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# Polo-like kinase 1 regulates mitotic arrest after UV irradiation through dephosphorylation of p53 and inducing p53 degradation

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**Abstract** **Ultraviolet (UV) irradiation can result in cell cycle arrest. The reactivation of Polo-like kinase 1 (Plk1) is necessary for cell cycle reentry. But the mechanism of how Plk1 regulates p53 in UV-induced mitotic arrest cells remained elusive. Here we find that UV treatment leads HEK293 cells to inverse changes of Plk1 and p53. Over-expression of Plk1 rescue UV-induced mitotic arrest cells by inhibiting p53 activation. Plk1 could also inhibit p53 phosphorylation at Ser15, thus facilitates its nuclear export and degradation. Further examination shows that Plk1, p53 and Cdc25C can form a large complex. Plk1 could bind to the sequence-specific DNA-binding domain of p53 and active Cdc25C by hyperphosphorylation. These results hypothesize that Plk1 and Cdc25C participate in recovery the mitotic arrest through binding to the different domain of p53. Cdc25C may first be actived by Plk1, and then its phosphatase activity makes p53 dephosphorylated at Ser15.**


*Keywords:* Polo-like kinase 1; p53; Cdc25C; UV; Mitosis arrest

## 1. Introduction

Ultraviolet (UV) irradiation can result in DNA damage [1,2]. The DNA damage checkpoint [3,4] can be regarded as a coherent signal transduction system that allows cells to transfer the information from a DNA lesion to the cell cycle. When DNA is damaged by UV irradiation, two critical effectors: the Cdc25 phosphatase and the p53 transcription factor are activated by the checkpoint kinases ATM/ATR (ataxia-telangiectasia mutated/ataxia-telangiectasia related protein) and Chk1/Chk2. The activation of the Cdc25 and the p53 affects at least three stages at cell cycle: the $G_1$/S transition, S phase progression and the $G_2$/M boundary [5,6].

During cell cycle progress, p53 is subject to tight regulation. Activation of p53 by DNA damage occurs at two levels: the stabilization of the p53 protein, leading to its accumulation in the nucleus, and activation of biochemical functions encompassed within the p53 protein [7]. The phosphorylation of human p53 on Ser20 and Ser15 is mediated by the Chk1/2 in response to UV radiation and γ-irradiation (IR) [8]. This phosphorylation weakens the affinity between p53 and its inhibitory factor Murine/Human double minute 2 (Mdm2/Hdm2), leading to the stabilization and activation of p53 [9,10]. The most important transcriptional target of p53 is the cyclin-dependent kinase (CDK) inhibitor $p21^{CIP1/WAF1}$, a potent inhibitor of several CDK complexes [11]. Cells lacking p21 have a defect in the DNA damage-induced $G_1$/S arrest [12,13].

Cdc25C phosphatase is an accelerator for $G_2$/M transition, whose major target is the Cdk1/cyclin B kinase. Cdc25C can active Cdk1/cyclin B1 complex by dephosphorylation at Thr14 and Tyr15. The protein Cdc25C phosphatase is itself regulated by phosphorylation at two major sites, at Ser216 and at its uncharacterized amino-terminal end. Ser216 phosphorylated by Chk1/2 after DNA damage [14,15] leads to Cdc25C inactivation. In contrast to the inhibitory phosphorylation by Chk1/2, Cdc25C also requires phosphorylation to become activated. Activating phosphorylation is thought to require both Cdk1/cyclin B complexes [16] and the action of Polo-like kinases (Plks) [17].

The Plks are conserved Ser/Thr protein kinases and regulate multiple processes in normal cell cycle progression [18,19]. Plks also seem to respond to DNA damage and are integrated into checkpoint control pathways. This finding suggested that DNA damage activated ATM and ATR both inhibit Plk1 kinase activity, a mechanism that could contribute to cell cycle arrest at $G_2$/M [20,21]. Very recently, another finding demonstrates a unique role for Plk1 acts in concert with Wee1 and Cdc25B during recovery from a DNA damage-induced arrest [22,23]. People also found that Plk1 physically binds to the p53 in mammalian cultured cells and inhibits its transcription activity [24]. But there is little report on how Plk1 act on p53 in UV-induced $G_2$/M arrest.

