Cancer Letters 454 (2019) 179–190

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

ZY0511, a novel, potent and selective LSD1 inhibitor, exhibits anticancer activity against solid tumors via the DDIT4/mTOR pathway

Yan Li[a,1], Lei Tao[a,1], Zeping Zuo[a,1], Yang Zhou[b], Xinying Qian[a], Yiyun Lin[a], Hui Jie[a], Chunqi Liu[a], Zhuoling Li[a], Huaqin Zhang[a], Hu Zhang[c], Xiaobo Cen[d], Shengyong Yang[a,**], Yinglan Zhao[a,*]

[a] State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, and Collaborative Innovation Center for Biotherapy, Sichuan University, Chengdu, 610041, China
[b] Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences, Chengdu, 610041, China
[c] Department of Gastroenterology, West China Hospital, West China Medical School, Sichuan University, Chengdu, 610041, China
[d] National Chengdu Center for Safety Evaluation of Drugs, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Collaborative Innovation Center for Biotherapy, Chengdu, 610041, China

ARTICLE INFO

Keywords:
Colorectal cancer
Cervical cancer
H3K4 methylation
Chemotherapy sensitivity

ABSTRACT

Lysine-specific demethylase1 (LSD1) plays a crucial role in cancer and has become a promising target for cancer therapy. However, the mechanism underlying the role of LSD1 in oncogenesis is poorly understood, and more effective LSD1 inhibitors are needed. Here we report the biological activity of a novel LSD1 inhibitor named ZY0511. ZY0511 specifically inhibited LSD1 activity and the proliferation of various human cancer cells especially the HeLa and HCT116 cells. ZY0511 significantly increased the expression of *DDIT4*, a known mTORC1 suppressor, which was a direct downstream target of LSD1 confirmed by ChIP-PCR. ZY0511-induced LSD1 inhibition upregulated the expression of DDIT4 by altering histone H3K4 methylation levels at its promoter, thus suppressing mTORC1 activity. Knockdown of DDIT4 attenuated the anticancer effect of ZY0511. Intraperitoneal administration of ZY0511 significantly prevented the growth of HCT116 and HeLa xenografts in mice and showed no detectable toxicity. Moreover, DDIT4 expression was correlated with the sensitivity of human cancer cells to chemotherapy. Taken together, ZY0511 showed therapeutic potential for solid tumors, the induction of DDIT4 may be used as a predictive biomarker of LSD1 inhibitors.

1. Introduction

LSD1 is a flavin adenine dinucleotide (FAD)-dependent oxidase that specifically removes the methyl group from mono- and dimethylated histone H3 at lysine 4 (H3K4) [1] or lysine 9 (H3K9) [2], thereby controlling gene expression. LSD1 is essential for mammalian development and is involved in many physiological and pathological processes, such as cancer [1–3].

LSD1 is overexpressed in many human cancers and is associated with poor patient prognosis [4], which provides evidence that inhibition of LSD1 may offer a therapeutic strategy for the treatment of cancers. Tranylcypromine (TCP), an FDA-approved anti-depression drug, was first reported to be an irreversible inhibitor of LSD1 that can unlock the all-*trans* retinoic acid (ATRA)-driven therapeutic response in non-acute promyelocytic leukemia [5]. Due to limitations in its potency and selectivity, TCP cannot be used alone as a specific LSD1 inhibitor. TCP-based LSD1 inhibitors have been identified for cancer therapy. For example, ORY-1001, GSK-2879552 and INCB059872 are currently undergoing clinical study for treatment of cancers, such as acute myeloid leukemia (AML) [6], acute lymphoblastic leukemia (ALL), myelodysplastic syndromes (MDSs), and small cell lung cancer (SCLC) [7]. Although they have high potency in above diseases, they exhibit low

Abbreviations: LSD1, lysine-specific demethylase1; mTORC1, mammalian target of rapamycin complex 1; DDIT4, DNA-damage-inducible transcript 4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AML, acute myeloid leukemia; ATRA, all-*trans* retinoic acid; TCP, tranylcypromine; APL, acute promyelocytic leukemia; HIF-1, hypoxia-inducible factor-1; MAO-A, monoamine oxidase A; MAO-B, monoamine oxidase B; SCLC, small cell lung cancer

* Corresponding author. State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, #17, 3rd Section, Ren min South Road, Chengdu, 610041, China.
** Corresponding author. State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, #17, 3rd Section, Ren min South Road, Chengdu, 610041, China.
E-mail addresses: yangsy@scu.edu.cn (S. Yang), zhaoyinglan@scu.edu.cn (Y. Zhao).
[1] Equal contributor.

https://doi.org/10.1016/j.canlet.2019.03.052
Received 8 November 2018; Received in revised form 9 March 2019; Accepted 29 March 2019

