ERK regulates HIF-1α-mediated platinum resistance by directly targeting PHD2 in ovarian cancer

Zhuqing Li[1,2,7], Wei Zhou[1,2,3,7], Yi Zhang[1,2,7], Wei Sun[4], Mingo MH Yung[5], Jing Sun[1,2], Jing Li[1,2], Chi-Wei Chen[1,2], Zongzhu Li[1,2], Yunxiao Meng[1,2], Jie Chai [1,2], Yuan Zhou [1,2], Stephanie S Liu[6], Annie NY Cheung[6], Hextan YS Ngan[5], David W. Chan[5,*], Wei Zheng[4,*], and Wenge Zhu[1,2,*]

[1] Department of Biochemistry and Molecular Medicine, The George Washington University School of Medicine and Health Sciences, Washington, DC 20037, USA
[2] GW Cancer Center, The George Washington University, Washington, DC 20052, USA
[3] Department of Colorectal Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China
[4] National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, MD, 20892, USA
[5] Department of Obstetrics and Gynecology, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China
[6] Department of Pathology, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China
[7] Co-first authors
[*] Co-corresponding authors:
David W. Chan, Tel: (852) 39179367; Email: dwchan@hku.hk
Wei Zheng, Tel: 301-827-5722; Email: wzheng@mail.nih.gov
Wenge Zhu, Tel: 202-994-3125; Email: wz6812@gwu.edu

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Translational Relevance

Identification of mechanisms regulating platinum resistance and new approaches for treatment of platinum-resistant ovarian cancer (PROC) will benefit cancer patients. The amount of HIF-1α plays an important role in platinum-resistance in ovarian cancer, but the mechanism regulating HIF-1α stability, in this context, remained largely unknown. This study demonstrates that PHD2 is a direct substrate of ERK and phosphorylation of PHD2 by increased ERK activity in PROC cells prevents PHD2 binding to HIF-1α, resulting in the inhibition of HIF-α hydroxylation and degradation. Further studies indicate that this ERK/PHD2/HIF-1α pathway is potentiated in PROC cells or tumors by TGF-β1. Inhibition of HIF-1α by YC-1, ERK by selumetinib, or TGF-β1 by SB431542 overcomes platinum-resistance *in vitro* or *in vivo*. These findings reveal an unexpected TGF-β1/ERK/PHD2/HIF-1α pathway regulating platinum-resistance in ovarian cancer. Additionally, this study points to three potential drugs to treat PROC patients.

Abstract

Purpose: Up to 80% of ovarian cancer patients develop platinum-resistance over time of platinum-based chemotherapy. Increased HIF-1α level is an important mechanism governing platinum-resistance in platinum-resistant ovarian cancer (PROC). However, the mechanism regulating HIF-1α stability in PROC remains largely unknown. Here, we elucidate the mechanism of HIF-1α stability regulation in PROC and explore therapeutic approaches to overcome cisplatin resistance in ovarian cancer.

Experimental Design: We first used a quantitative high throughput combinational screen (qHTCS) to identify novel drugs that could re-sensitize PROC cells to cisplatin. Next, we evaluated the combination efficacy of inhibitors of HIF-1α (YC-1), ERK (selumetinib), and TGF-β1 (SB431542) with platinum drugs by *in vitro* and *in vivo* experiments. Moreover, a novel TGF-β1/ERK/PHD2-mediated pathway regulating HIF-1α stability in PROC was discovered.

Results: YC-1 and selumetinib re-sensitized PROC cells to cisplatin. Next, the prolyl hydroxylase domain-containing protein 2 (PHD2) was shown to be a direct substrate of ERK. Phosphorylation of PHD2 by ERK prevents its binding to HIF-1α, thus inhibiting HIF-1α hydroxylation and degradation—increasing HIF-1α stability. Significantly, ERK/PHD2 signaling in PROC cells is dependent on TGF-β1, promoting platinum-resistance by stabilizing HIF-1α. Inhibition of TGF-β1 by SB431542, ERK by selumetinib, or HIF-1α by YC-1 efficiently overcame platinum-resistance both *in vitro* and *in vivo*. The results from clinical samples confirm activation of the ERK/PHD2/HIF-1α axis in PROC patients, correlating highly with poor prognoses for patients.

Conclusions: HIF-1α stabilization is regulated by TGF-β1/ERK/PHD2 axis in PROC. Hence, inhibiting TGF-β1, ERK, or HIF-1α is potential strategy for treating PROC patients.

INTRODUCTION

Most ovarian cancer patients initially respond well to platinum drug-based chemotherapy, but up to 80% patients relapse when becoming resistant to platinum. Resistance to platinum drugs is a big hurdle for treatment of ovarian cancer. Thus, treatments for **p**latinum-**r**esistant **o**varian **c**ancer (**PROC**) remain an unmet need; research into treatments will improve survival and quality of life for these PROC ovarian cancer patients (**1-4**).

Hypoxia-inducible factor 1α (HIF-1α) is a subunit of HIF-1, a heterodimeric transcription factor that is considered the master transcriptional regulator of multiple cellular pathways including cell proliferation, tumor migration, and angiogenesis (**5, 6**). Elevated levels of HIF-1α are associated with tumor metastasis and poor patient prognosis as well as chemo-resistance in several tumors including breast cancer, oropharyngeal cancer, and ovarian cancer (**7, 8**). HIF-1α contains an oxygen-dependent degradation domain (ODDD, residues 410-603) which is responsible for its rapid degradation under normoxic conditions (**9**). In the presence of oxygen, HIF-1α protein is hydroxylated by prolyl hydroxylase domain enzymes (PHDs) on two key proline residues (P^{402}/P^{564}) located within the ODDD, resulting in HIF-1α degradation by an ubiquitin-mediated pathway. Hydroxylation of HIF-1α by PHDs creates a binding site for the von Hippel-Lindau (VHL) E3 ligase, which promotes subsequent ubiquitylation and proteasomal degradation of HIF-1α. Therefore, PHDs are key factors regulating HIF-1α levels in cells; inhibition of PHDs' activity limits HIF1-α degradation (**10**). Nonetheless, the regulatory pathway governing hydroxylation of HIF-1α by PHDs remains largely unknown.