In light of these observations, our attention has focused on the interaction between Plk1 and p53 in recovery from mitotic progress in UV-induced $G_2$/M arrest cells. We found that in UV-induced $G_2$/M arrest cells, Plk1, p53 and Cdc25C can form into a large complex. Plk1 could active Cdc25C by hyperphosphorylation and the binding of Plk1 to p53 could affect the stability of p53 by dephosphorylation at Ser15. These results suggest that Cdc25C might mediate Plk1 negative regulation of p53 in rescue cell from mitotic arrest due to DNA damage by UV irradiation.

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## 2. Materials and methods

### 2.1. Plasmid construction and reagents

Plk1 gene was sub-cloned into pcDNA3.1 (Invitrogen Co., Carlsbad, CA, USA). The truncated Plk1 (303–603), lacking an $NH_2$-terminal kinase activity domain, was generated by PCR with the forward primer 5′-ATGAATTCAGGAGGCCTTGAGG-3′ and the reverse primer 5′-AAGCTAGCCATGAATGCAGTGG-3′. Amplified fragments were digested with *Nhe*I and *Eco*RI restriction enzymes. The construct was confirmed by sequence analysis. $p21^{CIP1/WAF1}$ luciferase reporter and L-galactosidase reporter plasmid were kindly gifted by Dr. Zhi-Ming Yin.

The mouse anti-Plk1 was from Zymed (South San Francisco, CA), mouse anti-p53 (DO-1), rabbit anti-p53 (FL-393), mouse negative control antibody Gal-4, rabbit negative control antibody Ob-R, rabbit anti-Cdc25C, anti-Cyclin B, anti-Actin, goat anti-Lamin B and goat anti-GAPDH were from Santa Cruz (Santa Cruz, CA), rabbit anti-phospho-p53 (Ser15) was from Cell Signaling (Beverly, MA). Conjugated anti-mouse or anti-rabbit secondary antibodies were purchased from the Calbiochem-Novabiochem Corp. (La Jolla, CA, USA).

### 2.2. Cell culture, transfection, synchronization, UV treatment and cell fractionation

HEK293 (human embryonic kidney 293 cell line) and $LO_2$ (normal human liver cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. Cells grown in the semi-confluence were transfected with indicated plasmids using phosphate calcium precipitation method. Directly after transfection, cells were incubated in thymidine (2.5 mM) for 24 h to arrest at the $G_1$/S transition. After released from a thymidine block for 6 h, a time at which the great majority of the cells had completed S-phase, irradiated cells with 30 J/$M^2$ UVB (254 nm, ultraviolet cross-linker). Then cells were collected after cultured for another 18 h in fresh medium to allow them enter the next mitosis stage.

Fractionation of HEK293 cell extracts was performed as follows. Cells were resuspended in hypotonic buffer (20 mM Tris–HCl at pH 7.4, 10 mM KCl, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 10 min on ice and lysed by dounce homogenization. Nuclei were pelleted by centrifugation at $1000 \times g$ for 10 min and washed with PBS twice. Both the cytoplasmic and nuclear fractions were mixed with SDS sample buffer and analyzed by SDS–PAGE and immunoblotting. The nuclear and cytoplasmic fractions were normalized to contain equal quantity of total proteins.

### 2.3. Cell cycle assay and mitotic index analysis

HEK293 cells were harvested and washed with PBS-0.5% Tween 20. Then stained with propidium iodide (50 μg/ml) for 30 min at 37 °C. Cell cycle was judged by flow cytometric analysis with FACScan (Becton Dickinson, Mountain view, CA, USA). Cells that are undergoing mitosis can be judged by the following two methods. First, the use of DIC microscopy can show the morphology the cells that are in mitosis. Cells will become round when enter mitosis. Second, stain cells with DAPI to show the morphology of their chromosomes. If those cells are indeed arrest in mitosis, the chromosome will condensed to specific position. Count the round cells and all the cells in each field, respectively.

$$\text{Mitotic index} = \frac{\text{number of cells in mitosis}}{\text{total number of cells}} \times 100\%$$

(All transfections were performed in triplicate. Each time we counted six fields and the total numbers of cells in each field were more than 150.)

### 2.4. Immunoprecipitation and Western blotting

Equal amount of total cell lysate were prepared for western blotting with appropriate antibody or were incubated with appropriate primary antibody overnight at 4 °C to do immunoprecipitation. The immunocomplexes were pulled down with the protein G–agarose (Roche, Mannheim, Germany) at 4 °C for 2 h. The precipitates were washed with the immunoprecipitation (IP) buffer (50 mM Tris–Cl, pH 7.5, 120 mM NaCl, 0.05% NP-40, 1 mM phenylmethylsulfonyl fluoride) for 10 min triple times at 4 °C. The pellets were boiled in the SDS sample buffer and resolved by 10% SDS–PAGE, then transferred to PVDF membrane (Roche, Mannheim, Germany). The membranes were incubated overnight with appropriate primary antibody. Actin was used as the loading control.

