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DUOXA1-mediated ROS production promotes cisplatin resistance by activating ATR-Chk1 pathway in ovarian cancer

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Author contributions

Y.M., C.C., W.S., W. Zheng, D.W.C., W. Zhu contributed to design experiments, interpret results, and write the manuscript. Y.M., C.C., W.S., W. Zhou, J.L., J.S., Zhuqing Li, Zongzhu Li, J.C.B., Y.K., W.P., A.T., Y.L., Z.D., contributed to perform experiments. M.M.Y., S.S.L., A.N.C., H.Y.N., D.W.C., contributed to collection and analyze the samples from patients. W. Zheng and W. Zhu supervised the project and acquired funding. All authors assisted in editing the manuscript and approved it before submission.

Conflicts of Interest

None to declare

Abstract

The acquisition of resistance is a major obstacle to the clinical use of platinum drugs for ovarian cancer treatment. Increase of DNA damage response is one of major mechanisms contributing to platinum-resistance. However, how DNA damage response is regulated in platinum-resistant ovarian cancer cells remains unclear. Using quantitative high throughput combinational screen (qHTCS) and RNA-seq, we show that dual oxidase maturation factor 1 (DUOX1) is overexpressed in platinum-resistant ovarian cancer cells, resulting in over production of reactive oxygen species (ROS). Elevated ROS level sustains the activation of ATR-Chk1 pathway, leading to resistance to cisplatin in ovarian cancer cells. Moreover, using qHTCS we identified two Chk1 inhibitors (PF-477736 and AZD-7762) that re-sensitize resistant cells to cisplatin. Blocking this novel pathway by inhibiting ROS, DUOX1, ATR or Chk1 effectively overcomes cisplatin resistance *in vitro* and *in vivo*. Significantly, the clinical studies also confirm the activation of ATR and DOUXA1 in ovarian cancer patients, and elevated DOUXA1 or ATR-Chk1 pathway correlates with poor prognosis. Taken together, our findings not only reveal a novel mechanism regulating cisplatin resistance, but also provide multiple combinational strategies to overcome platinum-resistance in ovarian cancer.

Keywords

ROS; high throughput screen; cisplatin resistance; ovarian cancer; DUOX1; ATR-Chk1

1. Introduction

Accumulating evidence has showed that most of advanced ovarian cancer patients have a good response to initial therapy with combination of cytoreductive surgery and platinum-based chemotherapy [1]. However, approximately 70% of patients will develop resistance to platinum drugs over the time of treatment [2, 3]. Multiple mechanisms have been identified to regulate platinum-resistance [4]. Among them, increased DNA damage response is positively correlated with platinum-resistance [5–7]. However, the mechanism of sustaining the high activity of DNA damage response in platinum resistant ovarian cancer cells remains largely unknown.

Reactive oxygen species (ROS) is composed of free radicals and reactive metabolites, such as superoxide, hydroxyl radical, and hydroxide [8]. Emerging evidence has shown that ROS act as a double-edged sword in living organisms [9]. Low dose of ROS is able to promote cell proliferation, mediate cell signal transduction, and under certain circumstance can promote cellular survival and tumor growth [10–12]. In contrast, excessive amount of ROS is capable of causing cellular damage on lipid, DNA, RNA and proteins [13]. In cancer cells, increased ROS accumulation can be resulted from activation of oncogenes [14–16]. Several proteins are well-known in regulating ROS production, including FOXM1, DPP4, PTPN11, ABL1, and Dual oxidase 1 (DUOX1) [15–19]. Dual oxidase 1 (DUOX1) is composed of DUOX1 and DUOX2 [20, 21]. DUOX1 has been shown to mediate thyroid hormonogenesis through production of hydrogen peroxide [22]. It was also reported that overexpression of DUOX1 inhibits murine muscle satellite cell differentiation by raising ROS production [19].

It is well documented that platinum drugs kill cancer cells by introducing DNA crosslinks via covalent bond between DNA bases and thereby, reducing cell viability [23]. FANCD2 and other members of Fanconi anemia family play a critical role in repairing DNA crosslink damage. Activation of FANCD2 is regulated through its mono-ubiquitination [24–27]. ATR (ataxia telangiectasia and Rad3-related protein) is a serine/threonine protein kinase that is essential to regulate DNA damage response in cells with DNA crosslinks [28]. In cells with DNA crosslinks, ATR activates Checkpoint kinase 1 (Chk1) by phosphorylation of at Ser-317 and Ser-345 [29]. Chk1 is important to mediate DNA damage repair by activating repair factors such as Rad51, FANCE and PCNA [30].

To identify the novel mechanism regulating cisplatin resistance, we conducted a quantitative high throughput combinational screen (qHTCS) together with RNA-sequencing (RNA-seq) in cisplatin resistant ovarian cancer cells [31, 32]. From this qHTCS, we identified two Chk1 inhibitors (PF-477736 and AZD-7762) that synergistically enhanced cisplatin efficacy against cisplatin resistant ovarian cancer cells. Significantly, we found DOUXA1 was up-regulated in platinum-resistant cells and elevated DOUXA1 increased ROS levels, which sustained the active ATR-Chk1 pathway. Inhibition of ROS, DUOX1, ATR or Chk1 effectively overcame cisplatin resistance *in vitro* or *in vivo*. The clinical studies also confirmed the activation of ATR and in recurrent and resistant ovarian cancer patients, and elevated ATR-Chk1 or DOUXA1 DUOX1 was correlated with poor prognosis in ovarian cancer patients who have received platinum drug-based therapy.

2. Methods and Materials

2.1. Cell lines and generation of resistant cells SKOV3 was from the American Type Culture Collection (ATCC). PEO14 and PEO23 were from Sigma-Aldrich. SKOV3 was cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). PEO14 and PEO23 were cultured in RPMI-1640 supplemented with 10% FBS. Establishment of cisplatin resistant cell line SKOV3 CR was as described previously [33]. Details were provided in Appendix S1 (Supplementary methods).

2.2. Antibodies and reagents were provided in Appendix S1 (Supplementary methods)

2.3. Cell viability assay Cell viability and IC₅₀ were measured as previous descriptions [34] [35]. Details were provided in Appendix S1 (Supplementary methods).

2.4. Quantitative high throughput combinational screen (qHTCS) Assay was followed as the previous study [32]. Details were provided in Appendix S1 (Supplementary methods).

2.5. RNA-sequencing (RNA-seq) Cells were collected at log growth phase. RNA isolation and RNA sequencing were performed by Q2 Solutions – EA Genomics. RNA sequence data have been submitted to Gene Expression Omnibus (GEO). The accession number is GSE98559. Details were provided in Appendix S1 (Supplementary methods).

2.6. Kaplan-Meier plotter Patients survival rates were analyzed with GSE and TCGA databases using platform (<http://kmplot.com>). Details were provided in Appendix S1 (Supplementary methods) [36].

2.7. Specimens of ovarian cancer patients Detailed information on human subject was provided in Appendix S1 (Supplementary methods) and Appendix S2 (Supplementary table).

2.8. Immunohistochemistry Details were provided in Appendix S1 (Supplementary methods).

2.9. siRNA interference siRNA targeting Chk1, DUOXA-1, and a negative control (siGL2) were as described previously [37–39]. Details were provided in Appendix S1 (Supplementary methods).

2.10. Flow cytometry and Modified comet assay Assays were performed as previous study [40]. Details were provided in Appendix S1 (Supplementary methods).

2.11. Clonogenic cell survival assay and Metaphase chromosome spread Details were provided in Appendix S1 (Supplementary methods).

2.12. Reactive Oxygen Species (ROS) detection, qPCR, and TUNEL assay Details were provided in Appendix S1 (Supplementary methods).