Extracellular regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPK) family and has been implicated in diverse cellular pathways including proliferation,

survival, differentiation, and motility (**11**). Although ERK was shown to control HIF-1α transcriptional activation (**12, 13**), it is unknown if ERK directly regulates HIF-1α protein stability and how ERK participates in platinum-resistance in PROC. The transforming growth factor β (TGF-β), which can activate the ERK pathway, belongs to the TGF superfamily comprising a large number of cytokines secreted by many cell types (**14**). A growing body of evidence indicates that TGF-β signaling is altered in cancer. A dual role for TGF-β in tumors, depending on tumor stage, cellular context, and tumor microenvironment has long been noted. TGF-β exerts tumor-suppressive effects; paradoxically, TGF-β can also act as a tumor activator because of its activity in suppressing immune and inflammatory responses (**15**).

We have established a quantitative high throughput combinational screen (qHTCS) to study platinum-resistance in PROC cells (**16**). Using this qHTCS, we identified the HIF-1α inhibitor (YC-1) and ERK inhibitor (selumetinib) that could overcome platinum-resistance in PROC cells. Further mechanistic studies revealed a novel ERK/PHD2/HIF-1α-mediated pathway regulating platinum-resistance in ovarian cancer cells. Significantly, we found that ERK directly interacts with and phosphorylates PHD2; the phosphorylated PHD2 compromises the interaction of PHD2 with HIF-1α, resulting in the reduction of HIF-1α hydroxylation, which stabilizes the HIF-1α protein in PROC cells. Importantly, both *in vitro* and *in vivo* studies indicated that this ERK/PHD2/HIF-1α pathway is activated in PROC cells and is a major driving force to promote platinum-resistance. Furthermore, our clinical studies confirmed the activation of ERK and HIF-1α in tumors from PROC patients.

Materials and methods

Antibodies and reagents

Antibodies used in immunoblotting: HIF-1α (BD, 610958, 1:1000) were purchased from BD Biosciences. BCL-X_L (ab32370, 1:1000) were from Abcam. β-actin (A5441, 1:1000) and anti-phospho-PHD2 (Ser 125) antibody (MABC1612, 1:1000) were from Sigma-Aldrich. p-ERK (CST, 4370S, 1:1000), ERK (4695S, 1:1000), hydroxy-HIF-1α (3434S, 1:1000), phospho-MAPK/CDK substrates (2325S, 1:1000), TGF-β (3711S, 1:1000) and PHD2 (4835, 1:1000) were from Cell Signaling Technology. Normal mouse IgG (sc-2025), mouse anti-goat IgG-HRP (sc-2354) and mouse anti-rabbit IgG-HRP (sc-2357) were from Santa Cruz Biotechnologies. *Cis*-diamineplatinum(II) dichloride (Cisplatin, Sigma-Aldrich, 479306), HIF-1α inhibitor YC-1 (Selleck Chemicals, S7958), MEK inhibitor, selumetinib (Selleck Chemicals, S1008), and TGF-β receptor inhibitor, SB431542 (Selleck Chemicals, S1067), were dissolved in DMSO and sterile 0.9% saline respectively for the *in vitro* and in *vivo* assay. Lambda Protein Phosphatase (Lambda PP) (P0753S) were purchased from New England Biolabs (NEB).

Quantitative high-throughput combinational screen (qHTCS)

Compound screen experiments were performed two rounds as previously described (**16**). Cisplatin resistant IGROV1 CR cells were used against 6,016 compounds from multiple compound libraries including NPC (NCGC Pharmaceutical Collection), MIPE (Mechanism Interrogation Plate) and LOPAC (The Library of Pharmacologically Active Compounds). Briefly, IGROV CR cells (1,500 cells/well) were plated in 1536-well polystyrene plates (Greiner Bio-One) with 5 µL DMEM medium (10% FBS) and incubated for 16 hr. In the first screen, drugs were tested at 11 different concentrations from 0.8 nM to 46 µM by serial dilution (1:3). After

incubation for 72 hr, total of 112 compounds that efficiently inhibited the proliferation of IGROV1 CR cells were identified. To further identify compounds that have synergy with cisplatin, these 112 compounds were screened at 11 different concentrations in combination with vehicle, or 8.5 μM cisplatin (IC50 of cisplatin). Then 4 μL/well ATP content cell viability assay reagent (Promega, G7570) was added into each well and incubated for 30 mins followed by detection of cell viability. Through the second screen, compounds that exhibited the increased cytotoxicity of cisplatin in IGROV1 CR cells were identified (**Supplementary Table S1**). The primary screen data and curve fitting were analyzed using software developed at the NIH Chemical Genomics Center (NCGC). The half-maximal inhibitory concentration (IC50 values) of the two-drug combinations was compared to that of a single drug.

Cell culture and establishment of resistance cell lines

Human ovarian cancer cell line SKOV3 (American Type Culture Collection) and IGROV1 (gift from Wei Zheng's lab) were cultured in DMEM containing 10% FBS. PEO1/4 cell lines (Sigma) were cultured in RPMI-1640 containing 10% FBS. All the cells were cultured at 37 °C in a cell incubator containing 5% CO_2. For hydroxylated HIF-1α protein analysis, all the cell samples were treated with 10 μM MG-132 (Selleck, S2619) for 8 hr before harvest (**17**). The cisplatin-resistant cell lines (SKOV3 CR and IGROV1 CR) were generated by six cycles of cisplatin treatment in our lab as described in published papers (**16**). The early-passages (less than 10 passages) of the resistant cell lines were used in our present study.

RT-qPCR

Cells were harvested and total RNA was isolated using an RNeasy Mini Kit (Qiagen, 74106) according to the manufacturer's instructions. Using an iScript™ cDNA Synthesis Kit (BioRad,

1708891), cDNAs were prepared and analyzed for the expression of genes by real-time PCR (qPCR) using SsoAdvanced™ Universal SYBR Green Supermi (BioRad, 1725271). The expression of each gene was normalized to the expression of β-actin. The sequences of the HIF-1α primers were: forward, 5’-CGGCGAGAACGAGAAGAA-3’ and reverse, 5’-GCAACTGATGAGCAAGCTCATA-3’ (**18**).

HIF-1α knockdown

Fifty nM HIF-1α siRNA (ThermoFisher, 106498) was transferred into cells using Lipofectamine™ RNAiMAX Transfection Reagent (ThermoFisher, 13778150) according to the manufacturer’s instructions. HIF-1α was stably knocked down using short hairpin RNA (shRNA, OriGene, TG320380) using Lipofectamine 2000 according to the manufacturer’s instructions. Cells were then treated with indicated agents for 48 hr after siRNA transfection or shRNA infection (**19, 20**).