### 2.5. Yeast two-hybrid interaction assays

The yeast two-hybrid screen was performed according to the MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech, Palo Alto, CA). The plk1 gene was sub-cloned into pGADT7 vector. The full-length p53 (1–393) and p53 truncation gene p53 (1–117), p53 (72–390), p53 (285–393) were sub-cloned into pGBKT7 vector. Co-transformation pGADT7-Plk1 with pGBKT7-p53 and a series of p53 deletion truncations into AH109 yeast cells, respectively, by standard lithium acetate (LiAc)-mediated method. Co-transformed yeast cells were then plated on an appropriate synthetic dropout (SD) medium, with tryptophan, leucine, histidine, and adenine (-T-L-H-A)-deficient selection plates, for 4 days at 30 °C.

### 2.6. Plk1 luminescent kinase assay

The Kinase-Glo™ Luminescent Kinase Assay (Promega. Madison, WI, USA) is a method of measuring kinase activity by quantifying the amount of ATP remaining in solution following a kinase reaction. Thus its luminescent signal is inversely correlated with the amount of kinase activity. Plk1 was immunoprecipitated and re-suspended in kinase reaction buffer (20 mM HEPES, pH 7.4–7.7, 10 mM $MgCl_2 \cdot 6$-$H_2O$, 50 mM KCl and 1 mM DTT) with 2 μM ATP and 4 μg casein as kinase substrate. Reactions were performed at room temperature for 1 h. Then equal volume of kinase-Glo™ Reagent was added to each tube and incubated at room temperature for 10 min to stabilize luminescent signal. The luminescence was measured on a luminometer (Lumat LB 9507).

### 2.7. $p21^{CIP1/WAF1}$ luciferase assay

HEK293 cells were subculture into six-well plates. Transiently co-transfected with luciferase reporter that carries the p53-responsive $p21^{CIP1/WAF1}$ promoter element (200 ng/well), β-galactosidase plasmid (50 ng/well) and together with pcDNA3, Plk1, and Plk1(303–603) (2 μg/well), respectively. 46 h after transfection, cells were treated with or without UV. After culture for another 2 h, cells were lysated and analyzed for their luciferase activities by luciferase reporter gene assay kit (Roche, Mannheim, Germany). All transfections were performed in triplicate. Results are shown as fold induction of the luciferase activity compared with control group.

### 2.8. Immunofluorescence staining

Immunofluorescence was performed as described [25]. Cells were co-stained for DNA with DAPI (Molecular Probes, Eugene, OR). The distribution pattern was revealed under a Leica DMR microscope equipped with a 40× (N.A. 0.65) objective. Images were captured with a cooled charge-coupled device camera (Dignostic Instruments, MI) and processed using SPOT software (Dignostic Instruments).

### 2.9. Protein cross-linking

Disuccinimidyl suberate (DSS) was purchased from Sigma. Synchronized HEK293 cells with Nocodazole (150 ng/ml) for 24 h to arrest the cells at $G_2$/M phase. After treat with UV for another 2 h, cells were washed with PBS. Freshly prepared DSS was then added to a final concentration of 0.25 mM to the cells in PBS. The dishes were incubated for 30 min on a rocker platform. After cross-linking, cells were harvested and subjected to immunoprecipitation, SDS–PAGE, and Western blotting as indicated.

## 3. Results

### 3.1. UV induces p53 activation and inhibits Plk1 kinase activity

Plk1 activity reaches its peak at $G_2$/M, the DNA damage induced by adriamycin could inhibit Plk1 activity and leads to cell cycle arrest [21]. Recent evidence further shows that p53 is also involved in controlling the $G_2$/M transition after DNA damage [2]. In accordance with their reports, it was not surprised to find that both the protein level and kinase

activity of Plk1 were decreased when the synchronized $G_2$ HEK293 cells were exposed to UV irradiation (Fig. 1A and B). Meanwhile, the protein level (Fig. 1A) and transcription activity (Fig. 1D) of p53 were increased. But over-expressed Plk1 in UV irradiated cells could inhibit p53 transactivation, while the kinase domain deficient Plk1 (303–603) failed to reduce the p53-mediated reporter expression (Fig. 1C and D). This suggested that there might be an inverse relationship between Plk1 and p53 during UV-induced DNA damage.