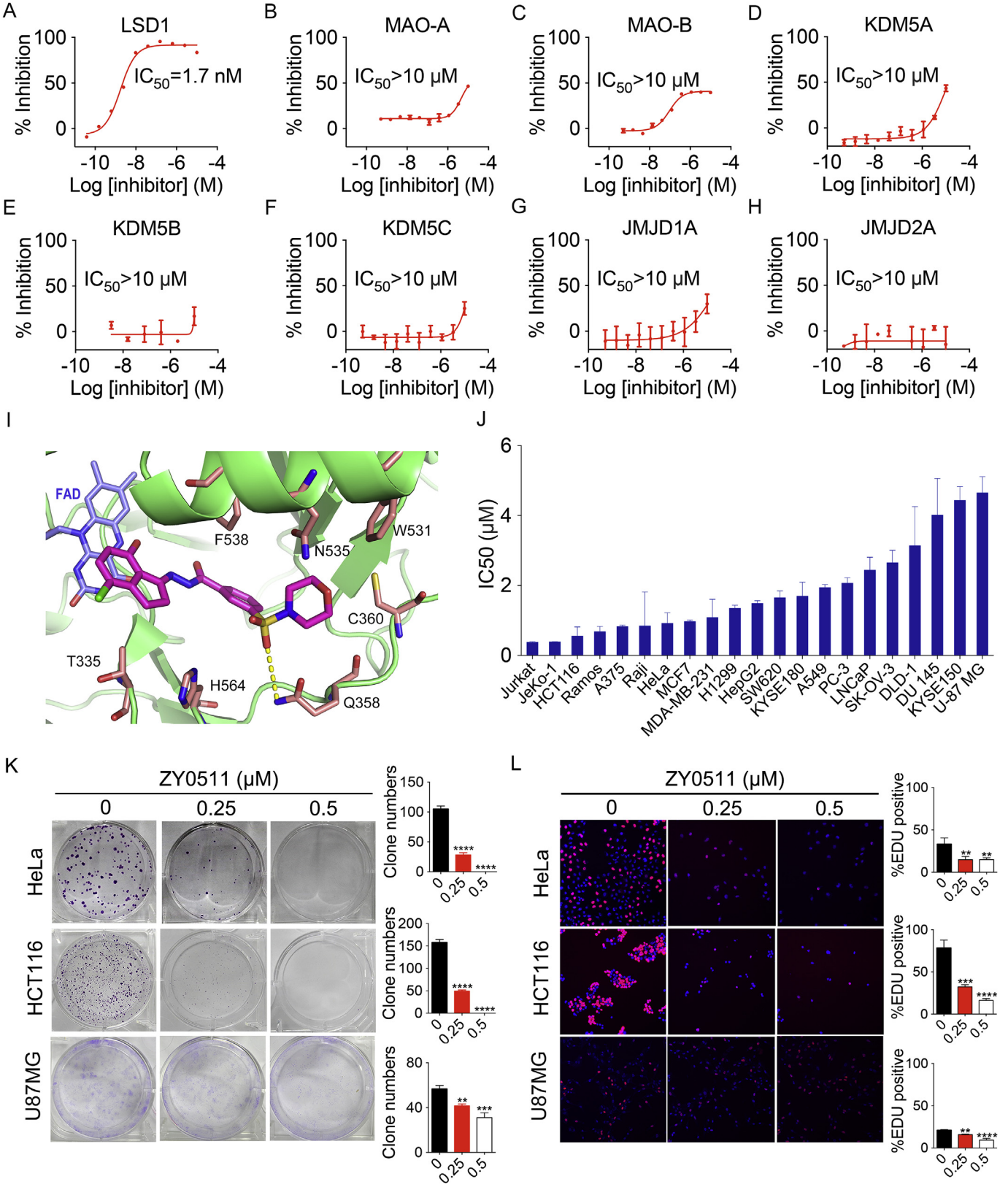

Fig. 1. ZY0511 is a novel LSD1 inhibitor that possesses good selectivity and antitumor activity *in vitro*. (A)–(H) The inhibitory activity of ZY0511 on LSD1, MAO-A, MAO-B, KDM5A, KDM5B, KDM5C, JMJD1A and JMJD2A enzymes. (I) ZY0511 docks into the active site of LSD1, and the π-π interactions between ZY0511 and FAD are shown in the 3-D structure. (J) The anti-proliferation effect of ZY0511 on multiple human cancer cell lines. (K) The inhibitory effect of ZY0511 on the colony formation of HeLa (top), HCT116 (middle), and U87MG (bottom) (data are shown as the mean ± SD, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA followed by Dunnett's test). (L) The anti-proliferation effect of ZY0511 on HeLa (top), HCT116 (middle), and U87MG (bottom) (data are shown as the mean ± SD, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA followed by Dunnett's test).

Table 1
Proliferation inhibitory effect of other LSD1 inhibitors on human cancer cell lines.

Cell lines	SP2509 IC_{50} (μM)	GSK2879552 Inhibitory ratio (10 μM)
HCT116	4.22 ± 0.53	0.72% ± 0.01
A375	1.68 ± 0.18	1.25% ± 0.02
HeLa	7.10 ± 0.53	4.48% ± 0.05
MCF7	0.51 ± 0.41	\
MDA-MB-231	0.94 ± 0.06	3.13% ± 0.04
H1299	2.66 ± 0.68	6.43% ± 0.06
HepG2	5.16 ± 0.21	0.47% ± 0.01
SW620	2.60 ± 0.27	0.04% ± 0.00
KYSE180	8.10 ± 2.90	1.41% ± 0.02
A549	3.87 ± 0.18	4.23% ± 0.03
PC-3	6.28 ± 1.68	0.46% ± 0.01
LNCaP	0.76 ± 0.38	\
SK-OV-3	7.08 ± 1.60	10.67% ± 0.03
DLD-1	1.88 ± 0.20	2.85% ± 0.03
DU 145	3.19 ± 0.54	4.46% ± 0.05
KYSE150	1.39 ± 0.04	2.09% ± 0.02
U-87 MG	8.84 ± 0.42	7.24% ± 0.13

IC_{50} values and inhibition ratios were determined by MTT assay as described in "Supplementary Materials and Methods", and were obtained from three independent experiments in triplicate. SD were calculated with STDEV in Excel. "\" represents no inhibition at this concentration.

efficacy against solid tumors other than SCLCs [4]. Pargyline, a peptide-based TCP derivative, has also been investigated. However, the delivery of peptide therapeutics to the nucleus is still an unsolved problem [8]. Great efforts have been devoted to developing LSD1 inhibitors against solid tumors. CBB1003 has been explored to treat colorectal cancer (CRC). Nevertheless, CBB1003 exhibits low potency [9], and its structure-activity relationships remain unknown. There is a large unmet clinical need to explore LSD1 inhibitors against solid tumors.

The mechanism by which LSD1 regulates cancer progression is the basis for LSD1-based cancer therapy. LSD1 sustains the leukemogenic potential of MLL-AF9 leukemia stem cells [10]. LSD1 inhibition induces the expression of myeloid differentiation-associated genes that promote the differentiation of leukemia cells [5]. LSD1 specifically interacts with androgen receptor [2], estrogen receptor [11] or large chromatin-modifying corepressor complexes, such as the CoREST [12] complex, and regulates prostate cancer and breast cancer progression by triggering androgen- [2] and estrogen-induced transcription [11]. LSD1 suppresses negative regulators of β-catenin signaling, which promotes the stemness and chemoresistance of $Lgr5^+$ hepatocellular carcinoma (HCC) [13]. By inhibiting LSD1, sorafenib-resistant stem-like cells are eliminated in HCC [14]. However, the underlying epigenetic mechanism is still poorly understood, and this lack knowledge has been a hurdle for the application of LSD1 inhibitiors.

Based on computer-aided drug design and high-throughput screening, we obtained a novel specific LSD1 inhibitor named ZY0511 and the structure was showed in the previous reference [15]. Our results show ZY0511 exhibits good anticancer activity against a panel of human solid tumor cells *in vitro* in terms of inducing cell cycle arrest and apoptosis. Furthermore, ZY0511 significantly inhibits solid tumor growth *in vivo*. ZY0511 is applied as a molecular probe, and the results demonstrate that LSD1 binds to the promoter of the DNA-damage-inducible transcript 4 (*DDIT4*) gene, which is a mTORC1 negative regulator and demethylase its H3K4 levels. *DDIT4* is a direct downstream target of LSD1 and may be correlated with cancer chemotherapy resistance.

2. Materials and methods

2.1. mRNA-seq

mRNA-seq was conducted using a profiler service provided by NOVELBIO Corporation (Shanghai, China). Total RNA was purified from HeLa cells after dimethyl sulfoxide (DMSO) or ZY0511 (2 μM) treatment for 2 days and stored in TRIzol (Invitrogen, USA). Triplicate samples were harvested for each group, and significant probe sets were filtered for detection using a fold-change > 1.5, $P < 0.05$ (Student's t-test) and FDR (false discovery rate) < 0.05. The differentially expressed genes were enriched in GO. Quality checks were also carried out by NOVELBIO Corporation (Shanghai, China).

2.2. Subcutaneous xenograft models

All animals experiments were approved by the Institutional Animal Care and Treatment Committee of Sichuan University in China (Permit Number: 20161025) and were carried out in accordance with the ARRIVE guidelines. The animals were kept at 21 °C, 55% humidity, on a 12 h light (SPF)/dark cycle and had food and water available *ad libitum*. Six-week-old BALB/c nude mice were purchased from HFK Biotechnology Company (Beijing, China), and all mice had Animal Quarantine Conformity Certificates. Detailed procedure are included in the *SI Materials and Methods*.

2.3. Chromatin immunoprecipitation assays

ChIP assays were performed according to the manufacturer's instructions for the Chromatin Immunoprecipitation Kit (Millipore, #17–10086). Cells were fixed with 1% formaldehyde, and cross-linked chromatin was sonicated to produce 200-1000-bp DNA fragments. The lysate was precleared with protein A/G agarose and incubated with specific antibodies, namely, anti-LSD1 (Millipore #17–10531), anti-H3K4me1 (CST #5326), anti-H3K4me2 (CST #9725) or IgG (Millipore #17–10086), at 4 °C overnight. Then, the protein/DNA complexes were eluted according to the instructions. Free DNA was purified and analyzed using real-time quantitative PCR.

2.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software. All data are represented as the mean ± SD or SEM as indicated in the figure legends. Comparisons between two groups, namely, the control group and the treatment group, were analyzed using an unpaired, two-tailed *t*-test. One-way ANOVA was utilized to compare the means of three or more unmatched groups with only one factor, such as drug treatment. A P value < 0.05 was considered statistically significant.

Detailed information about other assays are included in the *SI Materials and Methods*.

