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p62/SQSTM1 as an oncotarget mediates cisplatin resistance through activating RIP1-NF-κB pathway in human ovarian cancer cells

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Platinum-based therapeutic strategies have been widely used in ovarian cancer treatment. However, drug resistance has greatly limited therapeutic efficacy. Recently, tolerance to cisplatin has been attributed to other factors unrelated to DNA. p62 (also known as SQSTM1) functions as a multifunctional hub participating in tumorigenesis and may be a therapeutic target. Our previous study showed that p62 was overexpressed in drug-resistant ovarian epithelial carcinoma and its inhibition increased the sensitivity to cisplatin. In this study, we demonstrate that the activity of the NF-κB signaling pathway and K63-linked ubiquitination of RIP1 was higher in cisplatin-resistant ovarian (SKOV3/DDP) cells compared with parental cells. In addition, cisplatin resistance could be reversed by inhibiting the expression of p62 using siRNA. Furthermore, deletion of the ZZ domain of p62 that interacts with RIP1 in SKOV3 cells markedly decreased K63-linked ubiquitination of RIP1 and inhibited the activation of the NF-κB signaling pathway. Moreover, loss of the ZZ domain from p62 led to poor proliferative capacity and high levels of apoptosis in SKOV3 cells and made them more sensitive to cisplatin treatment. Collectively, we provide evidence that p62 is implicated in the activation of NF-κB signaling that is partly dependent on RIP1. p62 promotes cell proliferation and inhibits apoptosis thus mediating drug resistance in ovarian cancer cells.

Platinum (Pt)-based chemotherapies are frequently used as an adjuvant for ovarian cancer treatment.[1] However, drug resistance is still a major obstacle that limits efficacy of Pt-based therapeutic regimens.[2] Since the 1970s, tolerance to Pt has been blamed on effects unrelated to DNA damage. Recently, investigators have paid more attention to these "non-nuclear" effects and the molecular pathways that are involved in the cell survival and apoptotic escape.[3] Our previous study showed that sequestosome-1 (p62/SQSTM1) was highly expressed in cisplatin-resistant ovarian cancer cells. Furthermore, resistance to Pt was removed when we inhibited the p62 expression with the siRNA.[4] These results suggested that p62 may play a key role in the mechanism of platinum-resistance.

p62, also known as SQSTM1, was the first identified autophagy adaptor and is important for cellular detoxification and resistance to nutrient stress.[5] However, in contrast to other autophagy adaptors, p62 also functions as a multifunctional hub. It participates in many important cellular events that control proliferation and apoptosis, particularly during tumorigenic conditions.[6–9] The autophagy-independent role for p62 is reported in various cancers, including ovarian,[10] breast,[11] liver,[12] and kidney.[13] Increasing evidence suggests that elevated levels of p62 are correlated with poor prognosis in epithelial ovarian cancer.[14] Furthermore, knockout of p62 in mice significantly increases the sensitivity of pulmonary adenocarcinomas to doxorubicin which indicates that p62 may be involved in the regulation of survival signaling.[15] Our previous work suggested that p62 accumulation may activate transcription factor Nrf2 to confer cancer cells resistance to chemotherapy.[10] Recent advances in the understanding of p62 function suggest that it increases the activity of the nuclear factor kappa B (NF-κB) pathway in many cell systems.[9,16–18] High NF-κB activity may trigger several pro-survival genes expression that contributes to chemotherapeutic resistance. Thus, identifying the role of p62 in the activation of NF-κB may improve the understanding of the mechanism of drug resistance. Furthermore, given the complex roles of p62, research that goes beyond simple increases or decreases in levels will be necessary to understand the precise regulation of this molecule in pro-survival signaling pathways during tumorigenesis.

Receptor interacting serine/threonine kinase 1 (RIP1, also known as RIPK1) has emerged as a key upstream regulator that controls pro-survival signaling as well as the activation of multiple cell death pathways.[19–22] Furthermore, the ability of RIP1 to modulate these key cellular events is tightly

controlled by ubiquitination. The lysine (K) 63-linked polyubiquitin chains serve as a docking site to mediate the formation of a complex upstream of NF-κB that promotes the activation of pro-survival signaling.[20,23–25] Previous studies have indicated that deletion of the ZZ (Zinc finger) domain of p62 abolished binding of RIP1 to p62 and inhibited the activation of the NF-κB signaling pathway.[26] However, we still know little about the role of these key molecules in ovarian cancer. Here, we explored the effects of cisplatin on ovarian cancer cells and found that p62 could activated the NF-κB signaling pathway. And this activation was dependent on K63-linked ubiquitination of RIP1 that subsequently mediated drug resistance.

Materials and Methods

Reagents and antibodies. Cisplatin, trypan blue and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences (State of New Jersey, USA). ViaFect Transfection Reagent was purchased from Promega (Madison, MI, USA). Nuclear and cytoplasmic protein extraction kit was purchased from Beyotime (China). The following anti-body were used: anti-p62 (Abcam, Cambridge, MA, USA); anti-p50, anti-p65, anti-β actin (Proteintech, Chicago, IL, USA); anti-RIP1, anti-K63-linkage Specific Polyubiquitin (Cell Signaling Technology, Beverly, Massachusetts, USA); anti-Lamin A/C, anti-IκB, anti-pIκB (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell lines and cell culture. SKOV3 cells and their cisplatin-resistant clone SKOV3/DDP were purchased from the Chinese Academy of Medical Sciences and Peking Union Medical College. Both cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 culture medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2. SKOV3/DDP cells were cultured in the presence of 1 μg/mL cisplatin to maintain the resistance.