### 3.2. Recovery from a DNA damage induced mitotic arrest in mammalian cells requires Plk1

Plk1 play a crucial role in recovery from a DNA damage-induced arrest. This was confirmed by mitotic index analysis (Fig. 2A) and FACS analysis (Fig. 2B) in UV-induced mitotic arrest HEK293 cells. Compared with mock treatment cells, approximately 31.45% of the cells were arrested in mitosis by the UV treatment. Whereas this effect was overcome by the over-expression of Plk1 and cells proceeded through mitosis and entered $G_1$. In contrast, over-expression of Plk1 (303–603) could not break through mitotic arrest (Fig. 2A and B). In addition, compared with UV-induced arrest cells, the amount of Cyclin B1 protein was significantly reduced in the cells over-expression of Plk1, indicating that majority of the cells exited mitosis and entered $G_1$ (Fig. 2C). As a key regulator in $G_2$/M transition, Cdc25C is also a substrate of Plk1. Hyperphosphorylation during mitosis caused Cdc25C to migrate more slowly on SDS–PAGE gels [22]. We found that compared with UV treatment, over-expression of Plk1 in HEK293 cells could cause the hyperphosphorylation of Cdc25C, which is required for $G_2$/M transition (Fig. 2D). Thus, over-expression of Plk1 correlates with an ability to proceed through mitosis.

### 3.3. Plk1 regulates the p53 stabilization and localization through inhibit UV-induced p53 Ser15 phosphorylation

DNA damage leads to p53 stabilization and accumulation in the nucleus. We found that the Ser15 phosphorylation level of