3. Results

3.1. ZY0511 potently inhibits LSD1 activity and possesses high selectivity

We synthesized a series of novel small-molecule compounds against LSD1 and ZY0511 was the most potent and superior compound [15]. ZY0511 strongly inhibited LSD1 activity (IC_{50} = 1.7 nM) (Fig. 1A) and is high selective over FAD-dependent monoamine oxidases and histone demethylases such as JMJD1A, KDM5A, KDM5B, KDM5C (Fig. 1B–H). Using computer simulation and computer-based molecular docking methods (PDB ID: 5LHH), we found there was a hydrogen bond formed between ZY0511 and Q358 (glutamine358) of AOL domain (Fig. 1I). These data demonstrated that ZY0511 potently and selectively inhibited LSD1 activity.

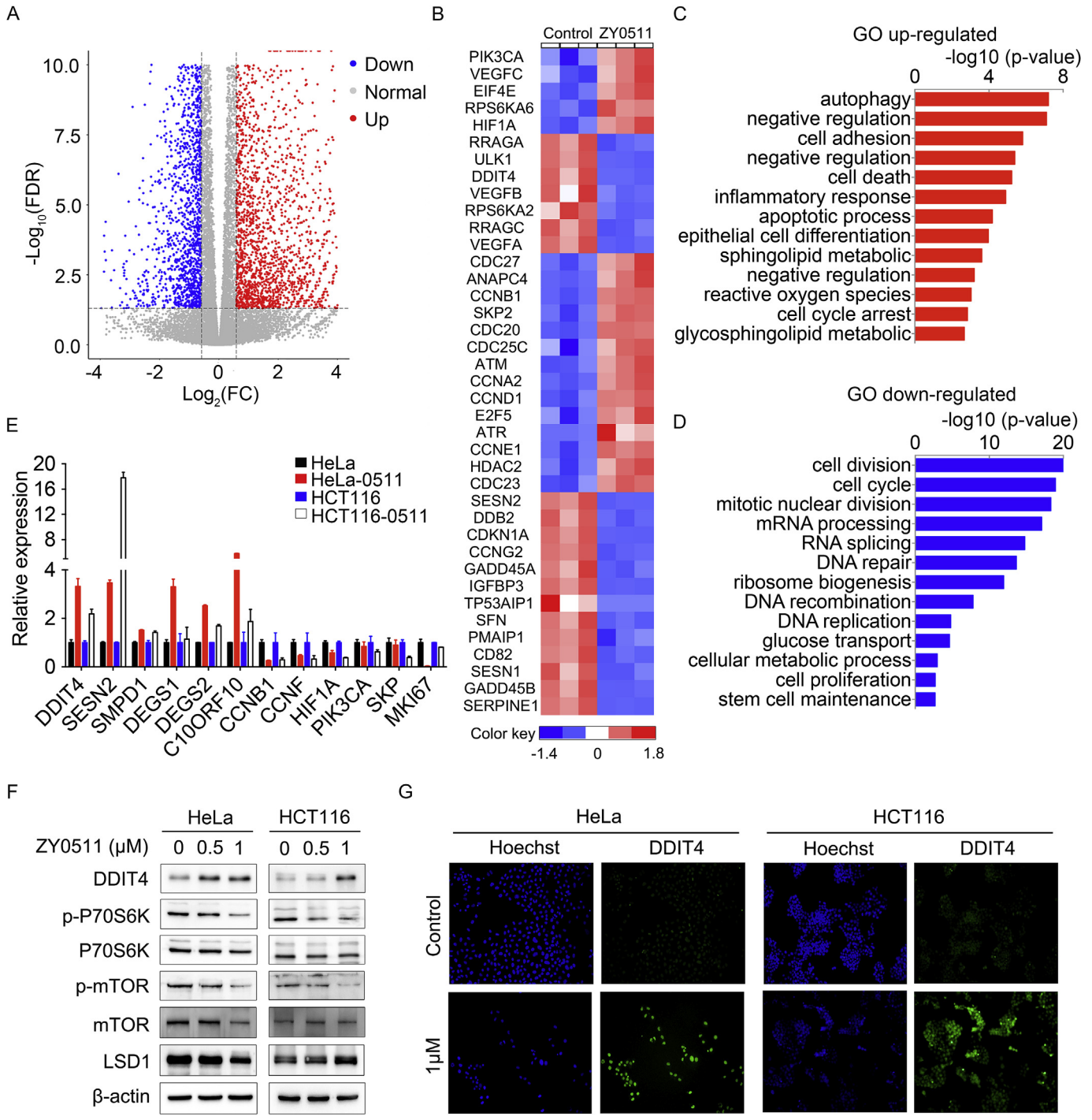

Fig. 2. Gene expression changes induced by ZY0511 in cancer cells. (A) The volcano plot of the mRNA-seq analysis of HeLa cells after ZY0511 exposure (red represents upregulation, blue represents downregulation, and gray represents no meaningful change). (B) Representative gene expression in the HeLa cells after ZY0511 exposure. (C) Representative GO term analysis of upregulated genes after ZY0511 exposure. (D) Representative GO term analysis of downregulated genes after ZY0511 exposure. (E) The qPCR validation of mRNA-seq gene expression in the HeLa and HCT116 cells. (F) Western blot detection of DDIT4 and mTOR signaling-related proteins in cancer cells after ZY0511 treatment; β-actin was used as the reference protein. (G) Immunofluorescence detection of DDIT4 in cancer cells after ZY0511 treatment; Hoechst was used to stain the nuclei. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. ZY0511 inhibits the proliferation of human cancer cells in vitro

ZY0511 significantly inhibited the proliferation of human cancer cells with IC_{50} values ranging from 0.2 to 3.3 μM (Fig. 1J). The sensitivity of cancer cells to ZY0511 may be associated with the cellular expression of LSD1. Jurkat, JeKo-1, Raji and Ramos, were chosen as the reference cell lines because they were sensitive to LSD1 inhibitors [5], and ZY0511 markedly inhibited their proliferation. In addition, we compared the anti-proliferative activity of ZY0511 with other LSD1 inhibitors such as SP2509 and GSK2879552, and ZY0511 showed superior activity than these inhibitors in most cell lines (Table 1) (Fig. S1). Though three LSD1 inhibitors are undergoing clinical study for AML and SCLC treatments, the efficiency of LSD1 inhibitors in solid cancers seldom be investigated [5,7,17–21]. Thus, we focused on the actions of