Cell transfection. p62-siRNA and non-target siRNA (Scramble) were obtained from Genechem (Shanghai, China).The p62-siRNA (si-p62) sequence was GAC-ATC-TTC-CGAAT C-TAC-A and the non-target siRNA (Scramble) was TTC-T CC-GAA-CGT-GTC-ACG-T. The pcDNA3.1-p62 and pcDNA3.1-ΔZZ-p62 (ZZ domain truncated mutation) and empty pcDNA3.1 vector (NC) were constructed by Sangon Biotech (Shanghai, China). 25×10^4 cells were placed into 6-well plates. After 36 h cells were transfected with siRNA or plasmids using transfection Reagent (Promega, Madison, MI, USA) according to the manufacturer's instructions.

Cell viability assays. 8×10^3 cells were seeded in 96-well plates in each well. After 36 h cells were treated with different concentrations of cisplatin after transfection. And the MTT assay was used to evaluate cell viability. We measured the absorbance at 570 nm using a Vmax Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Trypan blue staining. Cells were harvested and suspended in phosphate-buffered saline (PBS). For staining, Trypan blue was used at a concentration of 0.4%. Mix 90 μL cells and 10 μL Trypan blue in 1.5 mL tube for 2 min and count cells with the hemocytometer while dead cells have blue cytoplasm. Finally, we examined the percentage of cells that have clear cytoplasm (viable cells) versus total cells.

Luciferase assay. The cells were transfected with the NF-κB-Luc reporter plasmid using transfection reagent (Promega, Madison, MI, USA). After drug treatment luciferase activity was measured using the Dual-Luciferase assay kits (Promega, Madison, WI, USA) and normalized on the basis of Renilla luciferase activity.

Western blot analysis. Proteins were subjected to Western blotting as previously described.[27] Then incubated with primary antibodies overnight at 4°C. Horse radish peroxidase (HRP)-conjugated secondary antibodies were incubated (Proteintech, Chicago, IL, USA) according to the manufacturer's instructions. Then, immunodetection was performed using ECL reagent (Thermo Fisher Scientific) and visualized by a Syngene Bio Imaging (Synoptics, Cambridge, UK).

Immunoprecipitation assay. Cells were lysed in NP40 lysis buffer. Equal amounts of lysates were immunoprecipitated with 2 μg of the RIP1or K63-linkage Specific Polyubiquitin antibody overnight at 4°C. 25 μL of protein A and G agarose (Beyotime, China) were used each sample. Beads were washed with PBS three times with 1 mL each. The eluted proteins were examined by using western blot.

Immunofluorescence staining and confocal laser microscopy. Cells were washed and fixed in 4% (w/v) paraformaldehyde/PBS for 20 min and subjected to proteinase K digestion for 1 min. Then permeabilized with 0.1% Triton X-100 for 15 min. After blocking with bovine serum albumen for 30 min cells were incubated with primary antibody overnight at 4°C. The next day, stained with FITC/Texas Red-conjugated secondary antibodies (1:200 dilution; Proteintech, Chicago, IL, USA) for 30 min in the dark. Finally, Cells were treated with Hoechst 33342/H2O (1 μg/mL) for 2 min, and Images were acquired by an Olympus FV1000 confocal laser microscope.

Flow cytometry. Cells (25×10^4 cells/well) were seeded in 6-well plates. After exposure to different treatment, cells were collected and performed Annexin-V FITC/PI staining according to the manufacturer's instructions. Samples were analyzed by Accuri C6 Flow Cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

EDU. Cells were seeded in 96-well plate and exposed to 50 μM of 5-ethynyl-29deoxyuridine (EdU, RiboBio, Guangzhou, China) according to the manufacturer's instructions. Subsequently, the DNA contents were stained with Hoechst 33342 for 30 min and visualized under a microscopy (Olympus, Tokyo, Japan).

Caspase activity assay (Promega). Cells were collected and Caspase-Glo 3/7 Assay (Promega, Madison, MI, USA) was used as described in the manufacturer's instructions. Microplate reader (FLUOstar Omega, BMG LABTECH, German) was used for detecting.

RNA extraction and quantitative real-time PCR. Total RNA extracted and First-strand cDNAs synthesized were described as before.[4] Quantitative real-time PCR was done by using the MX300P instrument (Agilent, USA) followed by a 2-step PCR protocol. The primers sequences are as follows: primers for BCL2L1, 5′-TCCATCGATTCAGTCCCT-3′.Primers for CCL2 5′-CAATCAATGCCCCAGTCACC-3′, 5′- CCTGAA CCCAC TTCTGCTTG-3′. Primers for TGFB1 5′-GGATAACAC ACTGCAAGTGG-3′, 5′-GAGCTGAAGCAAT AGTTGG TG-3′. Primers for CSF3 5′-GCTTCCTGCTCAAGTGCTT A -3′, 5′-TTCCCAGTTCTTCCATCTGC-3′. Primers for IL-6 5′-CTGCAGCCACTGGTTCTGT-3′, 5′-CCAGAGCTGTGCA GATGAGT-3′. Primers for ACTB 5′-ATATCGCGTCGCT GGTCGTC-3′, 5′-AGGATGGCGTGAGGGAGAGC-3′. The relative expression was calculated by ΔCt among different experimental groups normalized to ACTB expression.