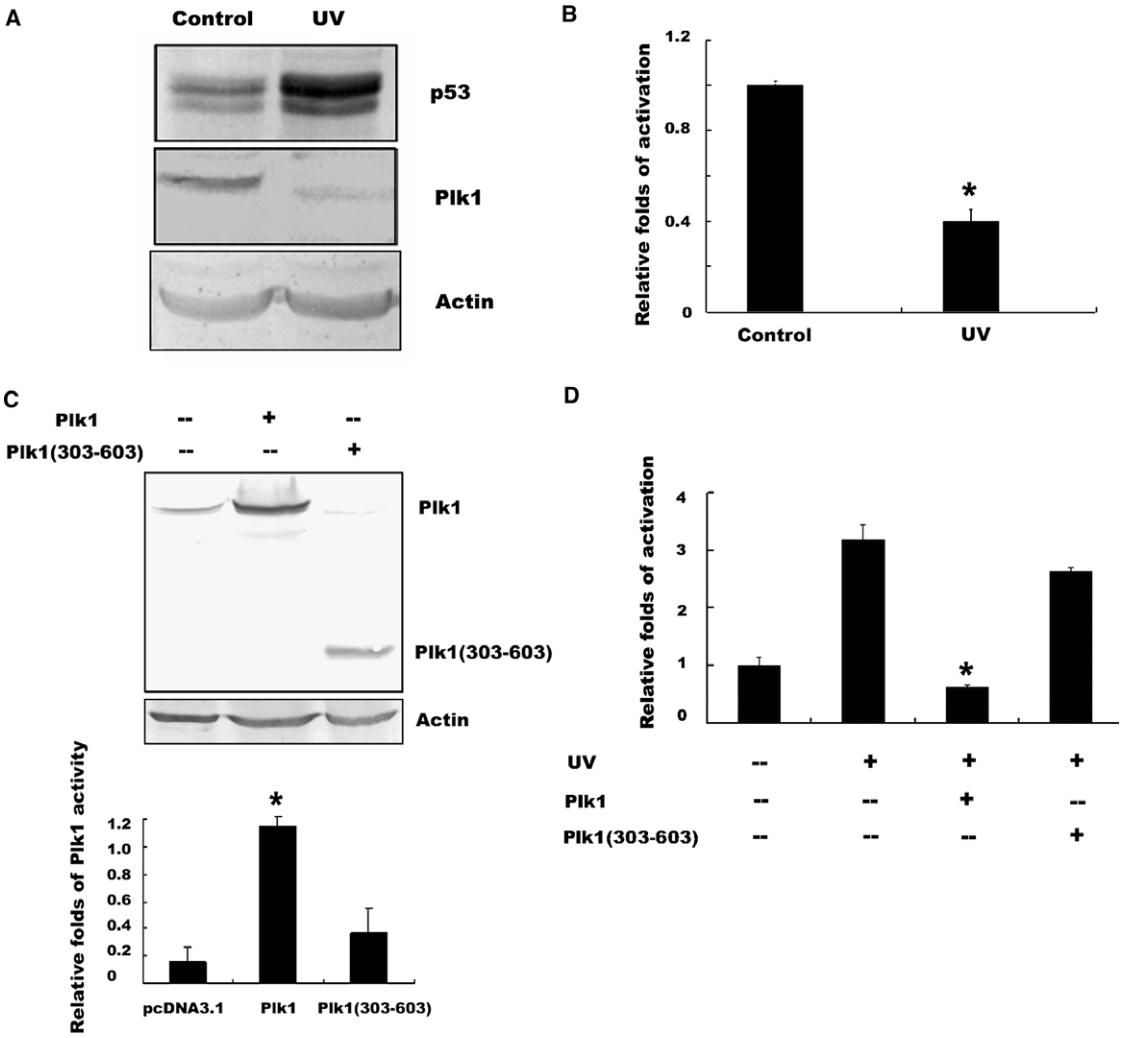


Fig. 1. The effect of UV irradiation on p53 activation and Plk1 kinase activity. HEK293 cells were blocked with thymidine for 24 h, after released from the block for 6 h, irradiated with UV. Harvest cells after culture for another 2 h. (A) Western blotting showed that the protein level of Plk1 was decreased and p53 level was increased after UV treatment. (B) The kinase activity of Plk1 was decreased after UV treatment (*, $P < 0.05$). (C) Western blotting and the kinase activity showed that, compared with endogenous Plk1, the protein level and kinase activity of Plk1 were increased if over-expression wild-type Plk1, while they were decreased if over-expression kinase domain deficient Plk1 (*, $P < 0.05$). (D) The $p21^{CIP1/WAF1}$ report gene activity was abrogated to only 19% of the control when overexpressed Plk1, but its activity was similar to the control when overexpressed Plk1 (303–603) (*, $P < 0.05$).