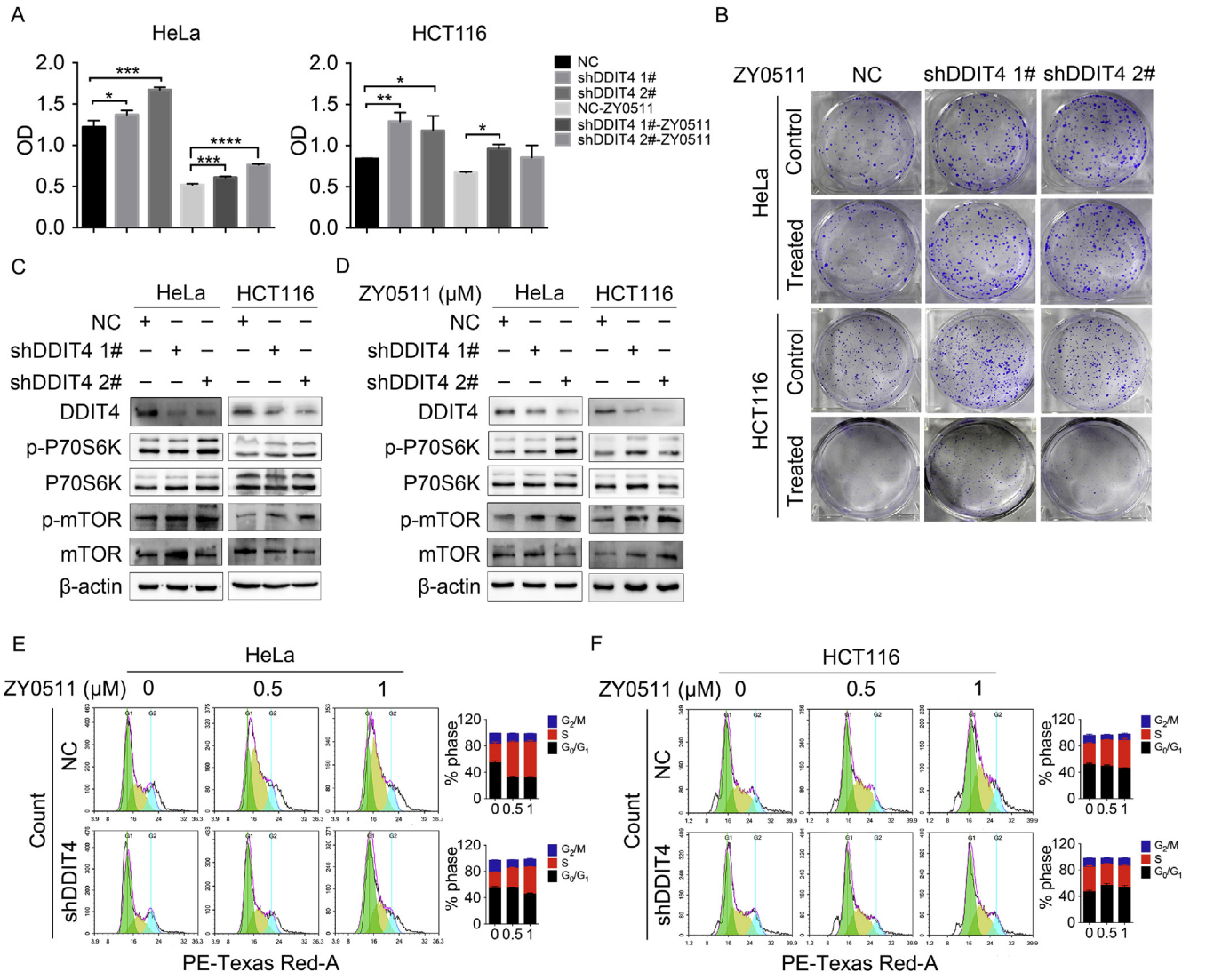

Fig. 3. DDIT4 mediates the inhibitory effect of ZY0511. (A) The proliferation activities of the DDIT4 stably depleted cells after ZY0511 treatment (data are shown as the mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA followed by Dunnett's test). (B) The effect of ZY0511 on the colony formation of DDIT4 stable knockdown cells. (C) Expression of mTOR signaling-related proteins after stable knockdown of DDIT4; β-actin was used as the reference protein. (D) Expression of mTOR signaling-related proteins in DDIT4 stable knockdown cells after ZY0511 treatment; β-actin was used as the reference protein. (E) (F) Cell cycle of HeLa-NC, HeLa-shDDIT4, HCT116-NC, and HCT116-shDDIT4 after ZY0511 treatment.

ZY0511 against solid tumors. HeLa and HCT116 cells which were sensitive to ZY0511, were used for further studies. U87MG cells with less sensitivity to ZY0511 were used for comparisons.

ZY0511 markedly inhibited the colony formation of HeLa and HCT116 cells, whereas the inhibition rate was only 26.5% for U87MG cells (Fig. 1K). ZY0511 also significantly decreased the number of EdU-positive HeLa and HCT116 cells while only slightly decreased the number of EdU-positive U87MG cells (Fig. 1L).

3.3. ZY0511 induces cell cycle S phase arrest and promotes apoptosis

ZY0511 treatment induced the cell cycle S phase arrest in HeLa and HCT116 cells (Fig. S2A), and ZY0511 had only a weak effect on U87MG cell cycle. In addition, ZY0511 exposure increased the numbers of Annexin V^+ cells of HeLa and HCT116 cells, while only weakly induced U87MG cells apoptosis (Fig. S2C). ZY0511 upregulated the pro-apoptosis protein Bax and cleaved-caspase 3 and downregulated the anti-apoptosis protein Bcl2 (Fig. S2D). These data suggested that the anti-tumor effects of ZY0511 is partly attributed to the cell cycle S phase arrest and apoptosis induction.

The methylation levels of H3K4 and H3K9 were measured and ZY0511 increased global cellular H3K9me2 levels, while H3K4me2 levels were altered slightly. This might be because other histone methylases and demethylases other than LSD1 maintain its level dynamically (Fig. S2B).

3.4. ZY0511 induces DDIT4 expression

mRNA-seq technology was applied to search for factors or genes that mediate the antitumor effect of ZY0511. We focused on the transcriptome change after 48 h of treatment. A total of 5277 genes were markedly altered in HeLa cells (Fig. 2A and B). The Gene Ontology (GO) Consortium was used to perform an enrichment analysis of the functions of the changed genes (Fig. 2C and D), and detailed information is shown in (Tables S1 and S2). ZY0511 upregulated genes closely associated with cancer autophagy, apoptotic process and cell cycle arrest (Fig. 2C). Genes correlated with cell division, cell proliferation and stem cell maintenance were decreased (Fig. 2D).

We then performed qPCR assays to verify the representative genes obtained from mRNA-seq results, including *CCNB1*, *CCNF*, *SKP*, *KI67*,

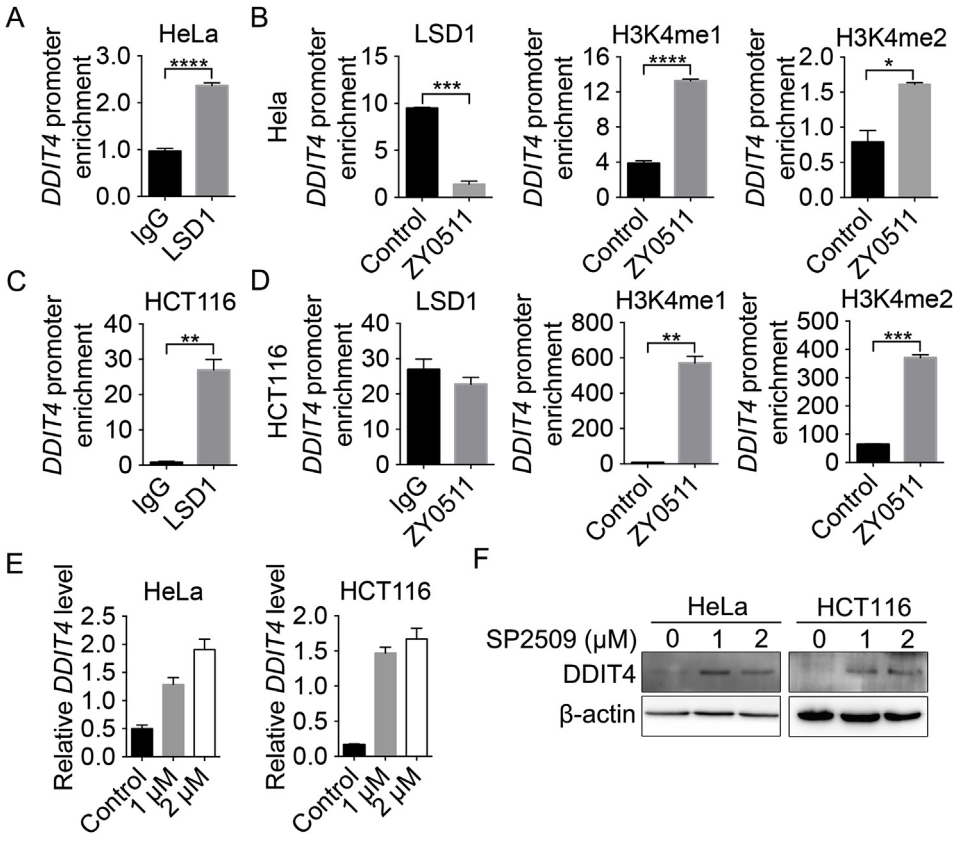

Fig. 4. *DDIT4* is a direct target of LSD1. (A) (C) The ChIP-PCR assay was applied to investigate the binding status of LSD1 to the *DDIT4* promoter. IgG was used as the negative control (data are shown as the mean ± SD, **P < 0.01, ****P < 0.0001, unpaired two-tailed t-tests). (B) (D) ChIP-PCR assay to detect the enrichment levels of LSD1, H3K4me1 and H3K4me2 at the *DDIT4* promoter. IgG was used as the negative control (data are shown as the mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, unpaired two-tailed t-tests). (E) The *DDIT4* mRNA levels in the HeLa and HCT116 cells after LSD1 inhibitor SP2509 treatment for 48 h (all displayed values are the means ± SDs). (F) DDIT4 protein expression in the HeLa and HCT116 cells after LSD1 inhibitor SP2509 treatment for 48 h; β-actin was used as the reference protein.