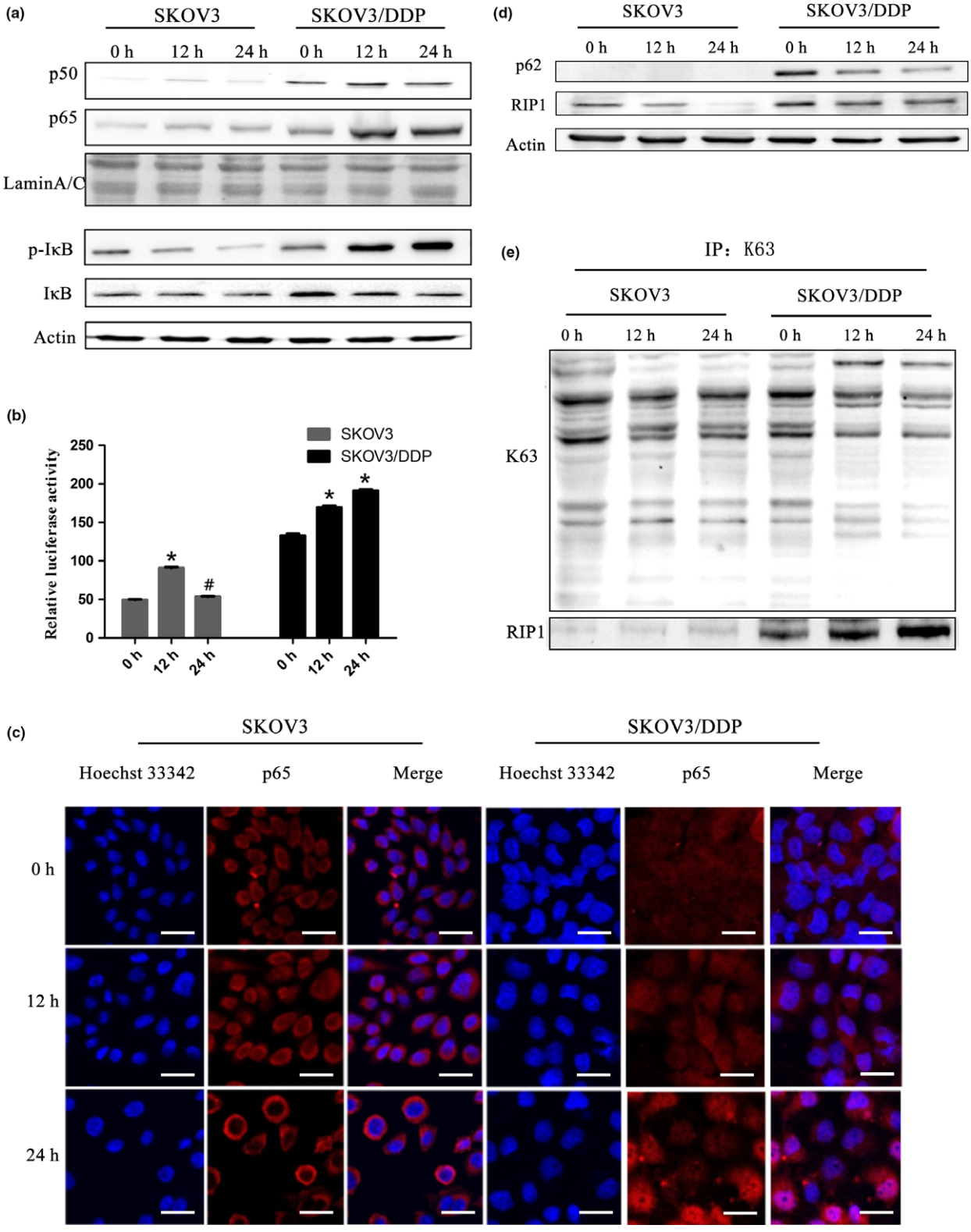

Fig. 1. Cisplatin treatment activates the NF-κB pathway and an increase in RIP1K63-linked ubiquitination in SKOV3/DDP cells. (a) Cells were treated with cisplatin (6 μg/mL) and expression of NF-κB p65 and NF-κB p50 in the nucleus and IκB, p-IκB in whole cell lysates was analyzed by western blotting. (b) SKOV3 and SKOV3/DDP cells were transiently transfected with a pNF-κB-Luc vector. The luciferase activity was assessed and normalized on the basis of Renilla luciferase activity (mean ± SD, $n = 3$. *$P < 0.05$, *vs* control). (c) SKOV3 and SKOV3/DDP cells were stained with Hoechst 33342 and antibodies against p65. They were observed under confocal laser microscopy (scale bar, 25 μm). (d) SKOV3 and SKOV3/DDP cells were immunoblotted (IB) with anti-p62 and anti-RIP1. (e) Immunoprecipitation (IP) was performed using the anti-K63 antibody followed by western blotting using anti-RIP1 antibody.