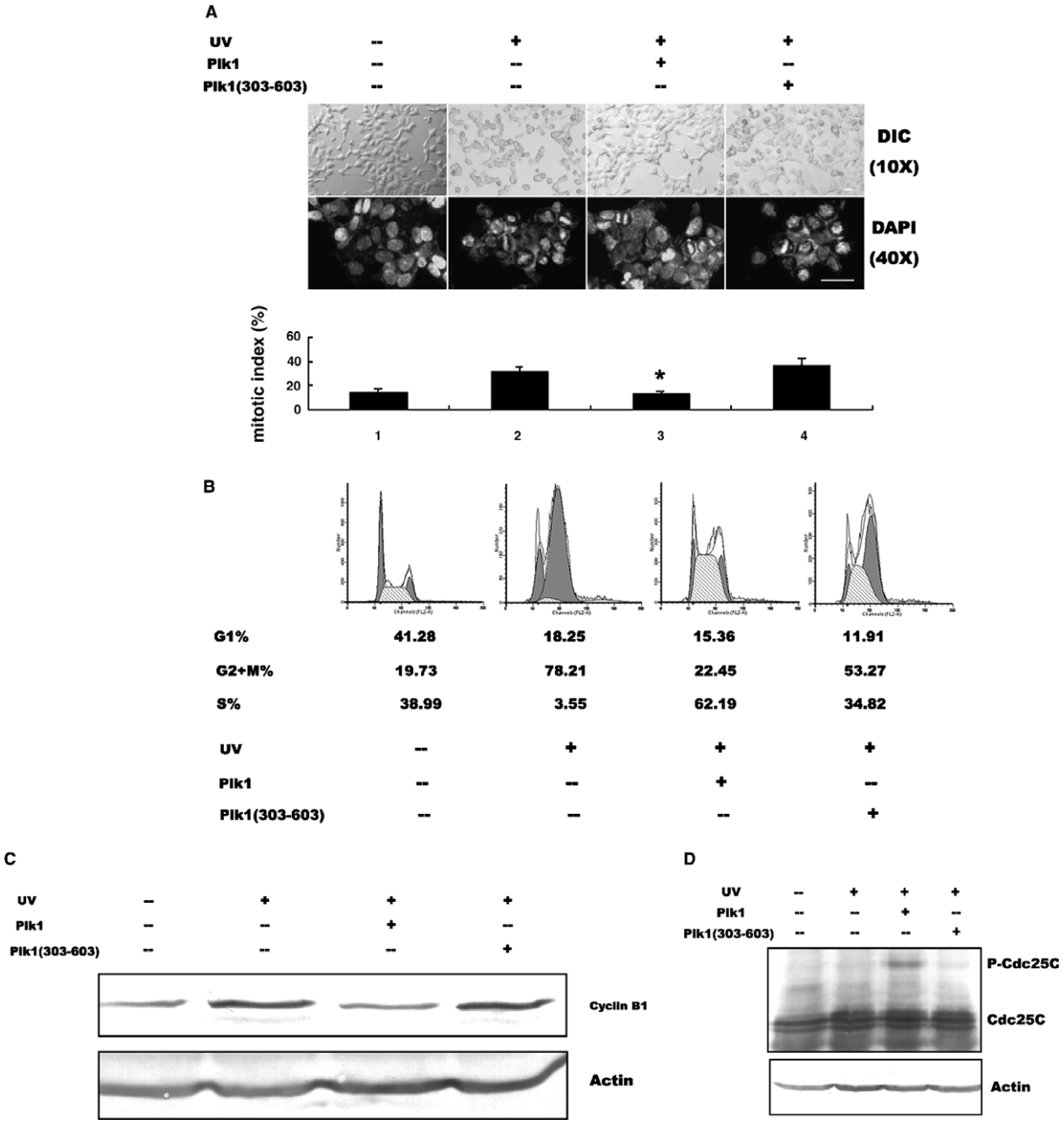


Fig. 2. The effect of Plk1 on the recovery of mitotic arrest. HEK293 cells transiently transfected with pcDNA3, Plk1, and Plk1 (303–603), respectively, were synchronized and treated with or without UV as described in material and methods. Then the cells were detected with microscopy (for A), collected for FACS analysis (for B) or collected for western blotting (for C) after cultured for another 26 h. Cells with the same treatment will culture for another 18 h (for D) in fresh medium for further study. (A) DIC and fluorescence microscopy showed the morphology of mitotic arrest cells. Compared with the control cells, the synchronized cells with UV treatment became round and the chromosome became condensed. Over-expression of Plk1 will recover mitotic arrest. Mitotic index results showed that about 31.45% of the cells were arrested in mitosis. Plk1 over-expression could rescue mitotic arrest and cells that remained in mitosis were declined to 12.88%, similar to normal control. While Plk1 (303–603) could have no effect (*, $P < 0.05$) (10× and 40×: objective; scale bar: 20 μm). (B) FACS assay showed that Plk1 could rescue the cells from mitosis arrest and about 55.76% cells were proceed into next cell cycle. While Plk1 (303–603) could only reduce 25% and about 53.27% remained in $G_2$ + M stage. (C) Compared with UV treatment, the amount of Cyclin B1 protein was remained high in the cells over-expression of Plk1 (303–603), while it was significantly reduced in the cells over-expression of Plk1. (D) Compared with UV-induced $G_2$/M arrest cells, Plk1 could recover endogenous Cdc25C to a hyperphosphorylation level. Kinase domain deficient truncate won't have such effect.

p53 was decreased when over-expressed Plk1 in UV-induced mitotic arrest cells, while over-expression of Plk1 (303–603) had no effect (Fig. 3A). The phosphorylation of Ser15 can block p53 binding with Mdm2, who regulates p53 stabilization. As shown in Fig. 3B, co-transfection of Mdm2 and Plk1 into HEK293 cells had more prominent effects on p53 degradation. Furthermore, in addition to acting as a binding site for Mdm2, the N-terminus of p53 is thought to contain a nuclear export sequence (amino acids 11–27). DNA damage-induced phosphorylation of Ser15 and Ser20 may also