2.13. Animal experiments Animal housing and all procedures were conducted with protocols approved by the Institutional Animal Care and Use Committees (IACUC) of The George Washington University. Details were provided in Appendix S1 (Supplementary methods).

3. Results

3.1. qHTCS and RNA-seq to identify Chk1 inhibitors that overcome cisplatin resistance in ovarian cancer cells

The main goal of this study is to integrate two platforms, qHTCS and genomic sequencing, to identify novel mechanisms regulating platinum-resistance in ovarian cancer and small molecules that can overcome platinum resistance in ovarian cancer cells. The identified pathways will be further confirmed by *in vivo* as well as clinical studies (Figure 1A). We first generated the cisplatin resistant cells using procedures as described previously and in methods [41, 42]. A resistant cell line, SKOV3 CR, was generated that exhibited 5-fold increase of IC₅₀ to cisplatin compared to sensitive SKOV3 cells (Figure 1B). To further confirm the pathway identified in the resistant cells generated in cell culture dish, we obtained another pair of platinum-sensitive and -resistant ovarian cancer cells, PEO14 and PEO23, which were derived directly from the same patient before and after development of platinum drug resistance [42]. Clearly, PEO23 exhibited a 4-fold increase of IC₅₀ to cisplatin compared to its sensitive counterpart PEO14 (Figure 1B).

We next conducted a qHTCS using SKOV3 CR cells to identify compounds that can re-sensitize SKOV3 CR cells to cisplatin. To accomplish this goal, we performed a two-round

screening. In the first-round screening with a total of 6,016 compounds from libraries of NPC (NCGC Pharmaceutical Collection), MIPE (Mechanism Interrogation Plate) and LOPAC (The Library of Pharmacologically Active Compounds), we identified 112 compounds that exhibited strong killing efficacy against resistant cells. To further discover compounds that have synergy with cisplatin, these 112 compounds were screened at 11 different concentrations in combination with cisplatin at 20 μ M. The second-round screening identified two Chk1 inhibitors, PF-477736 and AZD-7762 [43, 44], which re-sensitized resistant cells to cisplatin (Figure 1C). PF-477736 and AZD-7762 are on clinical trials for treating advanced solid tumors in combined with gemcitabine or irinotecan [45, 46]. These results suggest that activation of Chk1 pathway may play an important role in the regulation of cisplatin resistance in ovarian cancer cells.

3.2. Clinical evidence that ATR-Chk1 is activated in tumors from platinum drug recurrent or resistant patients

To explore the clinical relevance of Chk1 activation in the regulation of platinum drug resistance in ovarian cancer, we conducted RNA-Seq analyses to examine the gene expression profiles of SKOV3 and SKOV3 CR cells. A total of 3414 genes were found to be upregulated at least 1.5 folds in resistant cells. We next examined the correlation of Chk1 activation with the survival of ovarian cancer patients who have received platinum drug-based therapy. Since Chk1 is activated by its phosphorylation, we therefore investigated the correlation of patient survival with gene expression levels of Chk1 functional related genes. Specifically, using PathwayNet analysis (<http://pathwaynet.princeton.edu>), we identified a total of 392 genes that are functionally linked to Chk1 (Figure 1D). Comparing these genes with up-regulated genes identified by RNA-Seq, we identified a total of 108 genes linked to Chk1 function and were up-regulated in SKOV3 CR cells (Figure 1D). We named these genes as ATR-Chk1 signature genes. KEGG (<http://www.genome.jp/kegg/pathway.html>) pathway analyses indicated that these genes regulate cell-cycle, DNA replication, DNA repair, etc. (Figure 1E).

Since inhibition of Chk1 activity re-sensitized resistant cells to cisplatin, we hypothesized that the expression levels of ATR-Chk1 signature genes may be negatively correlated with survival of ovarian cancer patients. To test this hypothesis, we analyzed the data from ~500 ovarian cancer patients from TCGA database and compared expression levels of ATR-Chk1 signature genes with survival rate of these patients using Kaplan-Meier plotter [36]. Significantly, patients carrying higher expression levels of ATR-Chk1 signature genes displayed a poor overall survival rate and progression free survival rate (PFS) (Figure 1F).

The best way to evaluate the correlation of the ATR-Chk1 pathway with platinum-resistance in ovarian cancer is to examine and compare the activation of ATR-Chk1 in the tumor samples from the same patient before platinum drug treatment and after development of tumor recurrence or resistance. To this end, we identified a total of seven patients who have received platinum drug-based chemotherapy and had a recurrence or resistance 11–41 months post treatment. Immunohistochemistry (IHC) results indicated that the levels of phosphorylation of ATR at Thr-1989 (pATR) were increased in four out of seven recurrent tumor samples (Figure 1G, Supplementary table S1), suggesting that the upregulated ATR-

Chk1 pathway is an important mechanism contributing to cisplatin resistance of ovarian cancer. Significantly, one patient (Patient 1) has been confirmed clinically as a platinum-resistant ovarian cancer patient and the IHC analysis indicated that pATR levels were significantly increased in resistant tumors compared to sensitive tumor (Figure 1H).

3.3. Chk1 inhibition synergizes with cisplatin in cisplatin resistant ovarian cancer cells

Encouraged by the above clinical discovery, we examined whether inhibition of Chk1 by PF-477736 or AZD-7762 has synergistic effects with cisplatin in cisplatin resistant ovarian cancer cells. To this end, we first measured the cell proliferation and the synergistic effect between PF-477736 or AZD-7762 and cisplatin using SRB cytotoxicity assay. The combination index (CI) was employed to measure effects of the interaction between two drugs (synergism: $CI < 1$; additive effect: $CI = 1$; and antagonism $CI > 1$) [35]. Combined PF-477736 with cisplatin exhibited a good synergistic effect ($CI < 1$) on suppressing growth of SKOV3 CR cells (Figure 2A). AZD-7762 in the combination with cisplatin also displayed a good synergistic effect in SKOV3 CR cells at several combination windows (Figure 2B). Significantly, the combination of PF-477736 or AZD-7762 with cisplatin exhibited very good synergistic effects in PEO23 cells derived directly from the platinum resistant ovarian patient (Figure 2C–D).

To rule out the off-target effects of Chk1 inhibitors, we next examined whether knockdown of Chk1 by siRNA could re-sensitize resistant cells to cisplatin. We observed that Chk1 inhibition by siChk1 significantly reduced IC_{50} to cisplatin in resistant cells compared to siG12 control treated cells. The apoptotic analysis indicated that Chk1 inhibition significantly increased apoptotic cell death of resistant cells with cisplatin treatment (Figure 2F). Thus, Chk1 activation is critical to regulate cisplatin resistance in ovarian cancer cells.

3.4. ATR-Chk1 pathway is activated in resistant ovarian cancer cells

To elucidate the molecular mechanism of platinum-resistance in ovarian cancer cells, we examined the activation of ATR-Chk1 pathway in sensitive and resistant cells. Strikingly, phosphorylations of Chk1 at Ser-317 and ATR at Thr-1989 were increased in both cisplatin-resistant SKOV3 CR and PEO23 cells (Figure 3A), indicating that the ATR-Chk1 pathway was activated. Interestingly, FANCD2 protein level was also increased in both resistant cell lines (Figure 3A).

We next examined whether inhibition of ATR has synergistic effects with cisplatin in resistant cells. To this goal, we used ATR inhibitor (VE-821) to treat SKOV3 CR cells together with cisplatin and found that VE-821 re-sensitized SKOV3 CR cells to cisplatin (Figure 3B). Consistently, the combination of cisplatin with VE-821 exhibited a good synergistic effect in both SKOV3 CR and PEO23 cells (Figure 3C–D). Importantly, VE-821 indeed reduced the activation of both ATR and Chk1 (Figure 3E), and increased apoptosis as indicated by FACS in SKOV3 CR cells (Figure 3F).