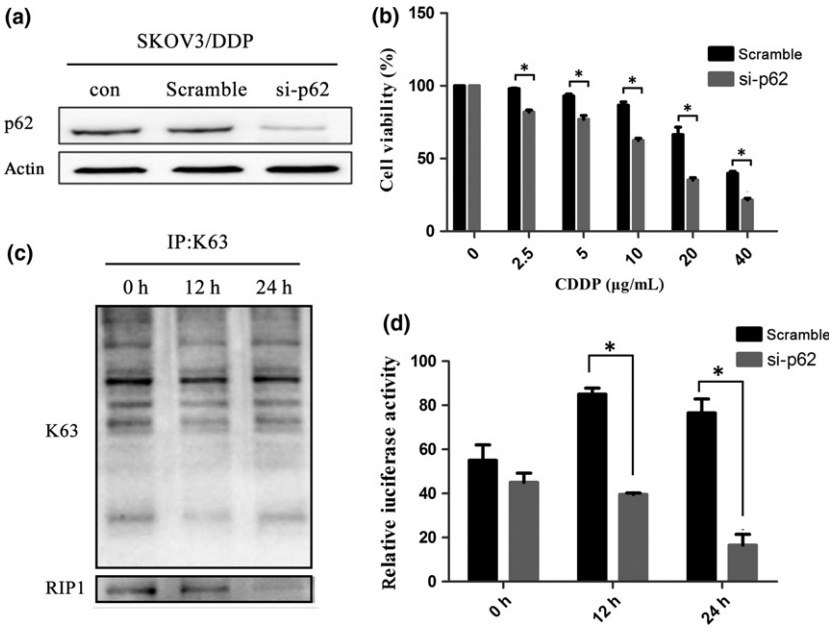

Fig. 2. Downregulation of p62/SQSTM1 inhibits the NF-κB pathway and RIP1K63-linked ubiquitination in SKOV3/DDP cells. (a) SKOV3/DDP cells were transfected with si-p62 or control siRNA (Scramble). After 24 h, cells were treated with cisplatin and the expression of p62 was analyzed by western blotting. (b) Cell viability was evaluated by MTT assay (mean ± SD, $n = 3$. $^*P < 0.05$, *vs* scramble). (c) SKOV3/DDP cells were transfected with si-p62. Protein lysates were used for immunoprecipitations with an anti-K63 antibody and analyzed by western blotting using anti-RIP1 antibody. (d) SKOV3/DDP cells with si-p62 or control siRNA were transiently transfected with a pNF-κB–Luc vector. The luciferase activity was assessed and normalized on the basis of Renilla luciferase activity (mean ± SD, $n = 3$. $^*P < 0.05$, *vs* scramble).

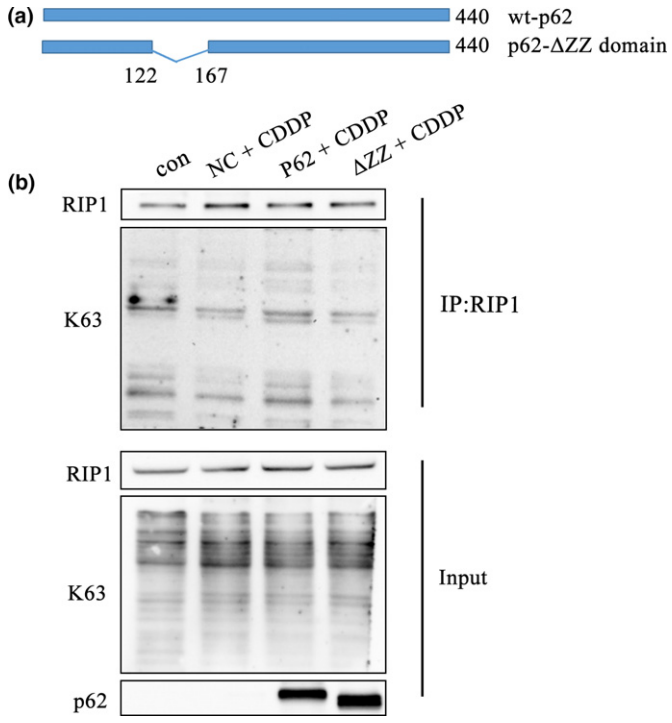

Fig. 3. Deleting the ZZ domain of p62 inhibits the K63 ubiquitination of RIP1 following the treatment of cisplatin in SKOV3 cells. (a) Schematic representation of the p62 wild type and mutant ZZ domain constructs. (b) After transfection with wt-p62 and ΔZZ-p62, SKOV3 cells were treated with cisplatin (6 μg/mL) for 24 h. Immunoprecipitation was performed using the anti-RIP1 antibody followed by western blotting using anti-RIP1 and anti-K63 antibody.

Statistical analyses. Statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA, USA). All the data are presented as means ± SD and carried out using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Cisplatin treatment activates the NF-κB pathway and an increase in RIP1 K63-linked ubiquitination in SKOV3/DDP cells. Through previous work, we determined that expression of p62 was different in parental SKOV3 human ovarian epithelial carcinoma cell lines and their established cisplatin-resistant subline SKOV3/DDP. SKOV3/DDP cells expressed higher levels of p62 and were less sensitive to cisplatin (CDDP) treatment.[4] To investigate whether the differential overexpression of p62 is correlated with a pro-survival mechanism, we evaluated the NF-κB signaling pathway in both cell lines. As shown in Figure 1a, p50/p65 levels markedly increased in the nucleus and phosphorylation of IκB also increased following cisplatin treatment in SKOV3/DDP cells. This result is in agreement with current knowledge on the activation of the canonical NF-κB pathway, which results in the phosphorylation of IκB, its subsequent degradation and the release of p50/p65 complex.[28–30] The luciferase reporter assay and immunofluorescence analysis confirmed our speculation that the NF-κB signaling pathway was activated in SKOV3/DDP cells following cisplatin treatment (Fig. 1b,c).

As noted above, RIP1 is involved in the canonical activation of NF-κB. Thus, we investigated the K63-linked ubiquitination of RIP1. We observed higher basal expression level of RIP1 in SKOV3/DDP cells (Fig. 1d). Furthermore, the co-immunoprecipitation results showed that cisplatin treatment increased K63-polyubiquitinated RIP1 over time in SKOV3/DDP cells but not in SKOV3 cells (Fig. 1e). These results prompted us to further explore the relationship between these molecules and cisplatin sensitivity in ovarian cancer cells.