Co-immunoprecipitation (Co-IP) and western blotting

For Co-IP, cells were harvested and lysed in Co-IP buffer (25 mM Hepes, 150 mM KAc, 5 mM $MgCl_2$, 1 mM Na_2EGTA, 10% Glycerol, 0.1% NP-40, and 1% complete protease inhibitor cocktail; Roche). Cell lysates were centrifuged and the supernatant was incubated with indicated antibodies or non-specific IgG as a negative control at 4°C overnight. Then the protein beads (protein A/G plus-Agarose sc-2003, Santa Cruz) were added into the tubes and incubated with the samples at 4°C for one hour. After that, the beads were washed at least three times using Co-IP buffer, and the precipitated proteins were used for further analysis after being mixed with NuPAGE™ LDS Sample Buffer (ThermoFisher, NP0007) and heated at 75°C for 10 minutes. For western blotting, cells were harvested and lysed with RIPA cell lysis buffer (ThermoFisher, 89900). Protein samples mixed with NuPAGE™ LDS Sample Buffer (ThermoFisher, NP0007) and

NuPAGE™ Sample Reducing Agent (ThermoFisher, NP0004) were then separated by NuPAGE™ 4-12% Bis-Tris Protein Gels (ThermoFisher, NP0335BOX) and were transferred to a nitrocellulose membrane (BioRad, 1620115) using transfer buffer (192 mM glycine, 25 mM Tris-HCl, 20% methanol) at 4°C for 2 hr at 100 Volt. The membrane was then blocked with blocking solution (3% Bovine Serum Albumin in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, TBST) at room temperature for 30 mins. Finally, the membrane was incubated with the indicated primary antibodies at 4°C overnight, followed by HRP-conjugated secondary antibodies at room temperature for 2 hr. Western blot analysis was performed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher, 34580).

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene and hydrated with ethanol solutions. After antigen-retrieval treatment (121°C for 10 mins in 10 mM sodium citrate buffer), slides were treated with a 3 % hydrogen peroxide methanol solution for 10 minutes to quench endogenous peroxidase activity. Ten percent normal goat serum was used to block nonspecific binding. Slides were then incubated with a primary antibody at 4°C overnight and the next day incubation of sections with IHC detection reagent (CST, mouse 8125, rabbit 8124) was carried out for 30 minutes at room temperature. An ImmPACT DAB peroxidase (HRP) substrate kit (Vector, SK-4105) was used to visualize the staining according to the manufacturer's instructions. The slides were lightly counterstained with hematoxylin. Staining was quantified for at least five fields at 200X magnification. IHC Score = summation (1+i) pi where i is the intensity score and pi is the percent of the cells with that intensity. In our experiment, we set i from 0-5 (no stain = 0; ≤1/100 cells stained = 1; ≤1/10 cells stained = 2;

≤1/3 cells stained = 3, ≤2/3 cells stained = 4; all cells stained = 5). The quantification of IHC staining was scored blindly by three independent observers (**21**).

Cell viability and clonogenic assay

Sulforhodamine B (SRB) assay was used to detect cell viability as in published papers (**22, 23**). For the clonogenic assay, cells were seeded in a 6-well dishes with a density of 500-800 cells per dish, then treated with 0.3 μM cisplatin, 0.15 μM YC-1, or both for 14 days to allow colony formation. Colonies were stained with crystal violet. Colonies with >50 cells were counted.

Cell apoptosis assay

In vitro cell apoptosis was analyzed by flow cytometry using a FITC Annexin-V Apoptosis Detection Kit (BD Biosciences, 556547) while *in vivo* apoptotic tumor cells were detected using an In Situ Cell Death Detection Kit (Roche, 11684817910) according to the manufacturer's instructions.

ELISA assay

TGF-β1 content in the cell culture supernatant was detected using a Human TGF-β1 Quantikine ELISA Kit (R&D, DB100B) according to the manufacturer's instructions.

Recombinant protein purification and GST Pull-down assay

His-tagged ERK1/ERK2 and GST-tagged PHD2 were expressed in *E. coli* BL21 treated with 0.1 mM IPTG at 16°C for 12 hr to induce protein expression. Bacteria were then harvested and resuspended in PBS containing 0.5% Triton X-100 and 1 mM PMSF, followed by ultrasonication for 20 min. The recombinant His-tagged and GST-tagged proteins were purified using Ni-NTA Agarose (25214, Thermofisher) and Glutathione Agarose (16100, Thermofisher) respectively. For the ERK1/2 and PHD2 GST pull-down assay, GST-tagged PHD2 was incubated with purified

ERK1 or ERK2 at 4°C for 2 hr. The elution was analyzed by western blot with indicated antibodies (**24**).

In vitro kinase assay

For the ERK *in vitro* kinase assay, the bead-bound ERK1/2 (His-tagged ERK1/ERK2 expressed from *E. coli* BL21) were washed at least three times with PBS. The proteins bound to histidine-tagged protein purification resin were separated by SDS-PAGE and visualized by Coomassie Blue staining. Immunoprecipitated IgG or His-ERK1/2 were incubated with recombinant human PHD2 Protein (H00054583, Novus Biologicals) and ATP in a kinase buffer. The supernatants containing phosphorylated protein were subjected to western blot assay (**25**).

Animal experiments

Five- to six-week old female BALB/c athymic nude mice of 20-25 g were purchased from the Jackson Laboratory. The mouse housing conditions and all the animal experiment procedures were performed in accordance with the Institutional Animal Care and Use Committees (IACUC) of George Washington University. After 10 days of recovery from the shipping process, mice were subcutaneously inoculated with IGROV1 or IGROV1 CR cells (suspended in 100 µL PBS, 5×10^6 cells/mouse) into the dorsal flank to establish the subcutaneous cisplatin sensitive and resistant xenografts. Mice were randomized into subsequent experiment groups when the average tumor volume reached 100-150 mm^3. To investigate the synergized effect of cisplatin and YC-1, four groups of mice (8 mice per group) with IGROV1 CR xenograft tumors were intraperitoneally treated with vehicle, cisplatin (4 mg/kg), YC-1 (30 mg/kg), or combination of cisplatin plus YC-1 twice a week for three weeks. To investigate the synergized effect of cisplatin and selumetinib, four groups of IGROV1 CR xenograft tumors-bearing mice (8 mice per

group) were intraperitoneally treated with vehicle, cisplatin (2 mg/kg), selumetinib (50 mg/kg) or combination of cisplatin plus selumetinib for 20 days, once each two days (n=8). For single cisplatin treatment, mice bearing IGROV1 xenograft tumors were intraperitoneally treated with vehicle or cisplatin (0.5, 1, 2 mg/kg intraperitoneally for 2 weeks, twice a week (n=8). Tumor size and body weight were measured every two days. The relative tumor volume was calculated using the formula: $a \times (b^2) / 2$, for which a and b represent the longest and shortest diameters, respectively. All the tissue and tumor samples were obtained 3 days after the final drug administration for subsequent evaluation.