3.5. G2/M phase checkpoint is activated in cisplatin resistant ovarian cancer cells

We noticed that resistant SKOV3 CR and PEO23 cells grew slowly compared to their sensitive counterparts SKOV3 and PEO14 cells. Given that ATR-Chk1 pathway was

activated in SKOV3 CR and PEO23 cells, we assumed that activated DNA damage response may interfere with cell-cycle progression. To test this hypothesis, we measured and compared the cell cycle progression of sensitive and resistance cells by FACS. Interestingly, compared to sensitive cell SKOV3, SKOV3 CR cells exhibited a G2/M phase accumulation (22% in SKOV3 cells and 33% in SKOV3 CR cells) (Figure 4A). Increased G2/M phase cell population indicates that G2/M phase checkpoint may be activated. Indeed, we detected the increased phosphorylation of Cdc2 at Tyr-15 (pCdc2), a marker of activation of G2/M phase checkpoint, in SKOV3 CR cells (Figure 4B). Activation of G2/M phase checkpoint prevents cells from entering mitosis [47]. We therefore examined the phosphorylation of H3 at Ser-10 (pH3), which is mitotic marker, and found a decreased phosphorylation of H3 at Ser10 in SKOV3 CR cells (Figure 4B), indicating an accumulation of cells at G2 phase before entering mitosis.

Up-regulated ATR-Chk1 pathway in platinum-resistant ovarian cancer cells suggests a higher DNA damage repair activity. To test this possibility, we measured and compared the DNA damage repair capability in both platinum-sensitive and -resistant cells. Using metaphase chromatin spread assay to examine the DNA breakage, we found that there was no difference of DNA breakage between SKOV3 and SKOV3 CR cells in DMSO treated cells (Figure 4C). However, cisplatin treatment significantly increased the percentage of breakage in SKOV3 cells compared to SKOV3 CR cells (35% in SKOV3 cells and 15% in SKOV3 CR cells) (Figure 4C). Micronucleus is the small nucleus that forms whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division and usually is regarded as a sign of genotoxic events and chromosomal instability [48]. Interestingly, Aphidicolin induced more micronuclei in SKOV3 cells (18%) than in SKOV3 CR cells (11%) (Figure 4D). Taken together, our data indicates that G2/M checkpoint is activated in resistant cells.

3.6. Elevated DNA damage repair activity in resistant ovarian cancer cells

To rule out the possibility that the acquired resistance of SKOV3 CR cells was due to reduced cisplatin uptake, we measured the DNA interstrand crosslinks (ICLs) in both sensitive and resistant cells after cisplatin treatment using a modified comet assay [49]. This assay is used to quantify amount DNA ICLs by the intensity of comet-like images (named as tail moment). After 2-hour incubation of cisplatin, cells received radiation of 20 Gy to shear DNA fragment randomly before analyzed with modified comet assay. As shown in Figure 5A, there was no difference in the amount of ICLs between SKOV3 and SKOV3 CR cells, indicating that failure to uptake cisplatin in resistant cells is not the case. Given that ATP7A and ATP7B are known for effluxion of cisplatin in cells [50, 51], we further measure the protein levels in SKOV3 and SKOV3 CR cells, and found the similar expression levels of ATP7A and ATP7B in both cells, suggesting that the resistance in SKOV3 CR cells is not due to the abnormal drug effluxion effect.

We next examined the DNA repair activity using the modified comet assay. Cells were treated with cisplatin and then released into cisplatin-free medium. We first measured the lengths of tail movements and found that the reduction of tail movements in SKOV3 CR was faster than SKOV3 cells (Figure 5C), indicating that SKOV3 CR cells have stronger ability

to remove DNA ICLs induced by cisplatin. FANCD2 plays a critical role in the repair of ICLs [24–27]. We next examined the activation of FANCD2 by measuring ubiquitination of FANCD2 levels in cells after cisplatin treatment. Strikingly, the ubiquitination of FANCD2 in SKOV3 CR cells decreased faster than that in SKOV3 cells (Figure 5D–E), indicating a stronger DNA repair activity in SKOV3 CR cells. Consistently, SKOV3 CR cells exhibited less Chk1 phosphorylation at Ser-317 compared to sensitive cells (Figure 5F–G).

3.7. DUOX1-mediated-ROS production sustains the elevated DNA damage response in resistant ovarian cancer cells

The next question is how ovarian cancer cells sustain the activated DNA damage response in platinum-resistant ovarian cancer cells. Since cisplatin can increase ROS levels [52], we hypothesized that after a long-term cisplatin treatment, the platinum-resistant ovarian cells may acquire a mechanism to sustain higher levels of ROS to activate DNA damage response. To test this hypothesis, we first examined ROS level and found that ROS level in both SKOV3 CR and PEO23 cells were increased compared to their sensitive counterparts SKOV3 and PEO14 cells (Figure 6A). To test whether ROS is a driving force for activating DNA damage response in resistant ovarian cells, we inhibited ROS production by using a ROS inhibitor called YCG063 [53]. Notably, YCG063 reduced ROS level in both SKOV3 CR and PEO23 cells (Figure 6B) and inhibition of ROS by YCG063 reduced levels of pATR, pChk1 and γ H2AX in both platinum-resistant ovarian cells (Figure 6C). Consistent with above results, inhibition of ROS by ROS inhibitors YCG063 or MnTmppy decreased pCdc2 level and increased pH3 level (Figure 6D), indicating that inhibition of ROS production abolishes G2/M checkpoint.

To investigate the mechanism of how ROS level is up-regulated, we analyzed the ROS related gene expression in SKOV3 CR cells from RNA-seq results and identified a total of 11 ROS related genes that were up-regulated in SKOV3 CR cells compared to SKOV3 cells (Figure 6E). Of these genes, DUOX1 draw our attention because it has been reported to be critical in the regulation of ROS and up-regulation of DUOX1 can promote ROS level [19]. qPCR and western blotting analyses indicated that DUOX1 was overexpressed in SKOV3 CR and PEO23 cells at both mRNA and protein levels (Figure 6F–G).

To clarify the role of DUOX1 in the regulation of cisplatin resistance, we depleted DUOX1 by siRNA and found that downregulation of DUOX1 reduced ROS levels (Figure 6H). Significantly, depletion of DUOX1 decreased the phosphorylation of ATR and Chk1 and re-sensitized SKOV3 CR cells to cisplatin (Figure 6I–J). Consistently, depletion of DUOX1 increased the apoptotic population in SKOV3 CR cells when treated with cisplatin (Fig. 6K). We next examined whether expression level of DUOX1 correlates with poor survival of ovarian cancer patients with platinum treatment history. As expected, ovarian cancer patients carrying higher expression levels of DUOX1 exhibited a poor overall survival rate (Figure 6L). To evaluate the whether DUOX1 is up-regulated in tumor samples from resistant ovarian cancer patients, we examined the expression levels of DUOX1 protein in tumor samples from the same patients before and after development of platinum drug resistance by IHC. Significantly, DUOX1 levels were increased in 6 of 7 patients (Figure 6M and supplemental Figure 2D).