Upregulation of NF-κB signaling and K63 ubiquitination of RIP1 are dependent on p62/SQSTM1 in SKOV3/DDP cells. To investigate whether p62 affected the activation of NF-κB or ubiquitination of RIP1, we used siRNA against p62 to inhibit its expression in SKOV3/DDP cells (Fig. 2a). In agreement with our previous study, cell viability decreased following cisplatin treatment when p62 expression was inhibited (Fig. 2b). Furthermore, using a luciferase assay we also observed p62

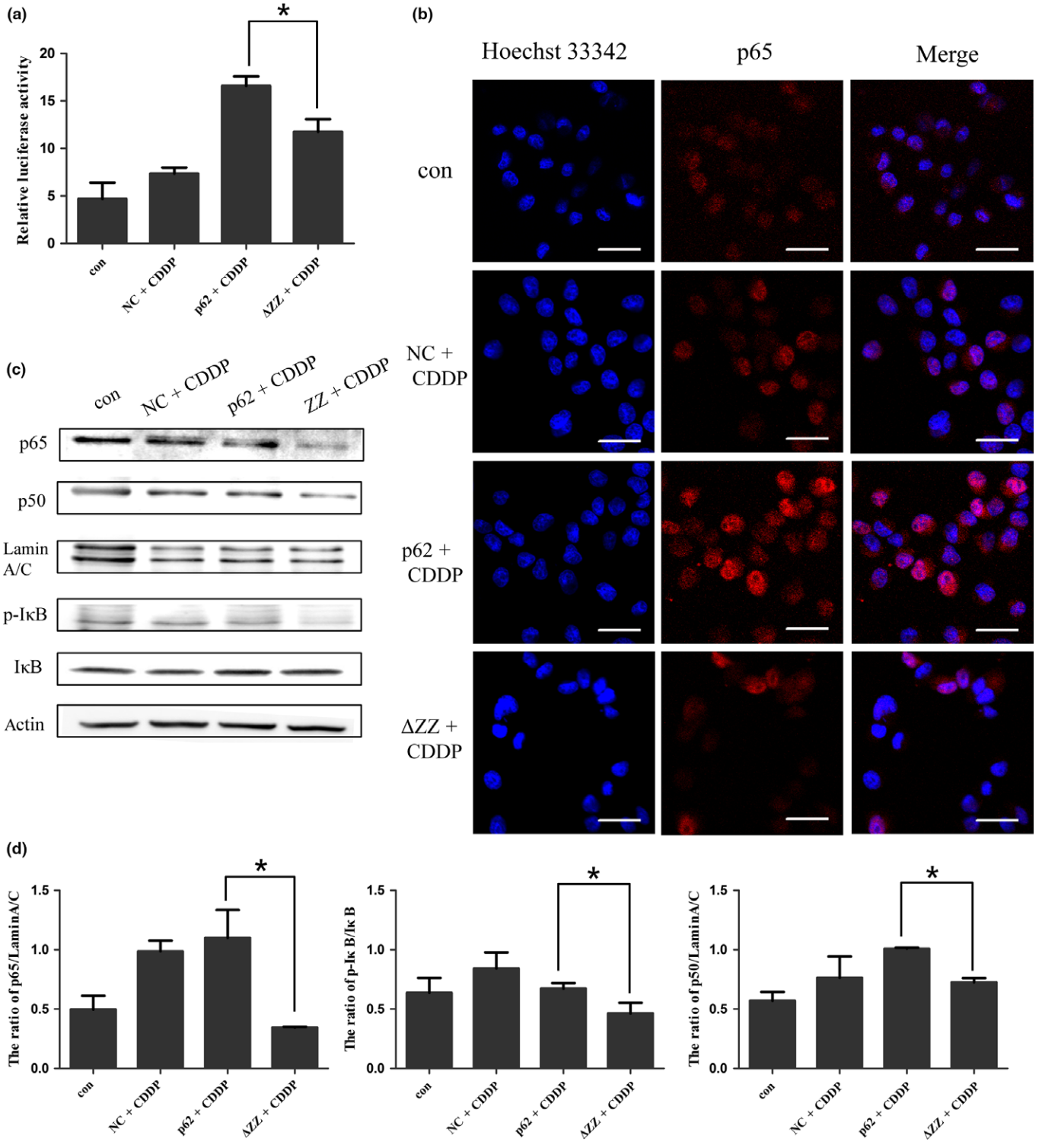

Fig. 4. Deleting the ZZ domain of p62 inhibited the activation of NF-κB signaling pathway in SKOV3 cells. (a) SKOV3 cells were transfected with wt-p62 and ΔZZ-p62. The luciferase activity was assessed and normalized on the basis of Renilla luciferase activity (mean ± SD, $n = 3$. *$P < 0.05$, *vs* wt-p62). (b) Localization of p65 and nuclear staining with Hoechst 33342 were observed with confocal laser microscopy (scale bar, 25 μm). (c) Western blotting was used to analyze the expression of NF-κB p65, NF-κB p50 in the nucleus and IκB and p-IκB in whole cell lysates of SKOV3 cells after transfection of different p62 plasmids. (d) Quantitation of NF-κB p65, NF-κB p50, IκB/p-IκB protein levels. Data were presented as mean ± SD, $n = 3$. *$P < 0.05$, *vs* wt-p62.

siRNA markedly induced downregulation of NF-κB transcriptional activity (Fig. 2d). Interestingly, the amount of co-immunoprecipitated K63-polyubiquitinated RIP1 also decreased (Fig. 2c). These data indicated that p62 functioned as a positive regulator for the activation of both the NF-κB pathway and RIP1.