SMPD1, *DEGS2, SESN2* and *PIK3CA* (Fig. 2E). The results were constent with mRNA-seq results. *DDIT4,* which is a negative regulator of mTORC1, was markedly upregulated among these altered genes (Fig. 2E–G). The expression of two critical proteins in the mTOR pathway such as p-mTOR and p-P70S6K were significantly decreased after ZY0511 treatment (Fig. 2F). Above results suggested that DDIT4 was involved in anticancer effect of ZY0511.

3.5. Upregulation of DDIT4 is a critical mechanism that mediates the anticancer effect of ZY0511

To further investigate the role of DDIT4 on effect of ZY0511, the experiment after *DDIT4* knockdown or overexpression were performed. *DDIT4* knockdown increased the proliferation of cancer cells (Fig. 3A and B) and the expression of p-mTOR and p-P70S6K (Fig. 3C). ZY0511-induced proliferation inhibition and downregulation of p70S6K and mTOR was abolished by *DDIT4* knockdown (Fig. 3D). DDIT4 knockdown also attenuated cell cycle S phase arrest induced by ZY0511 treatment (Fig. 3E and F). In addition, DDIT4 overexpression enhanced the activity of ZY0511 (Fig. S3A – S3C).

Then, we investigated whether the upregulation of DDIT4 by ZY0511 was due to the increased level of H3K4me1/2 at its promoter. Our results showed that LSD1 bound to the *DDIT4* gene promoter (Fig. 4A and C) and ZY0511 treatment significantly increased the level of H3K4me1/2 (Fig. 4B and D).

We also investigated the effect of SP2509, a selective LSD1 inhibitor [26], on DDIT4 expression. DDIT4 was markedly upregulated after SP2509 treatment (Fig. 4E and F). Collectively, our data indicate that *DDIT4* is a direct downstream gene of LSD1.

3.6. ZY0511 inhibits tumor growth in vivo

The *in vivo* antitumor activities of ZY0511 were evaluated with mouse subcutaneous xenograft models (Fig. 5). Nude mice received daily intraperitoneal injection of ZY0511. The results showed the tumor inhibition rate at 50 mg/kg of ZY0511 was 59.4% for the HeLa model and 51.3% for the HCT116 model (Fig. 5A and B), while the growth inhibition was weak for the U87MG model. The KI67-positive ra was markedly decreased *in vivo* by ZY0511 treatment (Fig. 5C). Moreover, DDIT4 expression was upregulated by ZY0511 treatment *in vivo* (Fig. 5D and Fig. S4A), H&E staining showed no lesions in main organs of ZY0511-treated mice, suggesting that ZY0511 treatment was well tolerated (Fig. S4B).

3.7. LSD1 depletion mimics the antitumor phenotype of ZY0511, and DDIT4 depletion blocks the effect of LSD1

We found LSD1 depletion indeed upregulated *DDIT4* expression, suppressed mTOR pathway (Fig. 6A and B) and inhibited tumor cells proliferation *in vitro* (Fig. 6C and D). ChIP-PCR assays showed increasing H3K4me1/2 levels at the promoter of the *DDIT4* after LSD1 depletion (Fig. 6E). DDIT4 knockdown weakened the tumor growth inhibitory effect of LSD1 knockdown (Fig. 6C and D).

Furthermore, knockdown of LSD1 repressed the growth of tumors *in vivo* (Fig. 6F and G) and DDIT4 silencing blocked the inhibitory effect of LSD1 depletion (Fig. 6F and G). Collectively, LSD1 deficiency inhibits cancer cells growth in a similar manner to ZY0511 treatment, and DDIT4 mediates the effect of LSD1.

3.8. DDIT4 expression is partly correlated with the sensitivity of cancer cells to chemotherapy

Chemoresistance is a key causal factor of cancer recurrence, and the dysregulation of epigenetic regulators plays a critical role in this process [27]. We speculated DDIT4 may mediate the sensitivity of cancer cells to chemotherapeutic agents. DDIT4 knockdown attenuated the inhibitory effect of paclitaxel, 5-FU and cis-platinum (Fig. 7A – 7C). The IC_{50} values indicated cells with DDIT4 knockdown were more resistant