Ovarian cancer patients

The tumor samples of all the chemo-sensitive and matched chemo-resistant ovarian cancer patients were kept as formalin-fixed paraffin-embedded sections in the Department of Pathology at the University of Hong Kong. Studies using human tissues were approved by the local institutional ethics committee (institutional review board reference No: UW 05-143 T/806 and UW 11-298). Written informed consent was received from each patient prior to their inclusion in the study. The histological types, disease stages, and cancer cell contents in each FFPE section were examined by experienced pathologists. Tumors from patients who had a total response to platinum-based therapy and no recurrence within 6 months were defined as platinum-sensitive, and tumors from patients who had the recurrence occur within 6 months following the completion of platinum-based therapy were defined as platinum resistant.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 Software. Data are represented as the mean ± the standard deviation (SD); p-values were calculated with Student's t-tests (unless

otherwise indicated) in all the data comparing control to treatment. *p<0.05, **p<0.01 and ***p<0.001. Progression-free survival (PFS) rates of patients were analyzed via Kaplan Meier analysis by online databases (http://kmplot.com) and a log-rank (Mantel-Cox) test was used in comparison of each arm (**26**).

RESULTS

qHTCS to identify HIF-1α inhibitor YC-1 overcoming cisplatin resistance

To explore new effective therapeutic strategies for treatment of PROC, we compared three paired platinum-resistant ovarian cancer cell lines with their platinum-sensitive controls (**Supplementary Fig. S1**). Among them, two paired cells (IGROV1 versus IGROV1 CR and SKOV3 versus SKOV3 CR) have been described previously (**16**). Another pair of platinum sensitive and resistant ovarian cancer cells (PEO1 and PEO4) was derived from the same patient before (PEO1) and after (PEO4) development of chemo-resistant to platinum-based drug treatment (**16**).

We carried out a two-round drug screen in the qHTCS format, using IGROV1 CR cells, against 6,016 approved drugs and bioactive compounds as previously described (**16**) (Data shown in **Supplementary Table S1**). HIF-1α inhibitor (YC-1) and MEK inhibitor (PD-0325901) were found to exert a very strong growth inhibition on IGROV CR cells when combined with cisplatin (**Fig. 1A**). The results suggest that ERK and HIF-1α may regulate platinum-resistance in ovarian cancer.

To validate the inhibitory efficacy of YC-1 (the validation for MEK inhibitor was included in **Fig. 3**), three PROC cell lines were treated with a combination of YC-1 and cisplatin for 72 hr and cell viability was measured by a sulforhodamine B (SRB) viability assay. Significantly, the combined

treatment of YC-1 with cisplatin showed an excellent synergy to inhibit the growth of PROC cells (**Fig. 1B and C**). The addition of 0.2 μM and 0.4 μM YC-1 reduced the IC50 of cisplatin in IGROV CR cells from 9.8 μM to 2.4 μM and 0.8 μM respectively (**Supplementary Figure S2A**). Consistently, the clonogenic assay indicated that combinatorial treatment with YC-1 and cisplatin significantly reduced the survival of IGROV1 CR cells compared to cisplatin or YC-1 treatment alone (**Fig. 1C**). Together, these results suggest that HIF-1α may regulate platinum-resistance in ovarian cancer cells.

Elevated HIF-1α levels in PROC were reduced by YC-1 treatment both *in vitro* and *in vivo*

To study how YC-1 regulates cisplatin-resistance in ovarian cancer, we examined the expression of HIF-1α and its target gene BCL-X_L in three paired sensitive and resistant cell lines. Strikingly, HIF-1α and BCL-X_L protein expression levels were increased in all three PROC cells compared to their sensitive counterparts (**Fig. 2A**). However, the HIF-1α mRNA levels in all three paired parental and resistant cell lines were not changed (**Supplementary Fig. S2B**), suggesting that increased HIF-1α protein levels are due to an alteration of the protein post-transcription pathway(s) in PROC cells.

We next depleted HIF-1α by using two independent short-hairpin RNAs (shRNAs) to explore possible HIF-1α regulation of cisplatin resistance in ovarian cancer. Downregulation of HIF-1α by shRNA redcued the BCL-X_L level and re-sensitized IGROV1 CR cells to cisplatin (**Supplementary Fig. S2C and Fig. S2D**). HIF-1α knockdown by siRNA in IGROV1 CR cells also reduced BCL-X_L levels and re-sensitized IGROV1 CR cells to cisplatin (**Supplementary Fig. S2E and Fig. S2F**). To further confirm HIF-1α's important role in cispaltin-resistance, we treated sensitive IGROV1 cell with $CoCl_2$, a chemical inducer of HIF-1α, followed by cisplatin treatment

(**Supplementary Fig. S2G**) (**27**). The cells with higher levels of HIF-1α induced by $CoCl_2$ exhibited stronger resistance to cisplatin compared to cells treated with vehicle (**Supplementary Fig. S2H**). Together, these observations suggest that HIF-1α is crucial for the regulation of platinum-resistance in ovarian cancer.

In addition, YC-1 treatment dramatically reduced both HIF-1α and BCL-X_L protein levels in a dose-dependent manner (**Fig. 2B**). Consistent with the synergistic effect of YC-1 and cisplatin on growth inhibition of resistant cells, the combination of cisplatin and YC-1 significantly increased the apoptotic population (**Supplementary Fig. S2I**), and promoted the cleavage of PARP-1 (**Supplementary Fig. S2J**), a hallmark of apoptosis (**28**).