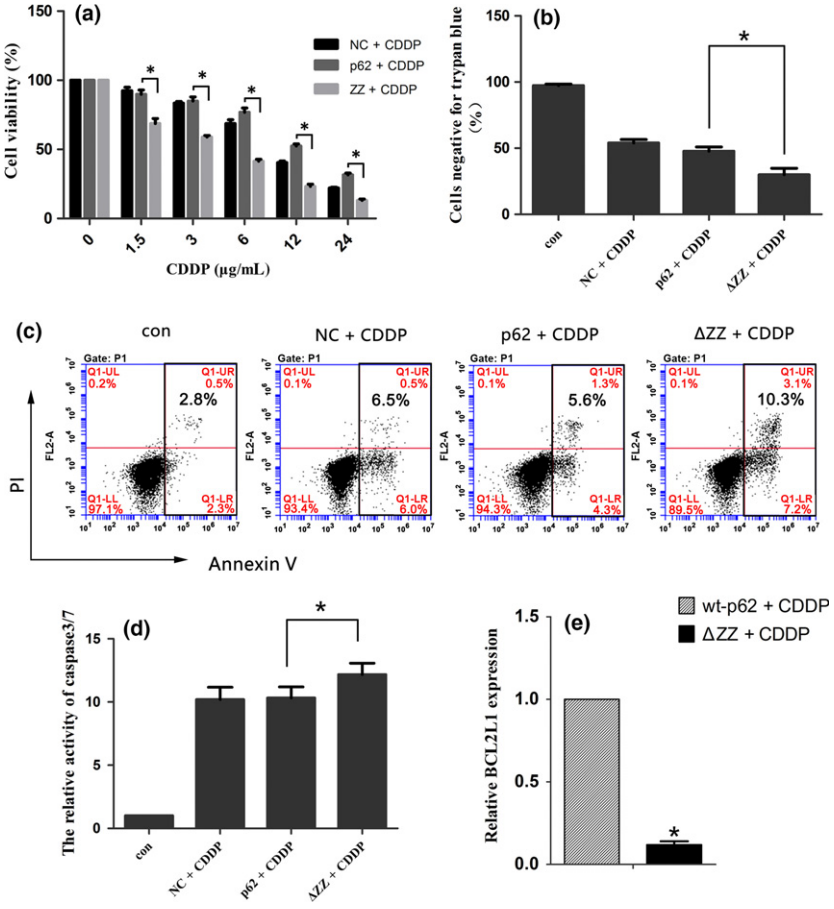

Fig. 5. Abolishing the regulation of RIP1 K63-linked ubiquitination by p62/SQSTM1 increases the sensitivity of SKOV3 cells to cisplatin. (a) The viability of cells was analyzed by an MTT assay. Data were presented as mean ± SD, $n = 3$. $^*P < 0.05$, *vs* wt-p62. (b) SKOV3 cells were treated as before. Cells were stained with Trypan blue and observed under a microscope. Data were presented as mean ± SD, $n = 3$. $^*P < 0.05$, *vs* wt-p62. (c) The apoptosis ratio of SKOV3 cells was detected by flow cytometry analysis. (d) Cells were analyzed using a caspase3/7 activity assay (Promega). Data are presented as mean ± SD, $n = 3$. $^*P < 0.05$ *vs* wt-p62. (e) The mRNA expression of BCL2L1 in SKOV3 cells after transfection of wt-p62 and ΔZZ-p62 plasmid. ACTB was used for normalization.Data are presented as mean ± SD, $n = 3$. $^*P < 0.05$ *vs* wt-p62.

p62/SQSTM1 activates the NF-κB pathway by increasing K63 ubiquitination of RIP1 following cisplatin treatment in SKOV3 cells. A previous study showed that in 293T cells p62 interacted with RIP1 through a specific structure known as the ZZ domain.[17] In this study, to investigate whether p62 upregulated NF-κB through regulating the ubiquitination of RIP1, a ZZ domain truncation mutation (ΔZZ) of p62 and a wild type (wt)-p62 were transfected into SKOV3 cells (Fig. 3a). Co-immunoprecipitation revealed that the K63-linked ubiquitination of RIP1 was downregulated when p62 no longer bound to RIP1 compared with cells overexpressing wt-p62 (Fig. 3b). These result suggested that p62 can truly affect the ubiquitination of RIP1.

We then investigated the activity of NF-κB in ΔZZ-p62 and wt-p62 expressing cells. As expected, deleting the ZZ domain decreased the luciferase activity compared with wt-p62 following the same dose treatment of cisplatin (Fig. 4a). The results of western blotting and immunofluorescence showed that the p50/p65 subunits were barely expressed in the nucleus when the p62 ZZ domain was absent (Fig. 4b–d). These results indicated that p62 was necessary to activate the NF-κB pathway by regulating the ubiquitination of RIP1.

Abolishing the regulation of RIP1 K63-linked ubiquitination by p62/SQSTM1 increases the sensitivity of SKOV3 cells to cisplatin. We have showed that inhibition of the K63-linked ubiquitination of RIP1 by mutating the key domain of p62 may downregulate the activity of NF-κB signaling pathway. Following this point, it is reasonable to speculate that loss of p62 ZZ domain may also affect the cell sensitivity to chemotherapy. In the following part, we examined cell viability using an MTT assay. As shown in (Fig. 5a), overexpression of ΔZZ-p62 decreased the sensitivity to cisplatin treatment compared with cells transfected with wt-p62. The trypan blue staining also suggested that transfection of the ΔZZ mutant decreased the number of viable cells (Fig. 5b).