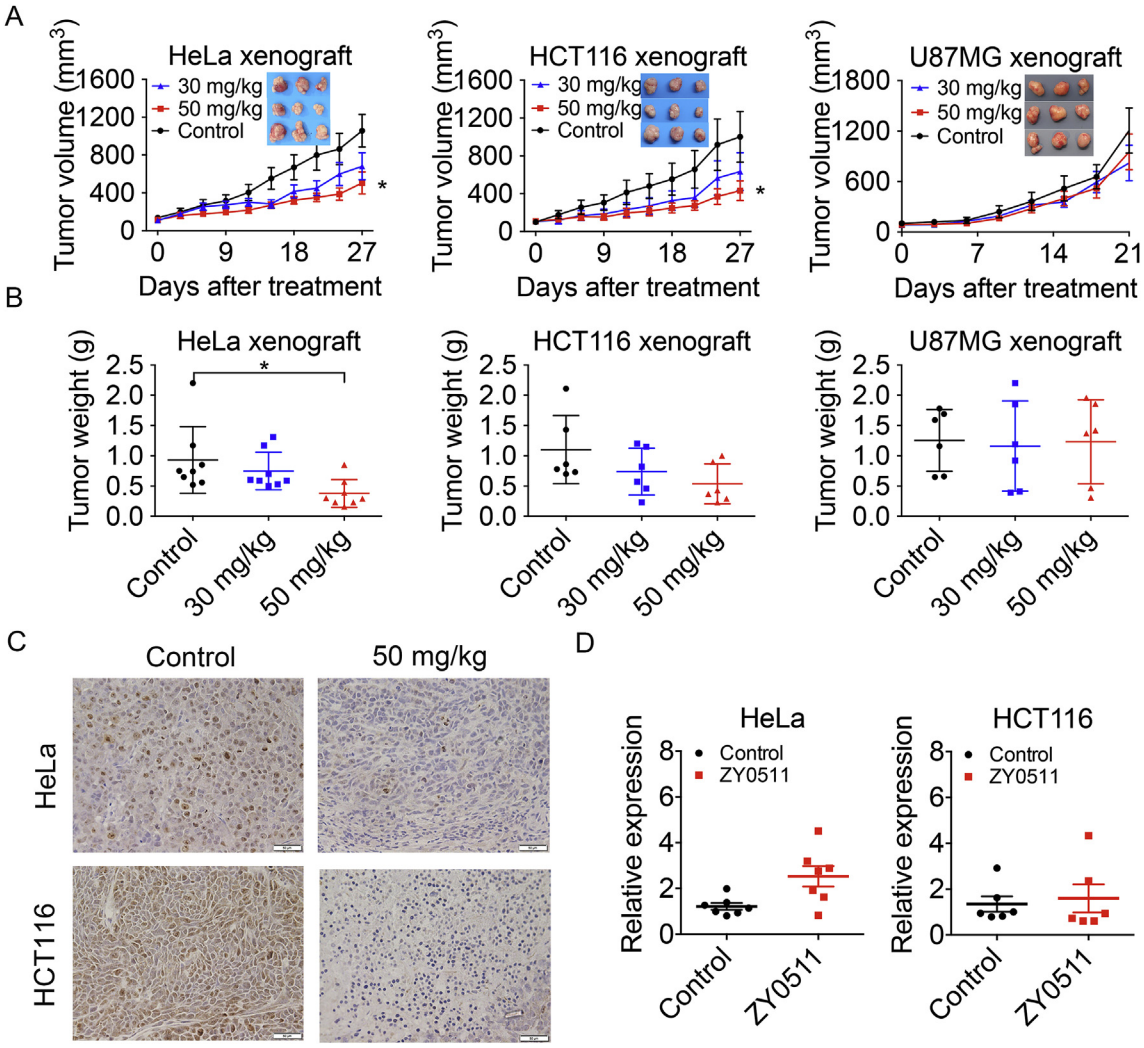

Fig. 5. The antitumor effect of ZY0511 *in vivo*. (A) The growth curve of the HeLa, HCT116 and U87MG cells *in vivo* after ZY0511 treatment (data are shown as the mean ± SEM, *P < 0.05, one-way ANOVA followed by Dunnett's test). (B) The tumor weights of HeLa, HCT116 and U87MG xenograft models after ZY0511 treatment (data are shown as the mean ± SEM, *P < 0.05, one-way ANOVA followed by Dunnett's test). (C) KI67 staining of tumors at the end of the experiment. (D) The mRNA level of DDIT4 *in vivo*.

than normal control cells. DDIT4 knockdown cells proliferated faster than the control cells under single-concentration exposures to paclitaxel or 5-FU, respectively (Fig. 7D and E). The colony formation ability after DDIT4 knockdown was improved (Fig. 7F and G). We extended the experiments to A375 and SK-OV-3 cell lines (Figs. S5A and S5C) and results also indicated the important role of DDIT4 expression on cancer cells' chemoresistance (Fig. S5B, S5D-S5F). Collectively, our data indicate that DDIT4 plays a critical role in modulating the sensitivity of cancer cells to chemotherapy.

4. Discussion

Histone modifications plays a critical role in cancer onset and progression which have become a major focus for pharmacological cancer interventions. We developed ZY0511, a novel LSD1 inhibitor, which markedly inhibits solid tumors growth without general toxicity. The anti-proliferation activity of ZY0511 to solid tumors *in vitro* is prominent compared with that of other LSD1 inhibitors. LSD1 inhibition by ZY0511 increases the H3K4me1/2 level of the *DDIT4* promoter and triggered its expression, thus inhibiting mTOR signaling. To the best of our knowledge, our findings are the first to demonstrate that *DDIT4* is a direct downstream target of LSD1 (Fig. 8), which is an important regulator of cancer cell sensitivity to chemotherapy. Our results lay a theoretical foundation for the clinical application of LSD1 inhibitors for solid tumor therapy.

TCP was the first identified LSD1 inhibitor that could suppress breast cancer [28], oral squamous cell carcinoma [29] and AML [10] which caused some toxicity in patients due to its nonselectivity [5]. High concentrations of TCP are necessary to achieve LSD1 inhibition, and clinical use has been very restricted due to food and drug interactions and to the risk of fatal hypertensive crisis in the case of tyramine ingestion [6]. GSK2879552 and ORY-1001 are TCP-based LSD1 inhibitors and both are used for leukemia treatment such as AML and lymphoma. Compared with GSK2879552 and SP2509, ZY0511 exhibited strong inhibitory effect on some solid tumors (Table 1). To improve the selectivity and avoid undesired activity, TCP analogues, such as RN-1 [30] and S2101 [31], that are known to be more selective than TCP were created. Nevertheless, the inhibitory effects of RN-1 and S2101 to LSD1 are low and the inhibitory effects against some solid tumors are also much weaker [32]. We speculated that the stronger inhibitory potency of ZY0511 for LSD1 is attributed to its better antitumor effect [30,31]. In addition to the above TCP analogues, new LSD1 inhibitors which combined aminothiourea and propargyl were discovered, especially compound 6b [33], and the IC_{50} of compound 6b to LSD1 was weaker than ZY0511, indicating a significantly improved ZY0511 activity. We also found the obvious inhibitory effect of ZY0511