To test the hypothesis of YC-1 inhibiting the growth of PROC cells *in vivo*, we applied a tumor graft model by subcutaneously implanting IGROV1 CR cells into nude mice to form tumors. Mice were randomized for treatment with vehicle, cisplatin (4 mg/kg), YC-1 (30 mg/kg), or a combination of cisplatin and YC-1. Three weeks after the treatment, cisplatin or YC-1 alone had little effects on IGROV1 CR xenograft tumors, but the combined treatment of YC-1 and cisplatin significantly reduced the tumor size (**Fig. 2C**). Immunohistochemistry (IHC) in these treated tumors indicated that treatment of YC-1, either in monotherapy or combination with cisplatin, significantly reduced the HIF-1α and BCL-X_L levels; furthermore, the combined treatment with YC-1 and cisplatin increased the apoptotic population as indicated by TUNEL staining (**Fig. 2D-F**). During the treatment course, there was no change in body weight or obvious histological change in heart, liver, kidney, or lung among the various treatment groups (**Supplementary Fig. S2K and L**).

ERK activation upregulates HIF-1α levels in PROC

MAPK has been shown to regulate HIF-1α expression (**29, 30**). Given the identification of a MEK inhibitor, PD-0325901, that overcame cisplatin resistance in IGROV CR cells from qHTCS (**Fig. 1A**) and the fact that PD-0325901 suppresses phosphorylation of ERK1/2 (**31**), we speculated that the ERK pathway may regulate HIF-1α as well as cisplatin-resistance in ovarian cancer cells. To test this hypothesis, we examined the activation of ERK in all three PROC cell lines. Significantly increased phosphorylated-ERK (p-ERK) as well as HIF-1α were seen in all three resistant cell lines compared to their sensitive counterparts (**Fig. 3A**), indicating the activation of the ERK pathway in PROC cell lines.

To test the ability of ERK1/2 to regulate HIF-1α protein levels, we used a selective MEK1/2 inhibitor, selumetinib, which has been tested in Phase II clinical trials and proved to significantly inhibit phosphorylation of ERK1/2 (**32**). As expected, selumetinib was able to reduce the p-ERK and HIF-1α levels in a dose-dependent manner (**Supplementary Fig. S3A**). Moreover, selumetinib efficiently inhibited the ERK/HIF-1α pathway and reduced cell viability when combined with cisplatin and the combination treatment exhibited an excellent synergistic effect in all three resistant cell lines (**Fig. 3B and C，Supplementary Fig. S3B-D**). These results demonstrate that ERK is critical to maintaining HIF-1α levels in PROC cell lines.

We next established the IGROV1 CR xenograft tumor-bearing mouse system to further examine the effect of cisplatin and selumetinib in combination on tumors *in vivo*. Although cisplatin alone had no effects on tumor growth due to resistance, selumetinib alone significantly inhibited tumor growth and combined drug treatments exhibited further inhibition on tumor growth (**Fig. 3D**). Immunoblot and IHC analysis of tumor samples indicated selumetinib could inactivate ERK/HIF-1α signaling in the xenograft tumors after the treatment

(**Fig. 3E, F and G**). Importantly, combined treatment of selumetinib and cisplatin had no effects on body weight with no histological changes in heart, liver, kidney, or lung among the various treatment groups (**Supplementary Fig. S3E and F**).

Since ERK can regulate protein expression either transcriptionally or post-transcriptionally (**33**), we therefore examined mRNA levels of HIF-1α by reverse transcription-quantitative real-time PCR (RT-qPCR) in IGROV1 CR cells after treatment with cisplatin, selumetinib, or both. The results indicated that selumetinib or selumetinib plus cisplatin did not change HIF-1α mRNA level in IGROV1 CR cells (**Supplementary Fig. S3G**), indicating that ERK regulates HIF-1α expression through a post-transcriptional regulatory pathway.

ERK increases HIF-1α stability by directly interacting and phosphorylating PHD2

HIF-1α protein stability is regulated by a ubiquitin-mediated pathway after hydroxylation by prolyl hydroxylases (PHDs) (**9**). Among all three PHDs, PHD2 is the most abundant form, we explored the possibility that ERK regulates HIF-1α expression by directly targeting PHD2 (**34, 35**).

We first examined the hydroxylation levels of HIF-1α in PROC cell lines and found that hydroxylated HIF-1α (OH-HIF-1α) was decreased in all three resistant cell lines, whereas higher levels of HIF-1α were detected (**Fig. 4A**). Given that the total PHD2 levels were unchanged in paired resistant cells (**Supplementary Fig. S4A**), we hypothesized that ERK might directly target PHD2 and regulate its activity. To test this hypothesis, we examined the phosphorylation levels of PHD2 at serine residues using a specific phosphorylated-MAPK substrate antibody against serine residues in PROC cells. Significantly, in all three PROC cell lines, phosphorylation of PHD2 was increased compared to their sensitive counterparts (**Fig. 4B and Supplementary Fig. S4B**). Strikingly, the interaction between HIF-1α and PHD2 in all resistant cells was decreased (**Fig. 4B**

and Supplementary Fig. S4B), suggesting that the phosphorylation of PHD2 by ERK may interfere the interaction between HIF-1α and PHD2.

We, therefore, tested ERK interactions with PHD2 by a co-immunoprecipitation (Co-IP) assay. From endogenous Co-IP of PHD2 in both IGROV1 and PEO1 cells, we could detect ERK proteins and vice versa (**Fig. 4C and Supplementary Fig. S4C**). Consistently, the *in vitro* GST pull-down assay indicated that both His-tagged ERK1 and ERK 2 interacted with GST-fused PHD2 (**Fig. 4D**), suggesting a direct interaction between PHD2 and ERK1/2.

We assumed that in resistant cells, enhanced phosphorylation of PHD2 by ERK may be due to the increased direct interaction between PHD2 and ERK. Indeed, the result showed that the interaction between PHD2 and ERK was significantlty increased in all resistant cells compared to their sensitive counterparts (**Supplementary Fig. S4D**). We next observed that inhibition of ERK by selumetinib reduced phosphorylated-PHD2 on serine residues and increased the interaction between PHD2 and HIF-1α (**Fig. 4E and Supplementary Fig. S4E**). Also, selumetinib treatment further increased HIF-1α hydroxylation as well as its degradation (**Fig. 4F and Supplementary Fig. S4F**).

