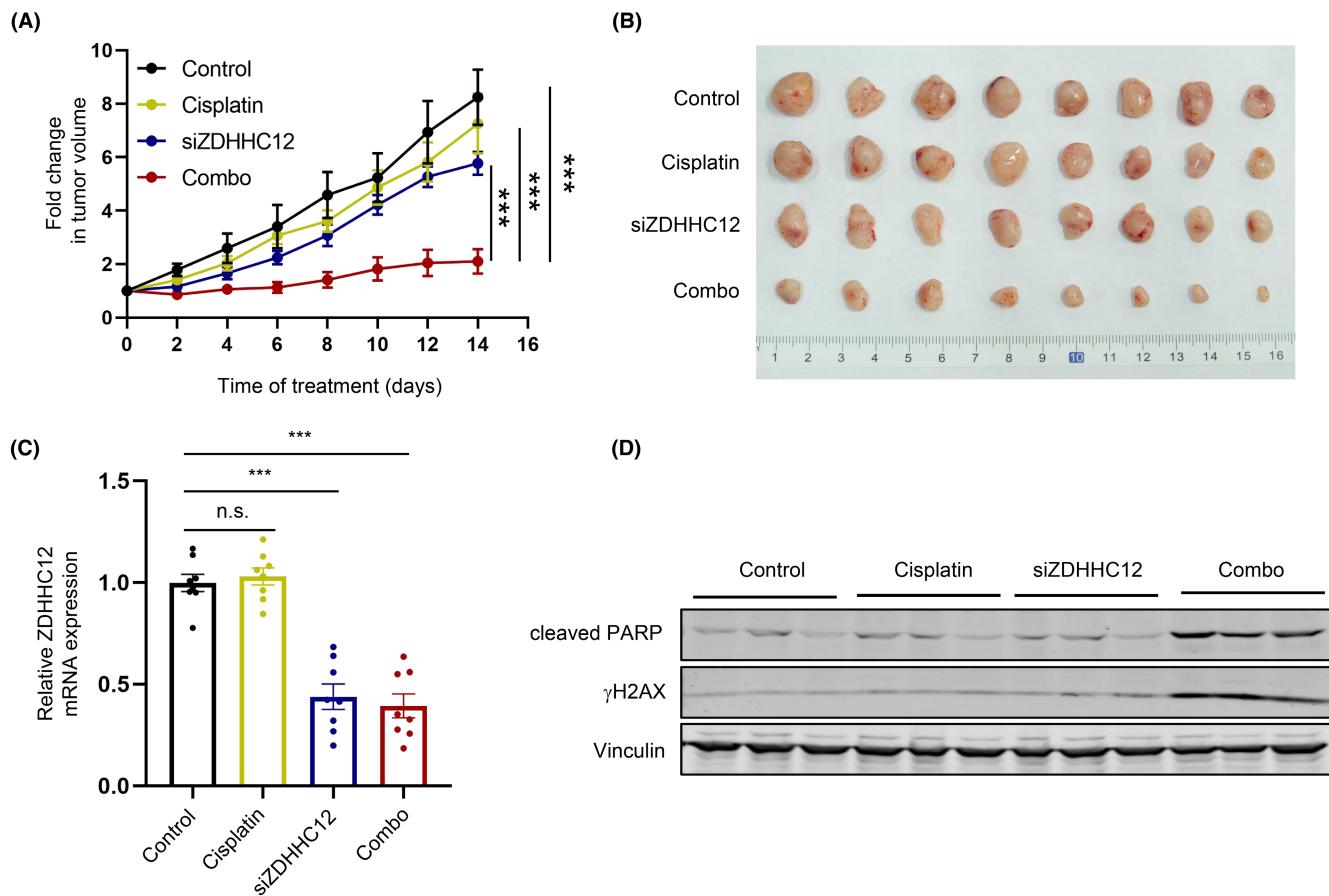


FIGURE 7 The combination of cisplatin and siZDHC12 is effective in vivo. (A) Tumor growth curve of SNU119-xenografted mice treated with siZDHC12 #1 and cisplatin either alone or in combination. The graph shows the fold change in tumor volume, with respect to the initial treatment at day 0. Control, Vehicle + siNC; siZDHC12, Vehicle + siZDHC12; cisplatin, cisplatin + siNC; Combo, cisplatin + siZDHC12. Data are shown as the mean \pm SEM ($n=8$ per treatment group). *** $p < 0.0001$ using a two-way ANOVA, with Turkey's multiple comparison tests. (B) The SNU119 xenograft tumor-bearing mice were treated as in (A). Representative gross images of SNU119 xenografted tumors isolated from mice in different treatment groups. (C) qRT-PCR analysis of ZDHHC12 mRNA expression in tumors. Data are shown as the mean \pm SEM ($n=8$ per treatment group). n.s. not significant, *** $p < 0.001$ (one-way ANOVA followed by a multiple comparisons test). (D) Western blot analysis of cleaved PARP and γ H2AX in tumors. Vinculin was used as a loading control.

of mitochondrial activity and ROS homeostasis in HGSOCs, a previously uncharacterized role for this protein. Inhibition of ZDHHC12 disrupts mitochondrial function and altered ROS levels as evidenced by increased cellular complexity, ATP levels, mitochondrial content and ROS accumulation. Future studies exploring the downstream targets of ZDHHC12 palmitoylation and their impact on mitochondrial bioenergetics hold great promise for the development of novel strategies against this aggressive cancer, potentially offering new hope for improved patient outcomes.

Cisplatin-induced cytotoxicity involves the generation of ROS and induction of oxidative stress.^{16,17} Our data suggest that inhibition of ZDHHC12 could be used to potentiate the cytotoxic effects of cisplatin by inducing excessive ROS accumulation. Notably, this effect is observed not only with ZDHHC12 silencing, but also with the general palmitoylation inhibitor 2BP,³³ a non-metabolizable palmitate analog that irreversibly blocks protein palmitoylation, and with the fatty acid synthase inhibitor C75, which is known to block the de novo synthesis of palmitate critical for protein