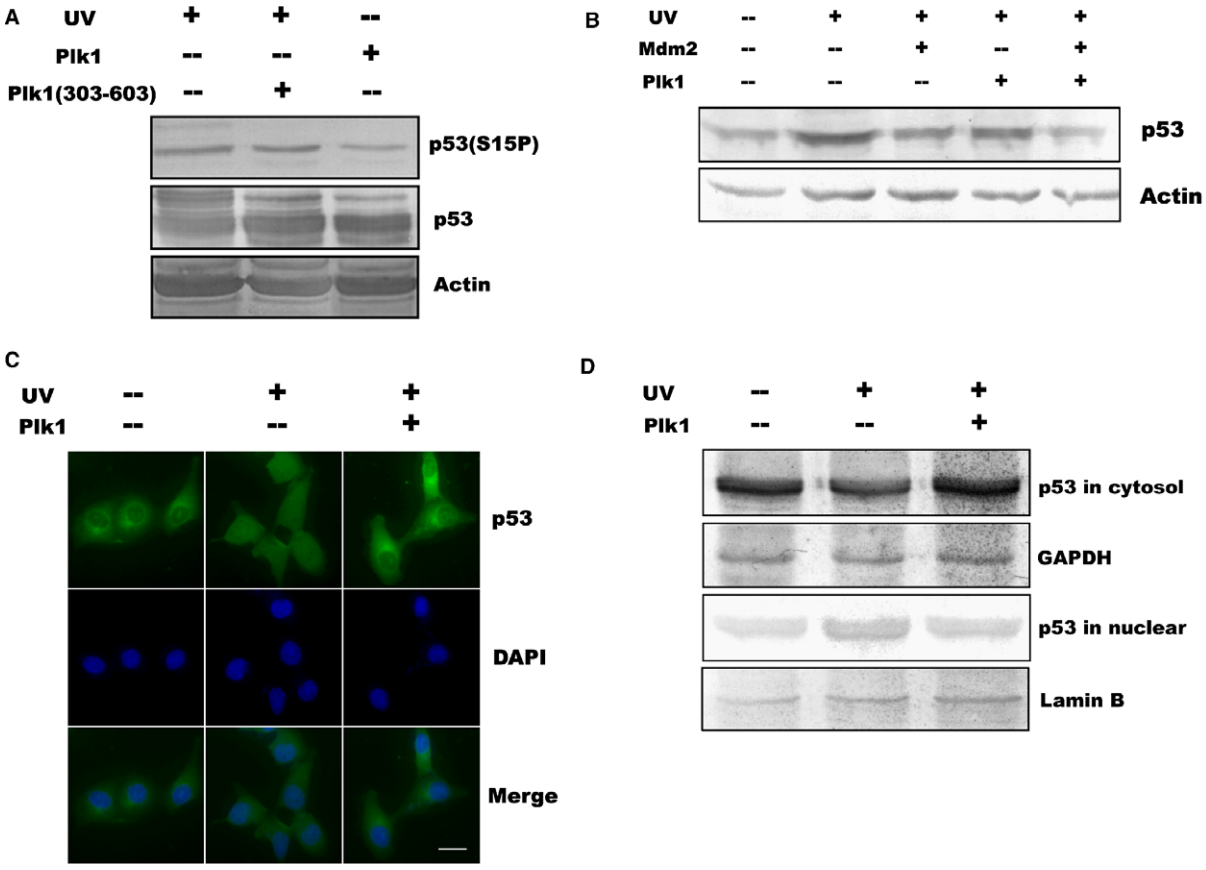


Fig. 3. Plk1 regulates the p53 stabilization and localization through inhibit UV-induced p53 Ser15 phosphorylation. HEK293 cells transiently transfected with indicated plasmids, respectively. 46 h after transfection, cells were treated with or without UV. Harvest cells after culture for another 2 h. (A) Plk1 can inhibit UV-induced p53 Ser15 phosphorylation. (B) Plk1 promote p53 degradation cooperated with Mdm2. (C) Immunofluorescence showed that Plk1 could facilitate p53 nuclear export during UV irradiation in LO2 cells (scale bar: 20 μm). (D) Fractionation experiments show that compare with UV treatment, Plk1 overexpression can cause p53 protein level increased in cytosol while decreased in nuclear.

block export of p53 mediated by this NES thereby maintaining p53 within the nucleus [26]. Under our experimental condition, UV irradiation could drive p53 import from the cytoplasm to the nucleus, while over-expression of Plk1 could inhibit this process and facilitate p53 nucleus export (Fig. 3C). Cell fractionation experiment also confirmed this result (Fig. 3D). The evidence we showed here suggested that Plk1 could regulate p53 transcriptional activity through corporation with Mdm2 and regulating its degradation.

### *3.4. Endogenous Plk1, p53 and Cdc25C interact with each other*

A recent study revealed that exogenous Plk1 physically binds to the tumor suppressor p53 in mammalian cultured cells [24]. In Fig. 4B, we confirmed that the endogenous Plk1 was co-immunoprecipitated with the endogenous p53 from HEK293 cell lysate, and vice versa. Yeast two-hybridization assay showed that among all the p53 truncations, only sequence-specific DNA-binding domain (72–390) retained the ability to bind with Plk1 (Fig. 4C). Co-immunoprecipitation showed that only the kinase domain of Plk1 could bind to p53 (Fig. 4D). Chemical cross-linking experiment using DSS shows that Cdc25C, p53 and Plk1 can form a large complex (Fig. 5A).