Since both ERK1 and ERK2 directly interact with PHD2, we hypothesized that PHD2 might be a phosphorylated substrate of ERK. To test this hypothesis, we screened the potential phosphorylation sites of PHD2 using an *in vitro* kinase assay, and found that PHD2 was phosphorylated at Ser125 by ERK1/2, as reconized by an anti p-PHD2 (Ser125) antibody (**Fig. 4G)**. Consistently, inhibition of ERK by MEK inhibitor selumetinib significantly reduced phosphorylation of PHD2 at Ser 125, increased HIF-1α hydroxylation as well as its degradation (**Fig. 4H)**. Furthermore, S125A mutation of PHD2 significantly increased the interaction

between PHD2 and HIF-1α compared to wild-type PHD2 (**Fig. 4I)**. Together, the results suggest that ERK directly phosphorylate PHD2 at Ser125 site and phosphorylation of PHD2 by ERK prevents its binding to HIF-1α, thus preventing HIF-1α hydroxylation and subsequently preventing HIF-1α ubiquitination and degradation.

Autocrine TGF-β1 activates ERK/PHD2/HIF-1α pathway to cause platinum resistance

We next sought to determine how ERK is activated in PROC cell lines. Since cytokines including TGF-β1 and growth factors are known activators of ERK (**36, 37**), we hypothesized that ERK may be activated by TGF-β1, resulting in platinum-resistance in PROC cell lines. We first collected conditioned medium from PROC cells (IGROV1 CR, PEO4, SKOV3 CR) and used the medium to culture platinum-sensitive cells (IGROV1, PEO1, SKOV3). Interestingly, we found that cells cultured with the conditioned medium from PROC cells exhibited a better survival when treated with cisplatin (**Supplementary Fig. S5A**), indicating that secreted factors from PROC cells may contribute to the acquired resistance in platinum-sensitive cells. We also found that TGF-β1 levels in the cell culture medium were increased in all three resistant cell lines (**Fig. 5A**). We next treated platinum-sensitive cells with recombinant human TGF-β1 (R&D, 7754-BH-005) which induced cisplatin resistance in sensitive cells (**Fig. 5B and Supplementary Fig. S5B**). The TGF-β1 levels were also increased inside all resistant cell lines (**Fig. 5C**). Moreover, recombinant TGF-β1 increased p-ERK and HIF-1α levels in both sensitive IGROV1 and PEO1 cells and this activation of the ERK/HIF-1α pathway was reversed by selumetinib treatment (**Fig. 5D and Supplementary Fig. S5C**).

To further test the notion that the TGF-β1/ERK/PHD2/HIF-1α pathway contributes to platinum-resistance in ovarian cancer cells, we treated all these resistant cells with a TGF-β1

inhibitor, SB431542, and found that inhibition of TGF-β1 decreased p-ERK and HIF-1α levels but increased hydroxylated HIF-1α (**Fig. 5E and Supplementary Fig. S5D**). Consistently, inhibition of TGF-β1 by SB431542 decreased PHD2 phosphorylation and increased the interaction between PHD2 and HIF-1α (**Fig. 5F and Supplementary Fig. S5E**). Similar to YC-1 and selumetinib, SB431542 also exhibited a synergistic effect with cisplatin on growth inhibition in PROC cell lines (**Fig. 5G and Supplementary Fig. S5F**).

Collectively, these findings demonstrate that secreted TGF-β1 acts as an autocrine factor to stimulate the activation of the ERK/PHD2/HIF-1α pathway, thereby inducing platinum-resistance in PROC cells.

Cisplatin induces the activation of TGF-β1/ERK/HIF-1α signaling *in vitro* and *in vivo*

Given that TGF-β1/ERK/HIF-1α signaling is up-regulated in cisplatin-resistant cells, we then assumed that cisplatin treatment may cause an activation of the TGF-β1/ERK/PHD2/HIF-1α pathway in sensitive ovarian cancer cells. To test this possibility, three sensitive cell lines were treated with multiple dosages of cisplatin for four days. The TGF-β1/ERK/HIF-1α signaling was dramatically activated in all the three cisplatin sensitive cell lines (IGROV1, SKOV3 and PEO1) after cisplatin treatment (**Supplementary Fig. S6A**). The TGF-β1/ERK/PHD2/HIF-1α activation was also observed in a time-dependent manner after 1 μM cisplatin treatment (**Supplementary Fig. S6B**). Consistent with these results, the cisplatin treatment increased phosphorylated-PHD2 levels and decreased the interaction of PHD2 with HIF-1α (**Supplementary Fig. S6C**).

We then established IGROV1 xenograft tumors that were treated with cisplatin for two weeks to determine the *in vivo* effect of cisplatin on the ERK/HIF-1α pathway. IHC analysis indicated a significant cisplatin-doses-dependent increase of p-ERK, HIF-1α, and BCL-X_L protein

levels in IGROV1 tumors (**Supplementary Fig. S6D and E**). Thus, the results indicate that cisplatin treatment induces the activation of the ERK/HIF-1α/BCL-X_L pathway in tumor cells *in vivo* which contributes to the development of cisplatin resistance.

Clinical evidence that TGF-β1/ERK/HIF-1α is activated in tumors from platinum sensitive or resistant patient tumors

To verify the TGF-β1/ERK/HIF-1α pathway is activated in platinum-resistant tumors from ovarian cancer patients, we used a Kaplan-Meier plotter to analyze the correlations of TGF-β1, ERK, or HIF-1α levels with the survival of ovarian cancer patients who received platinum-based chemotherapy. The results showed that the higher TGF-β1/HIF-1α levels and the higher the ERK signature genes expression levels, the worse the 5-year progression-free survival (PFS) (**Fig. 6A**). We also evaluated these correlations in the samples from the same patients before platinum drug treatment and after development of resistance to platinum drugs. To this end, IHC staining was performed to detect the p-ERK and HIF-1α levels in tumors (**Fig. 6B**). The HIF-1α level was significantly increased in all seven resistant tumors, while the p-ERK level was elevated in six of seven resistant tumors (**Fig. 6C**). The Pearson correlation coefficient ($r=0.729$, $p<0.01$) indicates a strong correlation between elevated levels of p-ERK and HIF-1α (**Fig. 6D**).

Therefore, our results indicate that the activation of the TGF-β1/ERK/HIF-1α pathway is highly correlated with platinum-resistance and poor prognoses for ovarian cancer patients.