3.8. ATR inhibition suppresses SKOV3 CR xenograft tumor growth

We tested whether inhibition of ATR-Chk1 pathway by using an ATR specific inhibitor VE-822 could suppress SKOV3 CR tumor growth in mice [54]. To this end, we first subcutaneously implanted SKOV3 CR xenograft tumors in nude mice and then administrated VE-822 to the animals. While SKOV3 CR tumors were resistant to cisplatin or VE-822 treatment, the combinatorial treatment of cisplatin with VE-822 significantly repressed tumors growth (Figure 7A). To evaluate effect and toxicity of VE-822 and cisplatin treatment, we collected tissues from xenograft tumors, livers, and kidney. Immunohistochemistry (IHC) analysis showed that VE-822 plus cisplatin did not cause obvious histopathological alterations, lesions of hepatotoxicity, and kidney toxicity (Figure 7B). Significantly, VE-822 treatment alone or in combined with cisplatin efficiently reduced the phosphorylation of ATR in tumors (Figure 7C). The combination of VE-822 with cisplatin induced the apoptosis in tumors as indicated by a positive TUNEL staining (Figure 7D).

4. Discussion

In this study, we used an unbiased qHTCS assay to identify Chk1 inhibitors that re-sensitize cisplatin resistant ovarian cancer cells to cisplatin. Subsequent analyses identified elevated DUOX1 as a driving force for activating and sustaining high level of ROS, which promotes cisplatin resistance by activating ATR-Chk1 pathway (Figure 7E). Importantly, we provided the clinical evidence showing that activated ATR and DUOX1 are found in platinum-resistant tumor tissues of ovarian cancer patients and the elevated ATR-Chk1 pathway and DUOX1 is correlated with poor survival of ovarian cancer patients who have received platinum-based therapeutic treatments. Although the activated ATR-Chk1 in the regulation of cisplatin-resistance has been reported [55, 56], the molecular mechanism of how platinum-resistant ovarian cancer cells sustain activated ATR-Chk1 is unknown. Therefore, our study provides with a new mechanistic insight of how ovarian cancer cells develop cisplatin-resistance over the time of treatment. This regulatory can be used as an intervention point for treatment of drug resistant ovarian cancer in future.

Using qHTCS, we identified two Chk-1 inhibitors, PF-477736 and AZD-7762, which synergistically suppress cisplatin-resistant ovarian cancer cells with cisplatin. A clinical trial at phase I ([NCT00437203](#)) has been conducted for determining the maximum tolerated dose of PF-00477736 in the combination with gemcitabine in patients with advanced solid malignancies. AZD-7762 showed its therapeutic potential in several clinical trials when combined with gemcitabine or Irinotecan [45, 46]. In our study, we demonstrated that the combination of two Chk1 inhibitors with cisplatin led to a promising and efficient treatment for cisplatin resistant ovarian cancer cells. Therefore, the combinational therapy of Chk1 inhibitors with platinum drugs is warranted for clinical trials to treat the cisplatin resistant ovarian cancer patients to evaluate the efficacy and safety.

It is exciting to find that ROS level is elevated in resistant ovarian cancer cells and increased ROS by overexpression of DUOX1 sustains the activation of ATR-Chk1 signaling. To our knowledge, this is the first evidence to link DUOX1-mediated ROS to ATR-Chk1 in the regulation of platinum-resistance. While it is reported that the ROS signaling can stimulate

cell survival factors [11], our study has revealed a novel alternative role of ROS signaling that promotes DNA damage response, resulting in cisplatin resistance. Although DUOXA1 is known to enhance ROS level by producing hydrogen peroxide [22], the detail mechanism of how DUOXA1 level is regulated in cisplatin resistant ovarian cancer cells is still unknown.

Over decades, platinum drugs are widely used as single or in combined with other anticancer agents to treat various types of cancers, such as lung cancer, color cancer, bladder cancer, head and neck cancer, ovarian cancer, and testicular cancers [57]. Therefore, targeting the DUOXA1-ROS-ATR-Chk1 signaling cascade would be a potential therapeutic approach for treatment of other platinum-resistant cancers that may have developed the same resistant mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

qHTCS	quantitative high throughput combinational screen
RNA-seq	RNA-sequencing
DUOXA1	dual oxidase maturation factor 1
ROS	reactive oxygen species

Reference

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Highlights:

- Providing multiple potential approaches for treatment of platinum resistant ovarian cancer
- Integrating qHTCS, RNA-seq and clinical studies to elucidate cisplatin resistant mechanism
- DUOXA1-mediated activation of ATR-Chk1 regulates cisplatin resistance in ovarian cancer

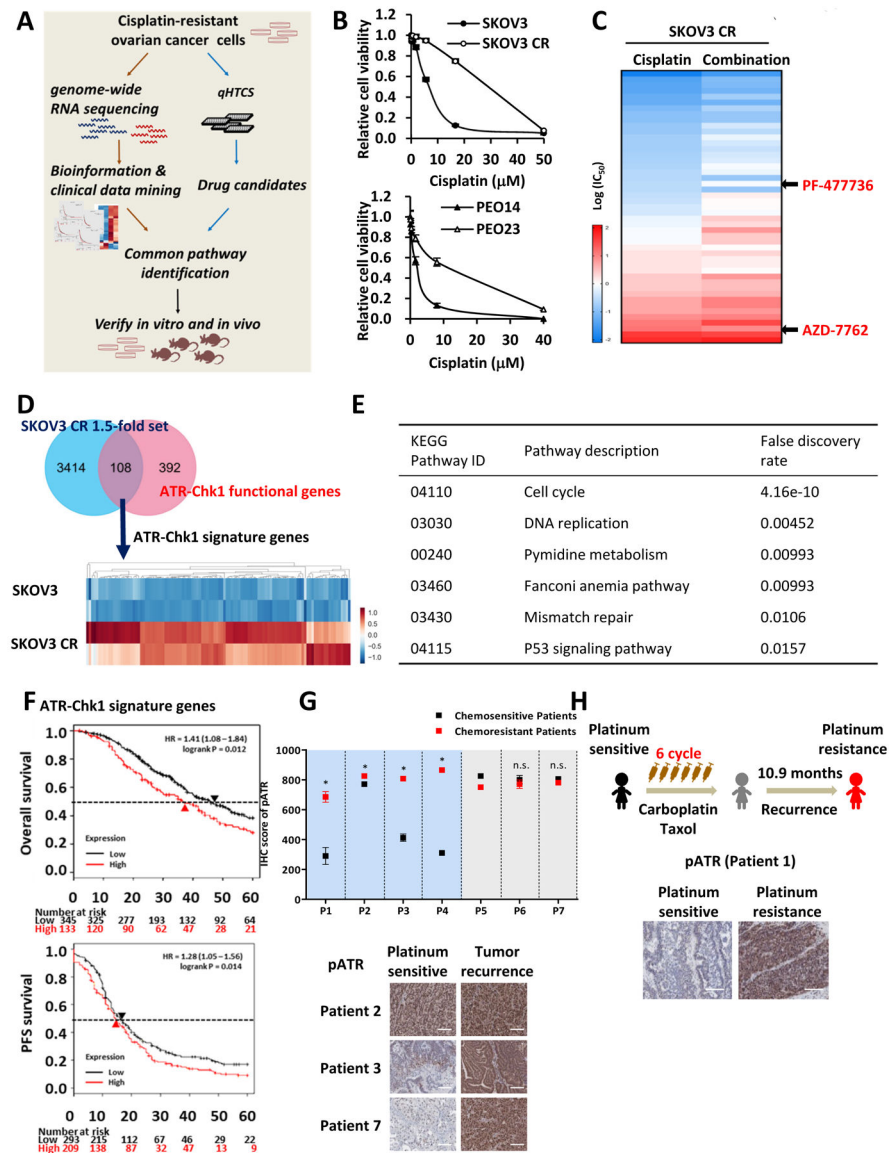


Figure 1. ATR-Chk1 is activated in cisplatin resistant or recurrent ovarian tumors from patients. (A) Schematics illustrating the combination of qHTCS and genomic sequencing to identify pathways regulating cisplatin resistance and compounds to overcome the resistance. (B) Viability of SKOV3 and SKOV3 CR cells (upper panel), PEO14 and PEO23 (lower panel) treated with increasing concentrations of cisplatin for 3 days. (C) The heatmap of second-round drug screening results based on drug-category-response scores of IC_{50} . The values are logarithm (base 10) of IC_{50} for the compounds or combined with 20 μM of Cisplatin. Two Chk1 inhibitors exhibit enhanced cell killing effect in resistant cells when combined with cisplatin (20 μM). (D) Venn diagram indicating the overlap of genes identified from the Chk1 functional gene set and up-regulated genes set in SKOV3 CR compared with SKOV3 cells (upper panel). The heatmap (lower panel) showed 108 overlapped genes (ATR-Chk1 signature gene set). Colors in the heatmap represent the gene expression levels after z-score normalization across different samples. (E) Cellular pathways regulated by ATR-Chk1