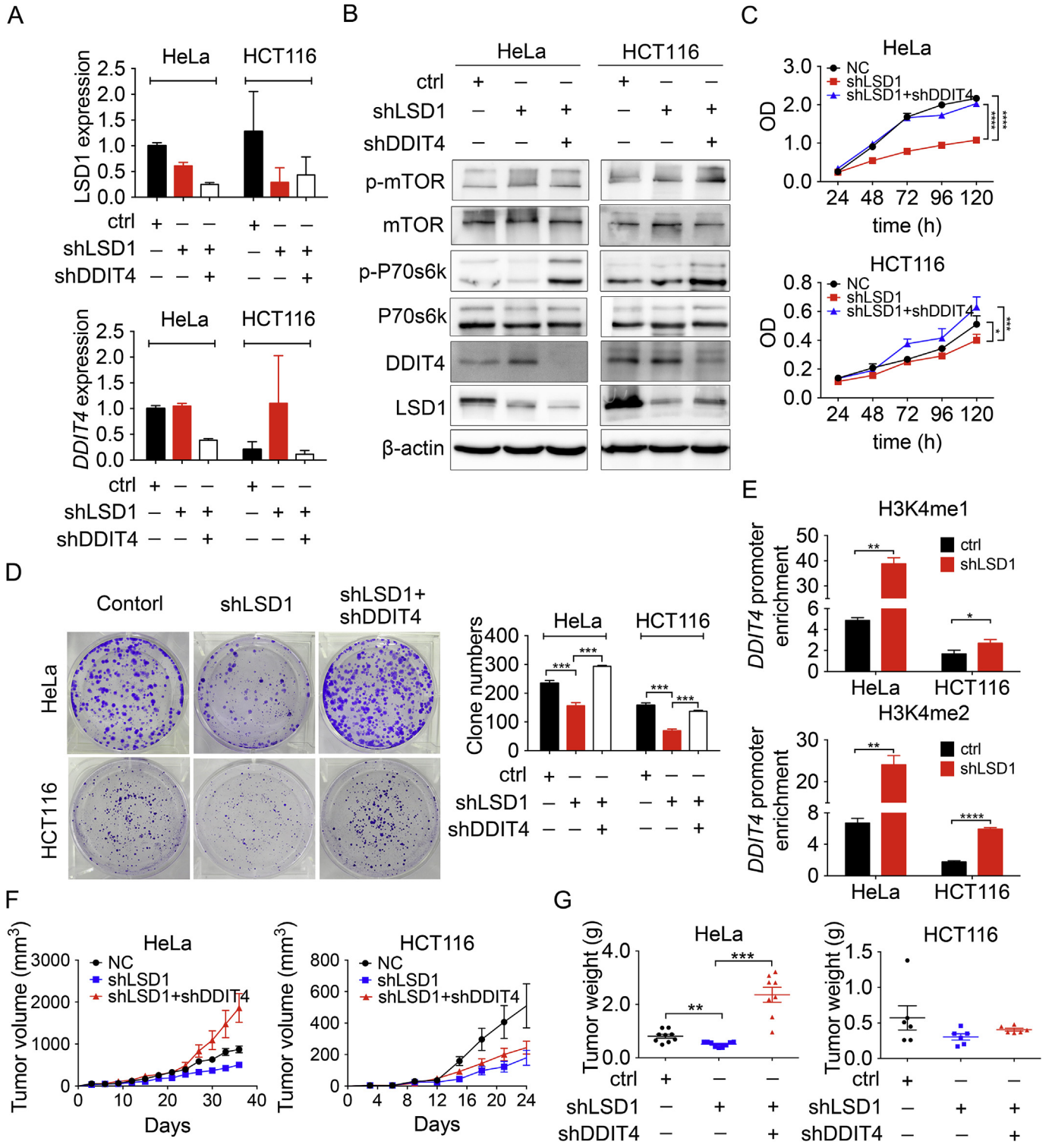

Fig. 6. Depletion of LSD1 inhibits cancer cells proliferation through up-regulation of DDIT4. (A) (B) Knockdown efficiency verification of LSD1 and DDIT4 and expression of mTOR-related proteins after knockdown of LSD1 and DDIT4 by Western blot; β-actin was used as the reference protein. (C) The proliferation of LSD1-depleted and DDIT4-depleted cells (data are shown as the mean ± SD, *P < 0.05, ***P < 0.001, ****P < 0.0001, unpaired two-tailed t-tests on 120 h). (D) The cell colony formation assay after knockdown of LSD1 or simultaneous knockdown of LSD1 and DDIT4 (data are shown as the mean ± SD, ***P < 0.001, unpaired two-tailed t-tests). (E) ChIP-PCR assay detection of the enrichment of H3K4me1 and H3K4me2 at the *DDIT4* promoter after LSD1 depletion (data are shown as the mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, unpaired two-tailed t-tests). (F) The tumor growth curves of subcutaneously transplanted HeLa and HCT116 cells with LSD1 and DDIT4 knockdown (all displayed values are the means ± SEMs). (G) The tumor weights of the subcutaneously transplanted HeLa and HCT116 cells with LSD1 and DDIT4 knockdown (data are shown as the mean ± SD, **P < 0.01, ***P < 0.001, unpaired two-tailed t-tests).

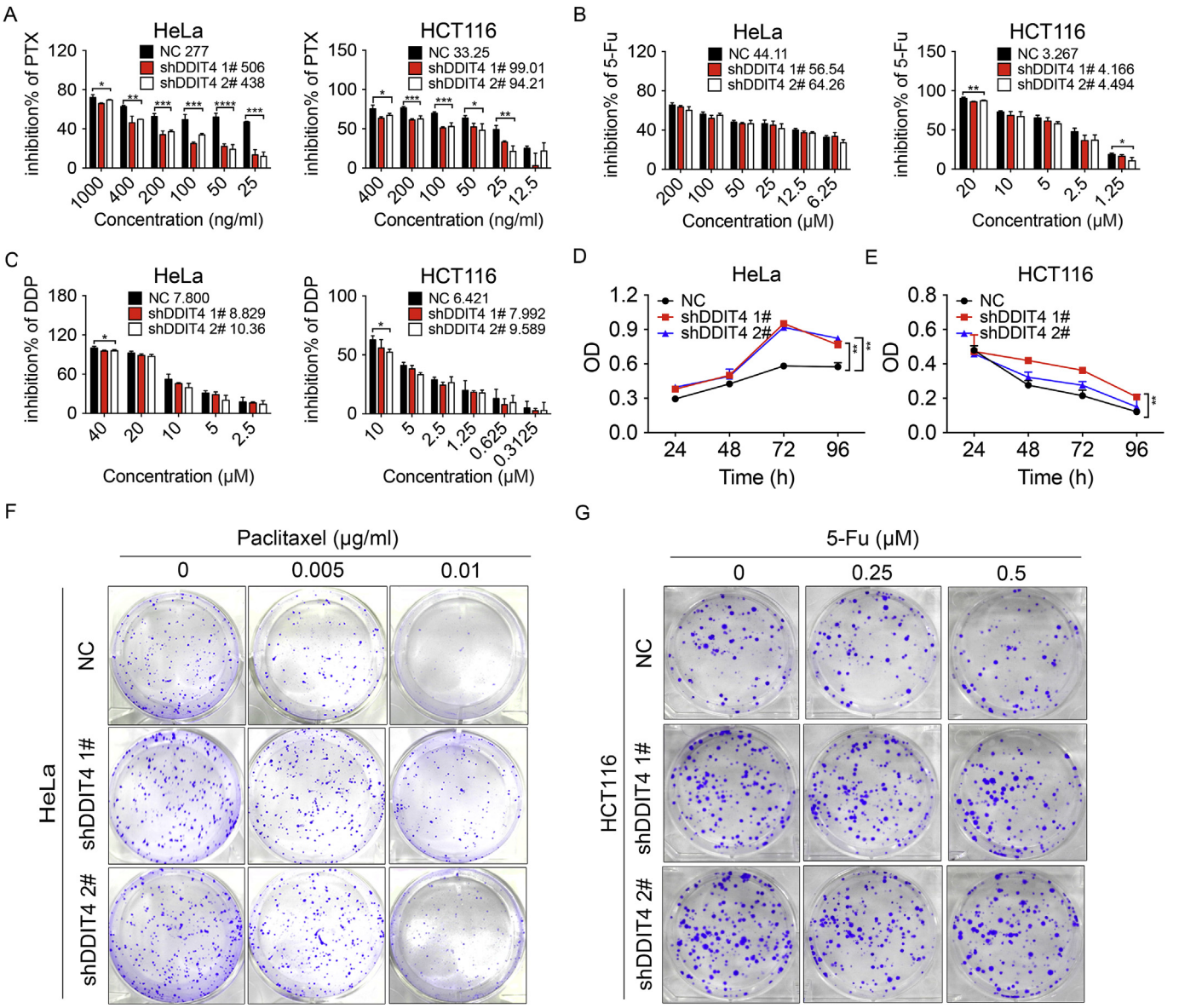

Fig. 7. The expression of DDIT4 is partly associated with the sensitivity of cancer cells to chemotherapy. (A) The proliferation inhibitory effect of paclitaxel on HeLa/HCT116 cells with DDIT4 knockdown. The IC_{50} values are indicated in the pictures (data are shown as the mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA followed by Dunnett's test). (B) The proliferation inhibitory effect of 5-Fu on HeLa/HCT116 cells with DDIT4 knockdown. The IC_{50} values are indicated in the pictures (data are shown as the mean ± SD, *P < 0.05, **P < 0.01, one-way ANOVA followed by Dunnett's test). (C) The proliferation inhibitory effect of cis-platinum on HeLa/HCT116 cells with DDIT4 knockdown. The IC_{50} values are indicated in the pictures (data are shown as the mean ± SD, *P < 0.05, one-way ANOVA followed by Dunnett's test). (D) The growth curves of HeLa/HeLa shDDIT4 cells with PTX exposure (12.5 ng/ml) (data are shown as the mean ± SD, **P < 0.01, unpaired two-tailed t-tests, 96 h of treatment). (E) The growth curves of HCT116/HCT116 shDDIT4 cells with 5-Fu exposure (20 μM) (data are shown as the mean ± SD, **P < 0.01, unpaired two-tailed t-tests, 96 h of treatment). (F) The colony formation of HeLa/HeLa shDDIT4 cells after paclitaxel treatment. (G) The colony formation of HCT116/HCT116 shDDIT4 cells after 5-Fu treatment.

to hematological cancer cells which proved the important role of LSD1 in hematological cancer as the previous studies [5,10]. ZY0511 possessed good selectivity and it did not significantly inhibit MAO-A and MAO-B activity or other histone demethylases. Moreover, ZY0511 is a compound different from the above LSD1 inhibitors which expands the chemical space where LSD1 inhibitor may be found. The chemical structure of ZY0511 makes it attractive for further structure–activity relationship studies of related molecules, which could be characterized for their ability to modulate particular LSD1-dependent functions in the progression of cancers. ZY0511 formed hydrogen bond with Q358 of AOL domain of LSD1, and a π-π reaction between ZY0511 and FAD that might enhance the anticancer effect of ZY0511.