Discussion

HIF-1α has been reported to promote cisplatin resistance in ovarian cancer (**38-40**); however, the mechanism regulating HIF-1α in ovarain cancer remains largely unknown. In this study, a

qHTCS assay was used to identify inhibitors of HIF-1α and MEK, which re-sensitized PROC cells to cisplatin. The follow-up mechanistic study not only revealed a novel TGF-β1/ERK/PHD2/HIF-1α pathway, the activation of which causes cisplatin resistance in PROC cells, but also points to three potential drugs to treat PROC patients. More importantly, for the first time, we provide clinical evidence confirming the link between the activation of ERK and HIF-1α in tumors from PROC patients.

We have demonstrated that in PROC cells activated ERK direclty phoshorylates PHD2; this phosphorylation reduces the interaction of PHD2 with HIF-1α, preventing HIF-1α hydroxylation and degradation (**Fig. 6E**). Importantly, this study shows targeting of TGF-β1 by SB431542, ERK by selumetinib, or HIF-1α by YC-1 overcame cisplatin-resistance *in vitro* and *in vivo*. Previously YC-1 was found to degrade HIF-1α, thus inhibiting tumor growth (**38**). Selumetinib is currently under Phase II clinical trials for cancer treatments of advanced hepatocellular carcinoma and multiple myeloma (**41, 42**). A phase I trial has shown low toxicity of selumetinib (**43, 44**). Thus, our study provides a solid mechanistic basis and preliminary clinic evidence, supporting the clinical application of combining selumetinib and platinum drugs for treatment of PROC.

Under normoxic conditions, HIF-1α is hydroxylated on two key proline residues P^{402} and P^{564} located within the ODDD domain by PHDs in the presence of oxygen, α -ketoglutarate, and iron. Hydroxylation of HIF-1α creates a binding site for the von Hippel-Lindau (VHL) tumor suppressor E3 ligase complex, resulting in polyubiquitylation and proteasomal degradation of HIF-1α (**45, 46**). PHD1/2 can be regulated by the post-transcriptional level, which affects their interaction with HIF-1α. For example, PHD1 phosphorylation at Ser-130 in CDK-dependent

manner lowers its activity towards HIF-1α (**47**). PHD2 phosphorylated on Ser-125 by rapamycin (mTOR), a downstream kinase of P70S6K increases its ability to degrade HIF-1α (**48**). JNK2-mediated phosphorylation of PHD1 at Ser-74 and Ser-162 increases the degradation of HIF-1α (**49**). Our study elucidates that phosphorylation of PHD2 by ERK stabilizes HIF-1α, thus leading to resistance to cisplatin in PROC cells. It is not known whether this mechanism exists in other physiological or pathological conditions. Our study sheds the light for further study to investigate whether this novel mechanism regulates tumorigenesis and chemo response in the future.

ERK/MAPK signaling is activated in many cancers in response to growth factors. TGF-β signaling has been reported to activate the MEK/ERK pathway; inhibition of TGF-β signaling restores drug responsiveness in MED12KD cells (**36**). Silencing TGF-β1 expression in lung cancer cells may deactivate the ERK, JNK, and p38 MAPK pathways (**37**). Consistant with these findings, the results of this study revealed the autocine signaling in TGF-β1 in PROC cell lines caused the activation of the ERK/PHD2/HIF-1α pathway, resulting in resistance to platinum treatments. The clinic evidence showed TGF-β1 levels in tumor samples strongly correlate with poor patient prognosis. Thus, determination of TGF- β1 levels may be used as a biomarker to predict platinum-resistance in ovarian cancer patients. Of course, studies using a large amount of clinical samples need be conducted to confirm it in future.

Platimun drugs have been used to treat several other cancers, including small cell lung cancer and pancreatic adenocarcinoma (**50**); HIF-1α has been shown to regulate cancer cell survival in many types of cancer (**38-40**). These two facts, along with our discoveries detailed in this paper, offer insights and hope for treating PROC. Future work will illuminate the role of the

ERK/PHD2/HIF-1α pathway in other drug-resistant cancers. These studies are expected not only to elucidate the mechanistic basis of cellular response to platinum treatments, but also provide potential new therapeutic approaches for the treatment of drug-resistant cancers.

Author contributions

Li ZQ, Zhou W, Zhang Y, and Zheng W contributed to designing experiments, interpreting results and writing the manuscript. Li ZQ, Zhou W, Zhang Y, Sun W, Yung MMH, Sun J, Li J, Chen CW, Li ZZ, Meng YX, Chai J, and Zhou Y contributed by performing experiments. Liu SS, Cheung ANY, Ngan HYS, Dai ZJ, and Chan DW contributed by the collection and analysis of the samples from patients. Zhu W supervised the project and acquired funding. All authors assisted in editing the manuscript and approved it before submission.

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Figure legends

Figure 1. Identification of YC-1 that synergizes with cisplatin to re-sensitize PROC cells to platinum drug

(**A**) qHTS to identify HIF-1α inhibitor YC-1 and MEK inhibitor PD-0325901 that re-sensitize IGROV CR cells to cisplatin. The heatmap represents the IC50 without (left panel) or with (right panel) cisplatin treatment based on the IC50 value from blue (the lowest) to red (the highest). Drugs from top to bottom are arranged based on the ratio of the IC50 (IC50 vehicle/IC50 with cisplatin) from high to low. (**B**) Synergy matrix (bottom) and surface plots (top) showing the synergy effect between YC1 and cisplatin in PROC cells (n=3) including IGROV1 CR, SKOV3 CR and PEO4. (**C**) Representative colony formation (top) and quantification (bottom) of IGROV1 and IGROV1 CR cells 14 days after treatment with 0.3 µM cisplatin, 0.15 µM YC-1 or together. Colonies were stained with crystal violet. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA. Data are mean ± SD from three independent experiments performed in triplicate. The same statistical analysis was conducted in the following figures.

Figure 2. HIF-1α signaling contributes to platinum-resistance in PROC *in vitro* and *in vivo*

(**A**) Immunoblot analysis of HIF-1α and BCL-X_L in parental and platinum resistant ovarian cancer cells. (**B**) Immunoblot analysis of HIF-1α and BCL-X_L in IGROV1 CR cells treated with the indicated concentrations of YC-1 for 24 hr. (**C**) Growth curves of IGROV1 CR xenograft tumors intraperitoneally treated with vehicle, cisplatin (4 mg/kg), YC-1 (30 mg/kg) or cisplatin plus YC-1 (combo) twice a week for three weeks (8 mice per group). A photograph a representative mouse tumor from each treatment arm is shown. Ruler scale is in cm. (**D**) Representative hematoxylin

and eosin (H&E), IHC, and TUNEL staining of tumor samples from mice treated with indicated drugs. Tumors were collected 3 days after the last treatment. Scale bar, 100 µm for H&E, BCL-X_{L}, and HIF-1α; 50 µm for TUNEL. (**E and F**) Quantification of IHC staining (**E**) and apoptotic cells (**F**) in tumor samples from mice treated with indicated drugs (5 mice per group).