signature genes. (F) Kaplan-Meier curves of 5-year overall survival rate (OS, upper panel) and progression-free survival rates (PFS, lower panel) based on clinical and molecular data for ovarian cancer patients. The patients were stratified by the expression levels in their tumors of the ATR-Chk1 signature genes. Median survival, Log-rank (Mantel-Cox) p values and hazard ratios (HR); 95 % confidence interval in parentheses) are shown. (G) Expression levels of pATR by IHC from the same ovarian patients (upper panel). The IHC quantification of pATR in platinum sensitive and recurrent tumor samples from seven patients. Data are represented as mean \pm SD. S, platinum-sensitive ovarian cancer patients; R, platinum-resistant/recurrent ovarian cancer patients. Representative IHC images of pATR in platinum sensitive and recurrent tumor samples (lower panel). Scale bar, 100 μ m. (H) Representative IHC images of pATR in platinum sensitive and resistant tumor samples from Patient 1.

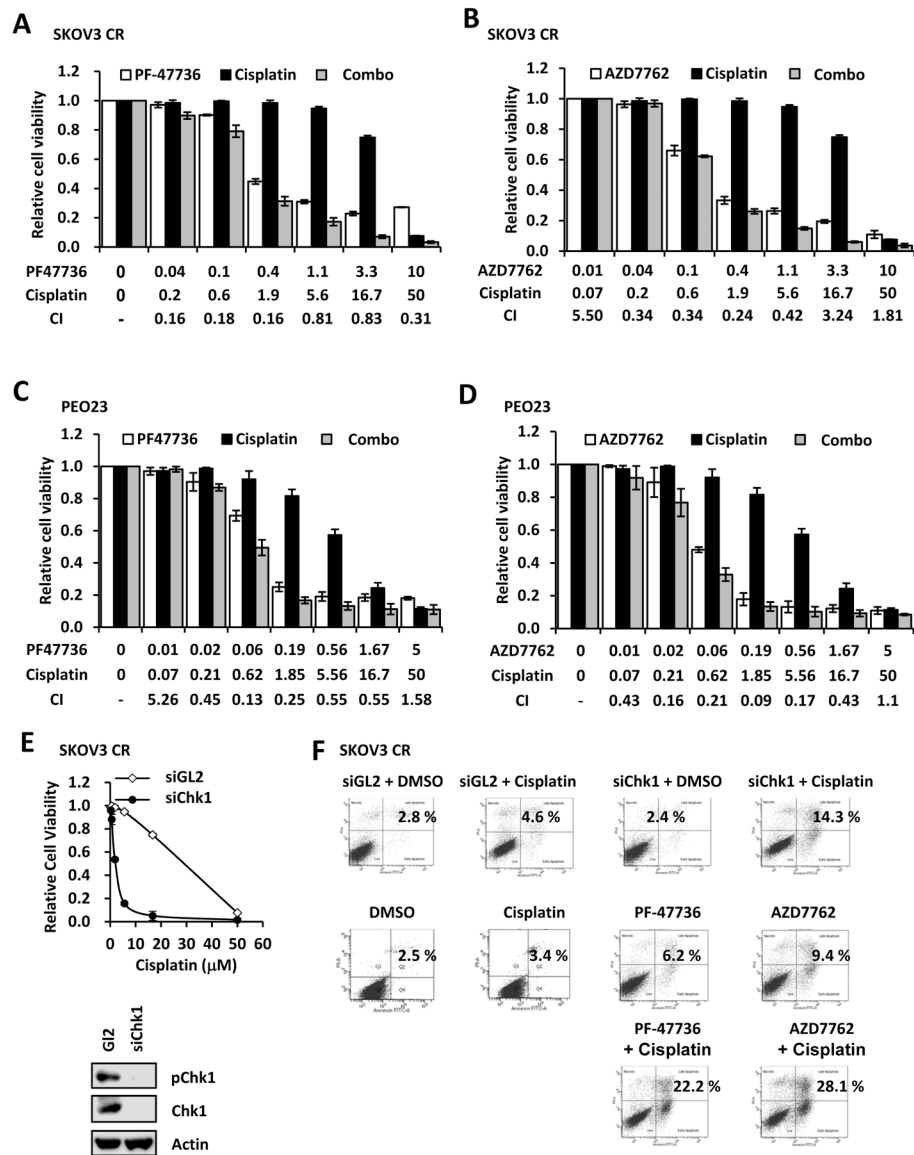


Figure 2. Chk1 inhibitors synergistically suppresses cisplatin resistant ovarian cancer cells growth.

(A) The synergistic effects of PF-47736 and cisplatin in SKOV3 CR cells. Concentrations and CI values were presented below the bars. (B) The synergistic effects of AZD7762 and cisplatin in SKOV3 CR cells. Concentrations and CI values were presented below the bars. (C) The synergistic effects of PF-47736 and cisplatin in PEO23 cells. Concentrations and CI values were presented below the bars. (D) The synergistic effects of PF-47736 and cisplatin in PEO23 cells. Concentrations and CI values were presented below the bars. (E) Viability of SKOV3 CR cells treated with increasing concentrations of cisplatin for 3 days after Chk1 depletion by siRNA. Data are represented as mean \pm SD from three independent experiments performed in triplicate. (F) SKOV3 CR cells treated as indicated were harvested for FACS analysis to examine apoptosis.