LSD1 is related to many types of cancers and participates in regulatory network of tumors progression. In AML, LSD1 acts at genomic loci bound by MLL-AF9 to sustain expression of oncogenes [10]. LSD1 is critical for survival and EMT in breast cancer because it modifies the TGF-β1 related pathway [18]. With the increasing development of LSD1 inhibitors, the complete understanding of the mechanism of LSD1 in cancers has become critical and urgent. Using ZY0511 as a specific LSD1 inhibitor, we observed that LSD1 promotes the proliferation of cancer cells through the mTORC1 pathway. Although research has reported a correlation between LSD1 and the mTORC1 pathway [24], we demonstrated that LSD1 directly binds to the promoter of *DDIT4* and regulates its expression, which is a novel epigenetic effect of LSD1 in cancer pathways.

The hyperactivation of the mTOR pathway leads to an increase in

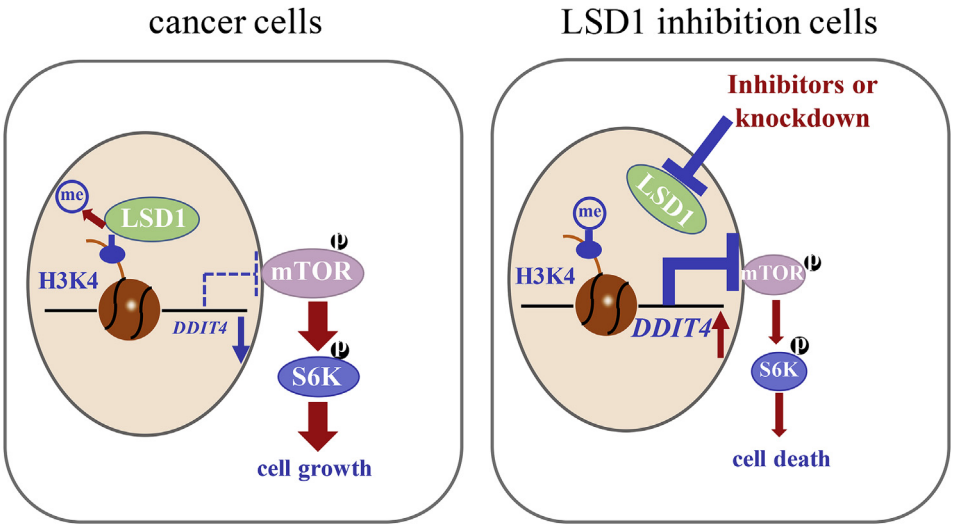

Fig. 8. The antitumor molecular model of the LSD1 inhibitor ZY0511.

cancer cell growth [34]. As a known mTORC1 negative regulator, DDIT4 is upregulated during multiple forms of cellular stress, including oxidative stress [35], starvation [36] and hypoxia [37], and DDIT4 inhibits mTORC1 activity by activating the TSC1/2 complex [36]. FAS inhibition elicits cell cycle arrest and apoptosis through upregulating DDIT4 [38]. However, DDIT4 promotes gastric cancer proliferation and tumorigenesis through the p53 and MAPK pathways [39] which indicates the dual roles of DDIT4 in cancer cells. In our results, cell cycle S phase arrest and apoptosis were induced markedly by ZY0511, which may be due to the increased expression of DDIT4. The histone demethylase inhibitor JIB-04 inhibits cancer growth through *DDIT4*, which also suggests multiple epigenetic regulators of *DDIT4* [40]. As mTORC1 is a critical regulator of autophagy that was found to be inhibited by ZY0511, we detected the expression of LC3A/B in the presence of ZY0511. The result showed LC3A/B was induced by ZY0511 which indicated that ZY0511 may also enhance cell death partly through inducing autophagy (Fig. S2D). Previous research has showed that AKT inhibition reduces expression of the H3K4 methylation specific histone demethylases KDM5 family, especially KDM5B, at the transcriptional levels [25]. We measured the AKT status in the presence of ZY0511. Surprisingly, the level of p-AKT was increased by ZY0511 (Fig. S2D). Because LSD1 inhibition by ZY0511 led to mTOR inhibition through the induction of DDIT4, we speculated mTOR inhibition may in turn induced a week feedback of up-regulation p-AKT. Thus, ZY0511 couldn't inhibit histone demethylases through AKT inhibition.

DDIT4 depletion promoted cancer cell growth but did not completely block the growth inhibitory effect of ZY0511, suggesting the diverse pathways or genes that are regulated by LSD1. WNT-β-catenin signaling [14], androgen signaling [41] and estrogen signaling [2,42] are reported to be correlated with LSD1 in cancer. There is a strong possibility that these pathways play a role in the anticancer effects of ZY0511. H3K9me2 levels were increased in cells after ZY0511 treatment. These results verified the multiple regulatory roles of LSD1 in tumorigenesis. LSD1 promoted carcinogenic gene expression by downregulating H3K9me2 levels and inhibited tumor suppressor gene expression by downregulating H3K4me2 levels. Our research mainly demonstrated that LSD1 inhibition upregulated tumor suppressor gene *DDIT4* by increasing the H3K4me2. LSD1 knockdown decreased the expression levels of the cell cycle-promoting genes SKP2 and CDC25A with significantly increasing levels of repressive marker H3K9me2 in their promoters [43], thus cell cycle S phase arrest induced by ZY0511 may be due to the increased expression of these genes, which needs further investigation.

The main obstacle of cancer chemotherapy is chemotherapy resistance, and combination therapy is considered to be an effective solution. Multidrug-resistant cancer cells show increased levels of JumonjiC demethylases followed by histone methylation status alteration in the body [44]. JumonjiC demethylase inhibitors were verified to have a synergistic effect with taxane-platin in NSCLC [44]. Knockdown of LSD1 prevents hypoxia-induced gefitinib resistance [45] and overcomes resistance to anti-PD-1 therapy in a mouse melanoma model [46]. Our results showed the reduction of DDIT4 could improve cancer cells tolerance capacity to chemotherapeutics treatments. DDIT4 mediates chemotherapeutic effects on cancer cells, which are the top 20 genes that are downregulated in cisplatin-resistant ovarian cancer cell lines [47] and are associated with resistance to topotecan, mitoxantrone, mitomycin C, etoposide and cyclophosphamide [48]. Our findings provide additional evidence for the critical role of DDIT4 in chemotherapy resistance.

We have developed a novel, potent and selective LSD1 inhibitor ZY0511, with potent anti-solid tumor activity. LSD1 knockdown or inhibition by ZY0511 induces cancer cell growth inhibition partly through the DDIT4-mTORC1 pathway. *DDIT4*, a direct downstream target gene of LSD1, negatively regulates cancer progression and plays an important role in cancer cell chemotherapy resistance. The LSD1/DDIT4 axis is a promising therapeutic target that would benefit solid tumor patients.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Yinglan Zhao and Xiaobo Cen conceived the study. Yan Li, Xinying Qian, Lei Tao, Yiyun Lin and Huaqin Zhang performed the experiments. Zeping Zuo and Yang Zhou synthesized compound ZY0511. Chunqi Liu, Hui Jie and Zhuolin Li performed the bioinformatics analysis of the mRNA-seq data. Yan Li wrote the paper and carried out almost the entire study.

Acknowledgments

This work was supported by a Project of the National Natural Science Foundation of China (81773198), National Keypoint Research and Invention Program of the China Ministry of Science and Technology (MOST-2016YFC1303200) and National S&T Major project (2018ZX09201018).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.052.

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