Figure 3. ERK1/2 regulate platinum-resistance by controlling HIF-1α protein level

(**A**) Immunoblot analysis of phosphorylated-ERK (p-ERK) and HIF-1α levels in parental and platinum resistant ovarian cancer cells. (**B**) ERK/HIF-1α signaling in PROC cells treated with cisplatin (5µM), selumetinib (10 µM) or a combination of both (combo) for 48 hr. (**C**) Synergetic effect of selumetinib (5 µM) and cisplatin (2 to 32 µM) in IGROV1 CR cells after 72 hr treatment. The combination index (CI) values are presented below the bars. (**D**) Tumor growth curves of IGROV1 CR xenograft tumor-bearing mice intraperitoneally treated with vehicle, cisplatin (2 mg/kg), selumetinib (50 mg/kg) or combination of cisplatin plus selumetinib for 20 days, once every two days (n=8). (**E**) Immunoblot analysis of ERK/HIF-1α signaling of tumor samples from mice bearing IGROV1 CR xenograft tumors intraperitoneally treated with indicated drugs as in Figure 3D. (**F and G**) Representative H&E and IHC staining (**F**) and quantification of IHC staining (**G**) of tumor samples from mice treated with indicated drugs. Tumors were collected 2 days after the last treatment. Scale bar, 100 µm for H&E, p-ERK, and HIF-1α.

Figure 4. ERK1/2 regulate HIF-1α signaling by directly interacting with and phosphorylating PHD2

(A) Immunoblot analysis of OH-HIF-1α and HIF-1α in parental and platinum resistant ovarian cancer cells. (**B**) Immunoprecipitation (IP) analysis revealed elavated phosphorylation of PHD2 (Ser residues) limited binding between PHD2 and HIF-1α, as well as increased HIF-1α protein levels in IGROV1 and IGROV1 CR cells. (**C**) Co-IP assays to detect the direct interaction between endogenous PHD2 and ERK in IGROV1 cells. (**D**) GST pull-down assay to detect the interaction of purified His-ERK1 or His-ERK2 with GST-PHD2. (**E and F**) Examination of phosphorylated-PHD2 (serine residues) protein levels and the interaction between PHD2 and HIF-1α by immunoprecipitation (IP) (**E**), and immunoblot analysis of hydroxylated-HIF-1α (OH-HIF-1α) and HIF-1α (**F**) in PROC cells treated with 10 μM selumetinib for 48 hours. (**G**) In vitro kinase assay of PHD2 phosphorylation by ERK1 or ERK 2, as recognized by anti-phospho-PHD2 (Ser 125) antibody. GST-PHD2 protein was incubated with His-ERK1 or His-ERK2 proteins in the presence of ATP. (**H**) In inhibition of ERK by selumetinib reduced phospho-PHD2 (p-PHD2) on Ser 125, increased HIF-1α hydroxylation as well as its degradation. (**I**) S125A mutation of PHD2 increased the interaction between PHD2 and HIF-1α. U2OS cells expressing HA-PHD2 or HA-PHD2 (S125A) were lysated and immunoprecipitated with anti-HA antibody, followed by immunoblotted for indicated proteins.

Figure 5. TGF-β1 regulates ERK/HIF-1α signaling in PROC cells

(**A**) Elisa assay of TGF-β1 levels (pg/mL) in platinum-sensitive/resistant cell culture supernates. (**B**) Relative cell viability of IGROV1 cells when treated with different doses of cisplatin (2 to 32 μM) or cisplatin plus 10 μg/mL TGF-β for 72 hr. (**C**) Immunoblot analysis of TGF-β1 and HIF-1α in parental and PROC cells. (**D**) Immunoblot analysis of TGF-β1/ERK/HIF-1α signaling in platinum-

sensitive cells (IGROV1) treated with 10 μg/mL TGF-β, 10 μM selumetinib, or both for 48 hr. (**E and F**) TGF-β1/ERK/HIF-1α signaling (**E**) and IP analysis of phosphorylated-PHD2 (serine residues) protein levels and the PHD2-HIF-1α interaction (**F**) in IGROV1 CR cells treated with SB431542 (TGF-β1 inhibitor) at 10 μM for 48 hr. (**G**) Synergetic effect of TGF-β1 receptor inhibitor SB431542 (5 μM) and cisplatin (2 to 32 μM) in IGROV1 CR cells after 72 hr treatment; the CI values for different concentrations are presented below the bars.

Figure 6. Clinical evidence of activated TGF-β1/ERK/HIF-1α pathway in PROC patients

(A) Kaplan–Meier analyses of 5-year PFS rate based on clinical and molecular data for ovarian cancer patients with platinum treatment history (http://kmplot.com/analysis/index.php?p=service&cancer=ovar). The patients were stratified respectively by the expression levels in their tumors of HIF-1α protein expression (left panel), the ERK signature genes (middle panel), and the expression levels in their tumors of the TGF-β1 protein (right panel). Medium survival, log-rank (Mantel-Cox) p values, and hazard ratio (HR) are shown. (**B**) Representative IHC images of HIF-1α and p-ERK in tumor samples from three patients (P1, P6 and P7) showing initial chemo-sensitivity and later chemo-resistance, respectively. (**C**) IHC quantification of HIF-1α and p-ERK in tumor samples from seven patients before and after development of resistance to platinum drug-based therapy. (**D**) The correlation between IHC HIF-1α and p-ERK protein levels in patients' tumors (Pearson's r from -1 to 0 indicates a perfect negative linear relationship, an r of 0 indicates no linear relationship, and an r from 0 to 1 indicates a perfect positive linear relationship between variables). (**E**) Proposed model for TGF-β1/ERK/HIF-1α signaling activation contributing to platinum-resistance

in PROC cells. Inhibiting the pathway with a TGF-β1 inhibitor (SB431542), ERK inhibitor (selumentinib), HIF-1α inhibitor (YC-1), or various combinations of these drugs, re-sensitizes platinum-resistant ovarian cancers.