palmitoylation.^{34,35} Importantly, the ROS scavenger NAC reversed the combinatorial treatment effects, suggesting that ZDHHC12-mediated palmitoylation of specific proteins affects cisplatin response in HGSOC through ROS-mediated mechanisms. We validated our in vitro findings by demonstrating the combinatorial therapeutic effect of siZDHHC12 and cisplatin in an ovarian cancer xenograft tumor model. We explored translational relevance in the context of cisplatin-resistant ovarian cancer. First, high ZDHHC12 expression correlated significantly with cisplatin-resistant OVCAR8 spheroids. Second, ZDHHC12 inhibition significantly enhanced the cisplatin cytotoxicity in an ascites-derived organoid line of ovarian cancer resistant to platinum-based chemotherapy. We further validated our in vitro findings by demonstrating the combinatorial therapeutic effect of siZDHHC12 and cisplatin in an ovarian cancer xenograft tumor model. These findings identify ZDHHC12 as a crucial modulator of cisplatin response, opening exciting avenues for the development of targeted therapies and overcoming resistance to significantly improve outcomes for ovarian cancer patients.

AUTHOR CONTRIBUTIONS

Xining Zhang: Data curation; formal analysis; investigation; visualization; writing – original draft; writing – review and editing. **Xingming Liao:** Data curation; formal analysis; investigation; validation. **Min Wang:** Funding acquisition; validation. **Jiao Liu:** Investigation; methodology. **Jiaxin Han:** Data curation; investigation; methodology. **Dong An:** Data curation; investigation; methodology. **Tiezheng Zheng:** Investigation; methodology. **Xuefei Wang:** Investigation; methodology. **Hailing Cheng:** Conceptualization; funding acquisition; supervision; writing – original draft; writing – review and editing. **Pixu Liu:** Funding acquisition; methodology; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: The study was approved by the Ethics Committee of The Second Hospital of Dalian Medical University, and conformed to the standards set by the Declaration of Helsinki.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All the animal experiments were approved by the Animal Research Committee of Dalian Medical University and conducted in accordance with institutional guidelines for animal care and handling.

ORCID

Hailing Cheng  <https://orcid.org/0000-0001-5618-8709>

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