We then determined if the reduction in cell number was the result of apoptosis induction or a failure in proliferation. First, we evaluated apoptosis using flow cytometry. As shown in Figure 5c, overexpression of ΔZZ-p62 in SKOV3 cells increased apoptosis with the treatment of cisplatin. Furthermore, the caspase3/7 activity assay also confirmed this speculation (Fig. 5d).Then we investigated proliferation by examining DNA replication using EdU Cell Proliferation Assay. EdU (5-Ethynyl-2′- deoxy uridine) is incorporated into cellular DNA during replication and newly synthesized DNA is quantitated by the levels of red stain.[31,32] As shown in Figure 6a, the cells in the replicative phase were inhibited after transfection with ΔZZ-p62 compared with wt-p62. Interestingly, staining with Hoechst 33342 also indicated an increase in apoptotic chromatin condensation.

To further understand the underlying molecular mechanisms of drug resistance induced by NF-κB activation, we then investigated several reported NF-κB target genes[33,34]: BCL2 like 1 (BCL2L1, also known as BCLX) is an anti-apoptotic protein regulated by NF-κB; Interleukin 6 (IL6) belongs to the cytokine/chemokine family; C-C motif chemokine ligand 2 (CCL2) is a regulator of cell proliferation and migration; Transforming growth factor beta 1 (TGFB1) and colony stimulating factor 3 (CSF3) are both well-studied growth factors.

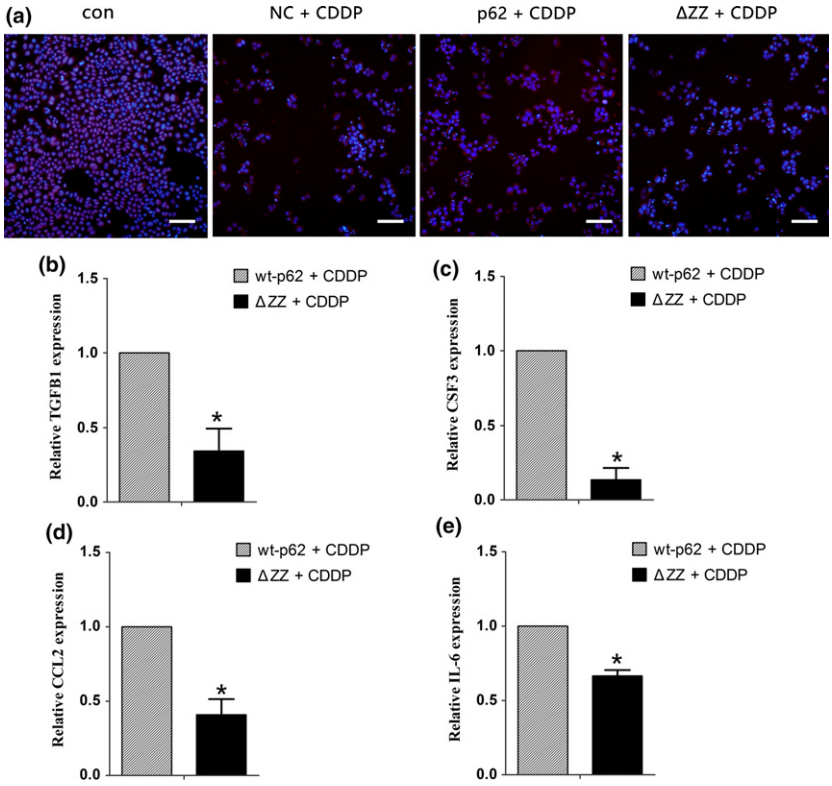

Fig. 6. The loss of the ZZ domain decreases cell proliferation in SKOV3 cells. (a) Merged pictures show the overlay of EDU stain and Hoechst33342 (scale bar, 50 μm). (b–e) Quantitative RT-PCR validation of well-known NF-κB regulated genes TGFB1, CSF3, CCL2, and IL6 in SKOV3 cells after transfection of wt-p62 and ΔZZ-p62 plasmid. ACTB was used for normalization. Data are presented as mean ± SD, $n = 3$. *$P < 0.05$ *vs* wt-p62.

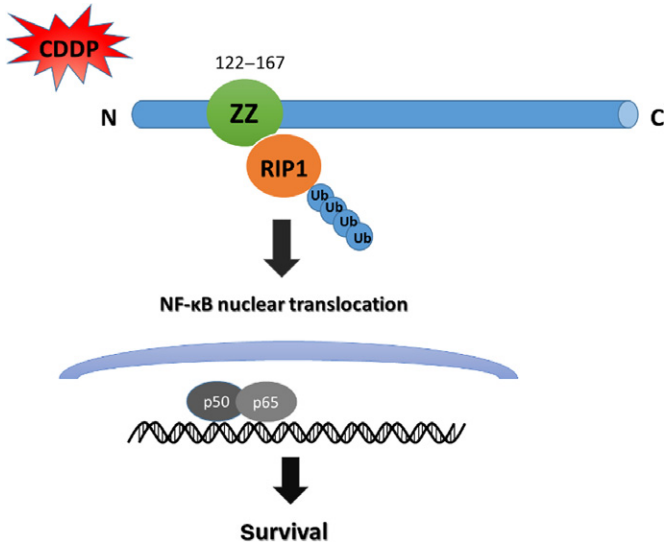

Fig. 7. p62-RIP1-NF-κB regulation pathway in ovarian cancer cells. p62 activating the NF-κB pro-survival pathway depends on K63-ubiquitinated RIP1 with cisplatin treatment. Furthermore, the activation of nuclear factor- κB (NF-κB) promotes the transcription of target pro-survival genes.