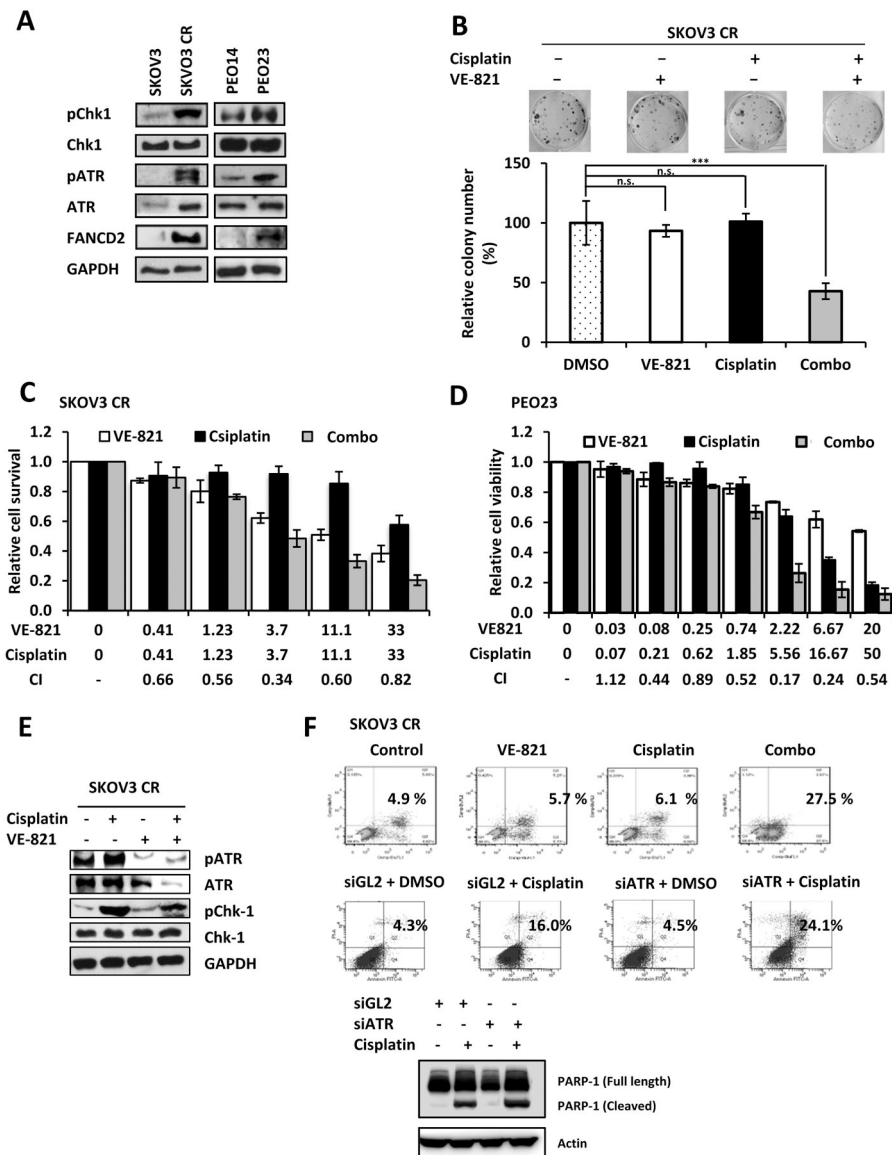


Figure 3. Inhibition of ATR overcomes cisplatin resistance in ovarian cancer cells
 (A) SKOV3, SKOV3 CR, PEO14, and PEO23 cells were harvested for Western blotting for pATR (Thr-1989), ATR, pChk1 (Ser-317), Chk1, and FANCD2. (B) Colony formation of SKOV3 CR cells 14 days after they have been treated with 0.5 μ M cisplatin and/or 0.1 μ M VE-821. Colonies were stained with crystal violet. Data are represented as mean \pm SD from three independent experiments performed in triplicate. ***, $p < 0.001$; n.s., not significant. (C) The synergistic effects of VE-821 and cisplatin on SKOV3 CR cells. Concentrations and CI values were presented below the bars. (D) The synergistic effects of VE-821 and cisplatin on PEO23 cells. Concentrations and CI values were presented below the bars. (E) SKOV3 CR cells treated as indicated were harvested for Western blotting for pATR (Thr-1989), ATR, pChk1 (Ser-317), and Chk1. (F) Cells treated as indicated were harvested for FACS analysis to examine the apoptosis (upper panel). SKOV3 CR cells treated as indicated were harvested for Western blotting for PARP-1 and Actin (lower panel).

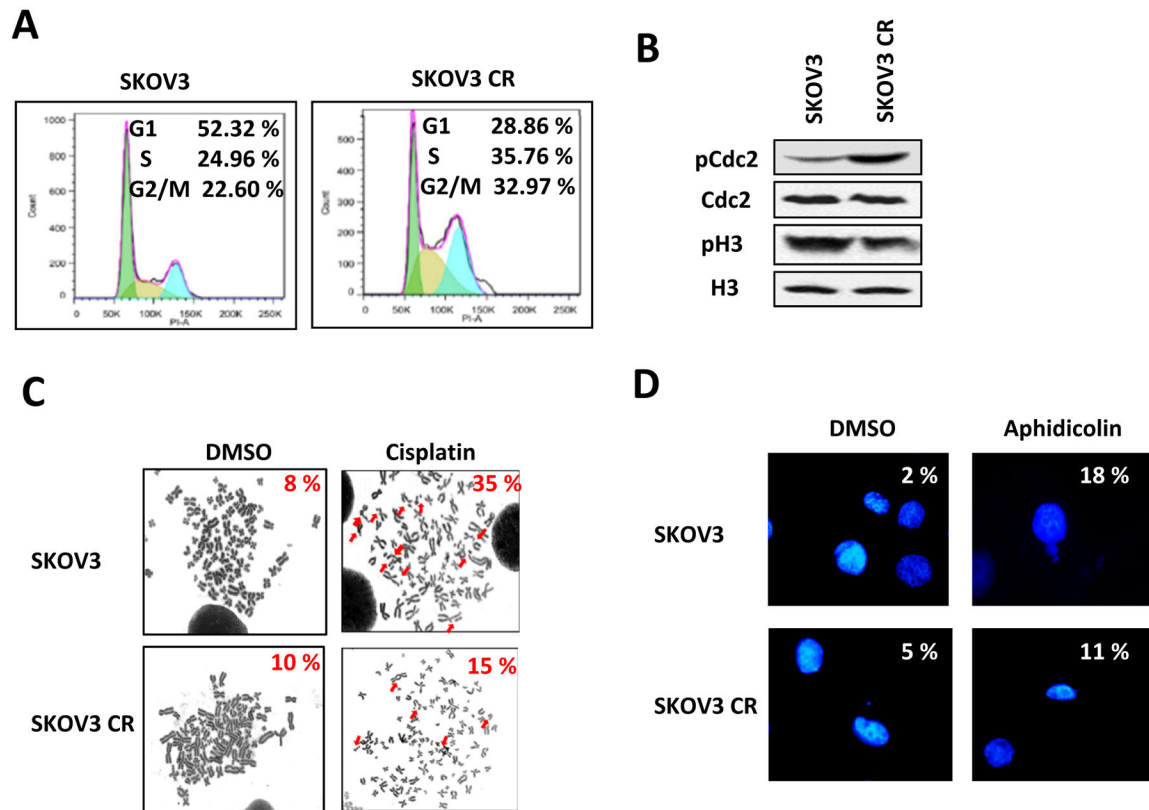


Figure 4. G2/M checkpoint is activated in cisplatin resistant cells

(A) SKOV3 or SKOV3 CR cells were harvested for cell-cycle analysis. The percentages of each cell-cycle stages were indicated. (B) SKOV3 and SKOV3 CR cells were harvested for Western blotting for pCdc2 (Tyr-15), Cdc2, pH3 (Ser-10), and H3. (C) Metaphase chromosome spread of SKOV3 or SKOV3 CR cells treated with control or cisplatin. The percentages of DNA breakage were indicated. (D) Micronuclei of SKOV3 or SKOV3 CR cells treated with control or Aphidicolin. The percentages of micronuclei population were indicated.