Our results indicated that compared with wt-p62, SKOV3 cells transfected with ΔZZ-p62 showed lower levels of BCL2L1 expression (Fig. 5e). Consistent with this result deleting the ZZ domain of p62 decreased expression of genes involved in proliferation (Fig. 6b–e). These results indicated that high levels of p62 in cisplatin-resistant ovarian cancer cells may protect them from death by evading apoptosis and promoting proliferation through activation of NF-κB signaling.

Discussion

Chemoresistance has been recognized as one of the crucial features of cancer whereby tumor cells escape the toxicity induced by drugs.[35] Although the antitumor effects of platinum compounds are related to DNA damage, this chemical feature means that Pt-based drugs also affect a variety of important cellular events including proliferation and apoptosis.[36,37] Therefore, understanding the role of these pathways may be helpful in combatting resistance. Our study suggests that the p62-RIP1-NF-κB pathway plays an important role in promoting cell survival and downregulating this pathway can significantly increase the sensitivity of ovarian cancer cells to cisplatin (Fig. 7).

We found that basal p62 expression was higher in cisplatin-resistant ovarian cancer cells (Fig. 1d). And p62 was truly related to resistant mechanism, not only cisplatin but other drug, in SKOV3/DDP cells during our previous study (Fig. S1). Although p62 protein levels decreased with cisplatin treatment in SKOV3/DDP cells, which was the result of autophagy, p62 mRNA transcripts still remained constant.[4] Furthermore, downregulating the expression of p62 increased the sensitivity to chemotherapy (Fig. 2b). Thus we believe that the mechanism of drug resistance in ovarian cancer cells is associated with the high level of p62. However, the precise molecular mechanisms of how p62 contributed to drug resistance in ovarian cancer remained poorly understood. p62 is overexpressed in many tumors and functions as a multi-functional hub that mediates its interactions with many binding partners. These include proteins involved in cellular transformation, proliferation and signaling such as NF-κB and MAPK.[38–40] We observed an increase in the transcriptional activity of NF-κB in drug-resistant SKOV3/DDP ovarian cancer cells when compared with parental SKOV3 cells. This result is in agreement with the previous findings in ovarian cancer cells.[41,42]

Therefore, it was reasonable to focus on the p62-mediated regulation of the pro-survival signaling complex NF-κB and explore the mechanism of chemoresistance in ovarian cancer cells.

RIP1 is a member of the RIP serine/threonine kinase family and binds with death receptors such as tumor necrosis factor (TNF) and Fas to promote apoptosis. Interestingly, cancer cells also show greater sensitivity to TNF-induced death when RIP1 is absent because of inactivation of the NF-κB survival pathway.[23,43] Recent studies suggested that the K63-linked polyubiquitin chains of RIP1 can rapidly recruit the IKK complex to promote phosphorylation of NFKBIA and activate NF-κB signaling, which contributed to cell survival in lymphoma glioblastoma, and colorectal cancer cells.[44–46] Furthermore, Sanz established the relationship between RIP1 and p62 in 293T cells using co-immunoprecipitation. She found that deletion of the ZZ domain of p62 significantly inhibited the binding of RIP1. This interaction increased the expression of pro-survival genes through upregulating the NF-κB signaling pathway.[17] In the current study, cisplatin treatment resulted in an increase of K63-linked ubiquitination of RIP1 in drug-resistant ovarian cancer cells. When we deleted the ZZ domain that interacts with RIP1, the K63-ubiquitinated form of RIP1 was reduced. Furthermore, the transcriptional activity of NF-κB was also inhibited. We then evaluated sensitivity to cisplatin in cells harboring the deleted domain. These cells showed a reduced proliferative capacity and an increase in apoptosis when they lost regulation of RIP1. They are supported by prior studies from Teramachi's laboratory who also observed that cell growth was inhibited by using the inhibitor XRK3F2 that specifically blocks the ZZ domain of p62 in myeloma cells.[47] These results confirm our hypothesis that p62 can regulate the NF-κB signaling pathway through the ubiquitination of RIP1 in ovarian cancer cells.

However, there are still many questions to be answered. We observed that overexpression of wt-p62 did not completely block apoptosis in ovarian cancer cells. We speculate that in SKOV3/DDP cells the escape from apoptosis was partly dependent on the activation of p62-RIPK1-NF-κB survival pathway under the stress of chemotherapy. However, overexpression of the multi-functional p62 protein is likely to have pleiotropic effects.[48–50] Furthermore, p62 may also function as a switch that regulates opposing pathways depending on its interaction with different molecules in physiological and pathological conditions.

Collectively, our study provides evidence that, in ovarian cancer cells, p62 participates in the mechanism of cisplatin-resistance by activating the NF-κB pro-survival pathway, and this process depends on K63-ubiquitinated RIP1. These data contribute to understanding the role of "non-nuclear" targets in chemotherapy and may aid development of an effective therapeutic strategy to overcome Pt-based drug resistance in ovarian cancer.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CDDP/DDP	cisplatin
NF-κB	nuclear factor kappa B
p50	NFKB1 (nuclear factor kappa B subunit 1)
p62/SQSTM1	sequestosome-1
p65	RELA proto-oncogene
RIP1	Receptor interacting serine/threonine kinase 1
ZZ	Zinc finger

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 p62 participated in the mechanism of VK3-resistance in SKOV3/DDP cells (a) SKOV3 and SKOV3/DDP cells were treated with varying doses of VK3 for 8 and 16 h. Cell viability was determined by MTT assay. Data were presented as a mean ± SD, $n = 3$. (b) Western blot analysis for the nuclear Nrf2 protein in transfected SKOV3/DDP cells.