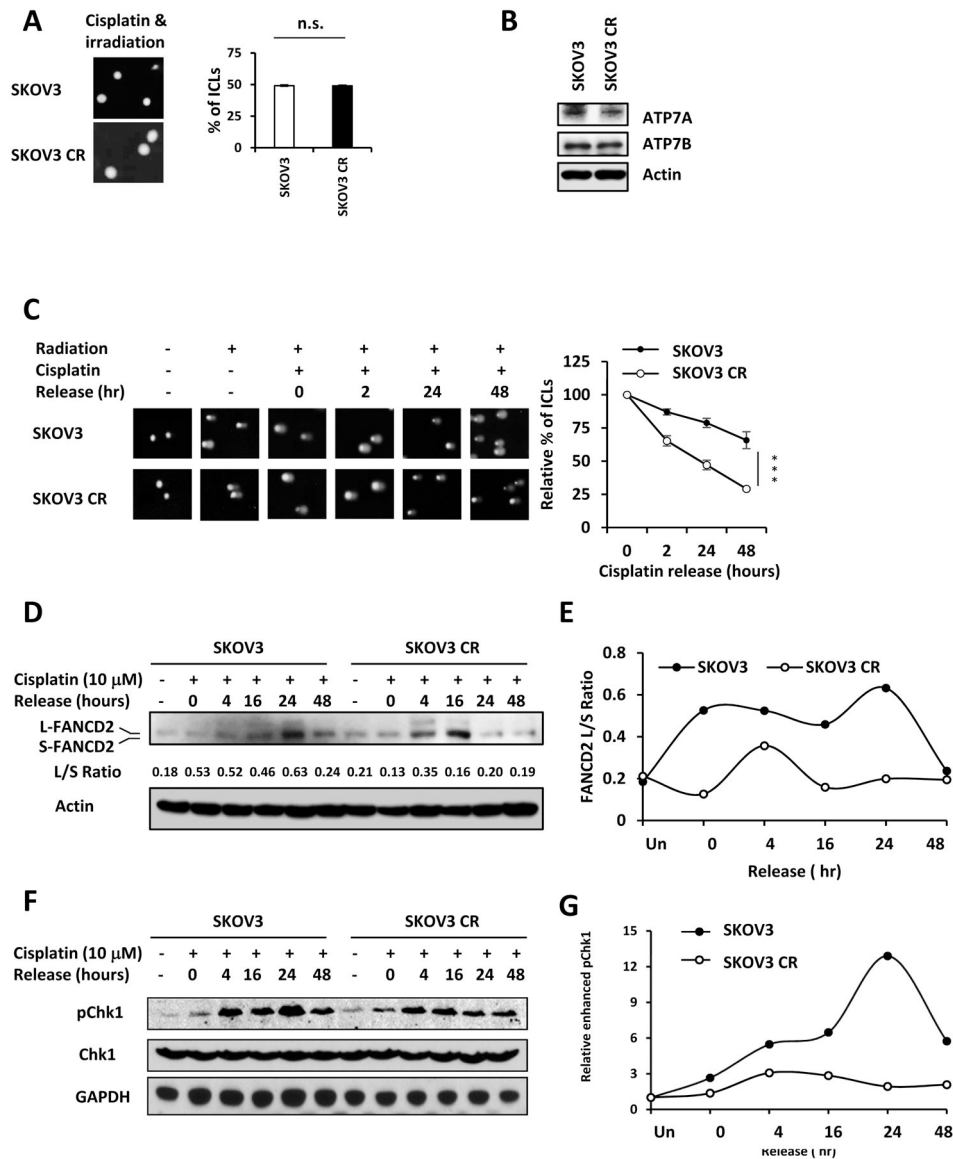


Figure 5. Cisplatin resistant ovarian cancer cells exhibited elevated ability to repair DNA damage.

(A) Modified comet assay to examine ICLs in SKOV3 or SKOV3 CR cells. (B) SKOV3 CR cells treated as indicated were harvested for Western blotting for ATP7A and ATP7B. (C) Cells treated with 10 μ M cisplatin were released into cisplatin-free medium and then collected for modified comet assay at indicated time points. Representative images from modified comet assay (left panel). Relative percentage of ICLs in cells treated with cisplatin at indicated time points (right panel). (D) Cells treated with 10 μ M cisplatin were released to cisplatin-free medium and then harvested at indicated time points for western blotting for FANCD2 (L-FANCD2, mono-ubiquitinated form; S-FANCD2, unmodified form). (E) Quantification of ratio of L/S-FANCD2 in cells treated as in C. (F) Cells treated with 10 μ M cisplatin were released to cisplatin-free medium and then harvested at indicated time points for Western blotting for indicated proteins. (G) Quantification of the levels of phosphorylation of Chk1 in cells treated as in F.

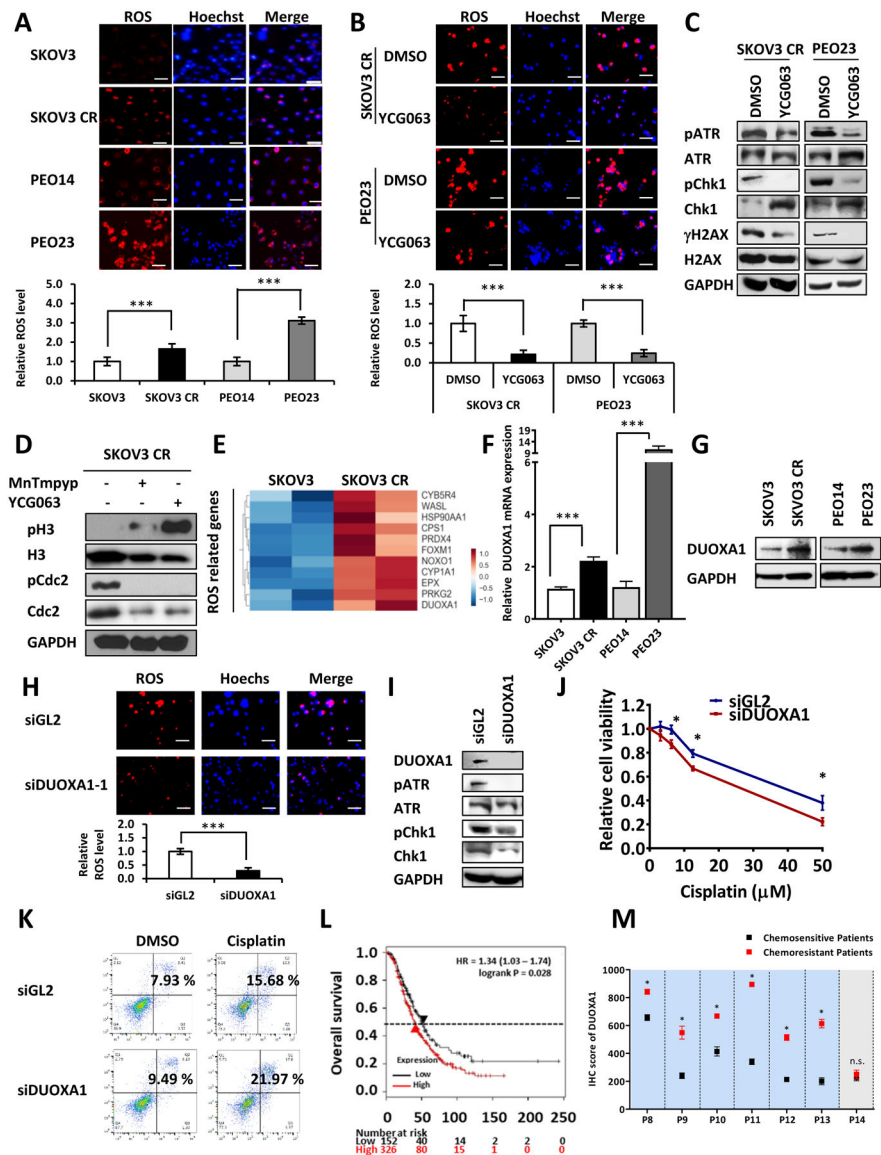


Figure 6. DUOX1 overexpression sustains increased ROS level in cisplatin resistant ovarian cancer cells.

(A) Representative images of reactive oxygen species (ROS) production in SKOV3, SKOV3 CR, PEO14 and PEO23 cells (upper panel, scale bar, 100 μ m). Quantification of relative ROS level in tested cells (lower panel). Data are represented as mean \pm SD from three independent experiments performed in triplicate. ***, $p < 0.001$. (B) ROS production in SKOV3 CR and PEO23 cells after treated with the ROS inhibitor YCG063 20 μ M for 24 hours (upper panel; scale bar, 100 μ m). Quantification of relative ROS level in tested cells (lower panel). Data are represented as mean \pm SD from three independent experiments performed in triplicate. ***, $p < 0.001$. (C) SKOV3 CR and PEO23 cells treated with YCG063 20 μ M for 24 hours were harvested for Western blotting for indicated proteins. (D) SKOV3 CR cells treated with indicated compounds were harvested for Western blotting for indicated proteins. (E) Heatmap of genes that are involved in ROS pathway and up-regulated in resistant cells. Colors in the heat-map represent the gene expression levels after z-score

normalization across different samples. (F) qPCR to examine DUOX1 expression in indicated cells. Data are represented as mean \pm SD from three independent experiments performed in triplicate. ***, $p < 0.001$. (G) SKOV3, SKOV3 CR, PEO14 and PEO23 were harvested for Western blotting for DUOX1. (H) SKOV3 CR cells transfected with control siGL2 or siDUOX1 were examined for ROS production (upper panel; scale bar, 100 μ m). Quantification of relative ROS level in tested cells (lower panel). Data are represented as mean \pm SD from three independent experiments performed in triplicate. ***, $p < 0.001$. (I) SKOV3 CR cells treated as indicated were harvested for pATR (Thr-1989), ATR, pChk1 (Ser-317), and Chk1. (J) Viability of SKOV3 CR cells treated with increasing concentrations of cisplatin for 3 days after DUOX1 knockdown. *, $p < 0.05$. (K) SKOV3 CR cells treated as indicated were harvested for FACS analysis to examine apoptosis. (L) Kaplan-Meier curves of 5-year overall survival rate (OS) based on clinical and molecular data for ovarian cancer patients. The patients were stratified by the expression levels of DUOX1 in their tumors. Medium survival, Log-rank (Mantel-Cox) p values and hazard ratios (HR); 95 % confidence interval in parentheses) are shown. (M) Expression levels of DUOX1 by IHC from the same ovarian patients. The IHC quantification of DUOX1 levels in platinum sensitive and platinum resistance tumor samples from seven patients. Data are represented as mean \pm SD.

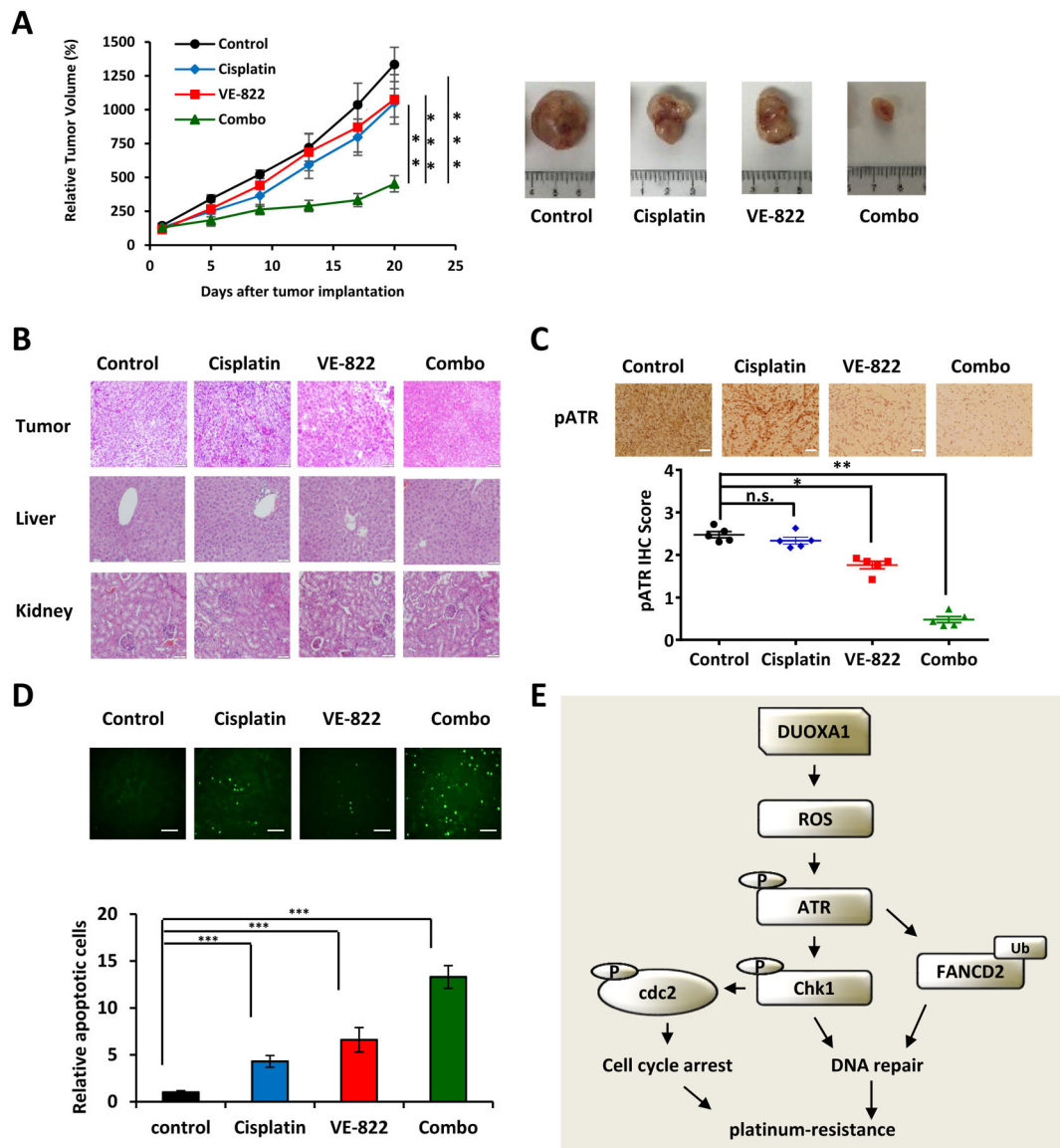


Figure 7. ATR inhibition suppresses SKOV3 CR xenograft tumor growth

(A) Growth curves of SKOV3 CR xenograft tumors treated with vehicle, VE-822 (oral gavage at 60 mg/kg on days 1, 2, 3, 5, 6, 7, 9, 10, and 11), cisplatin (6 mg/kg intraperitoneally on days 1, 5, and 9), or VE-822 plus cisplatin (Combo) for 2 weeks. Data are represented as means \pm SEM, $n = 6$ mice/group. **, $p < 0.01$, ***, $p < 0.001$ by two-way ANOVA. The right panel is the photograph of the representative tumors from mice at treatment on day 24. Ruler scale is in cm. (B) Representative hematoxylin and eosin (H&E) staining of samples of xenograft tumors, livers, and kidneys from mice treated with cisplatin and/or VE-822. Specimens were collected on day 20. (C) IHC staining of pATR in samples of xenograft tumors collected on 4 days after the last treatment. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. (D) *In situ* apoptosis analysis of xenograft tumors collected on 4 days after the last treatment using TUNEL assay. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Scale

bar, 100um for H&E and pATR IHC, 50 µm for TUNEL. (E) Working model of DUOXA1-mediated ROS production in the regulation of cisplatin resistance in ovarian cancer.

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