Plk1 is a protein kinase that could phosphorylate its target protein, but here we show that Plk1 could affect the dephosphorylation of p53. There must be an intermediator who made p53 dephosphorylation. We have shown that Plk1 could hyperphosphorylate Cdc25C in Fig. 2D. In Fig. 4A, we showed that Cdc25C could co-immunoprecipitated with both Plk1 and p53. Thus the Cdc25C might be the mediator between Plk1 and p53.

## 4. Discussion

UV-induced DNA damage can cause cell cycle arrest. It is report that Plk1 reactivation before the other positive cell cycle regulators [27] is necessary for cell cycle reentry after DNA damage induced $G_2$ arrest [22]. As a major regulator for various cell cycle checkpoints, Plk1 directly target many key cell cycle regulators such as p53, Cdc25C, Cyclin B, etc. [28]. Here we present that in UV-induced mitotic arrest cells, Plk1, p53 and Cdc25C can form into a large complex. Plk1 can active Cdc25C phosphatase by hyperphosphorylation and the binding of Plk1 to p53 could dephosphorylated p53 at Ser15 and facilitate its degradation through Mdm2 pathway.

Despite the reports that Plk1 could phosphorylate p53 in vitro [29], however, in present study, we found that the Ser15 phosphorylation was decreased when Plk1 was overexpressed in UV-induced mitotic arrest cells. Plk1 could also

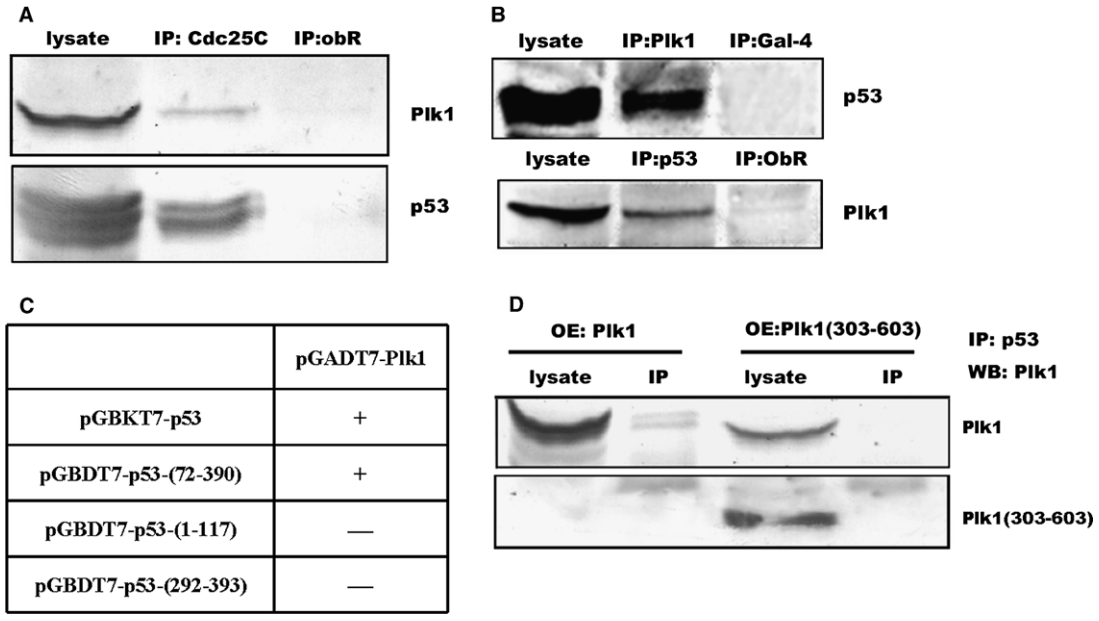


Fig. 4. Endogenous Plk1, p53 and Cdc25C interact with each other. The HEK293 cells were synchronized with nocodazole (150 ng/ml) for 24 h to arrest the cells at $G_2$/M phase. After transfection for 46 h, treat with UV. Harvest cells after culture for another 2 h. (A) IP show that both Cdc25C and Plk1 could bind with p53. (B) Co-immunoprecipitation showed that endogenous Plk1 interact with p53 directly. (C) The yeast two-hybrid screen showed Plk1 interacts with p53 DNA-binding domain. (D) IP showed that it was Plk1 kinase domain that binds with p53.

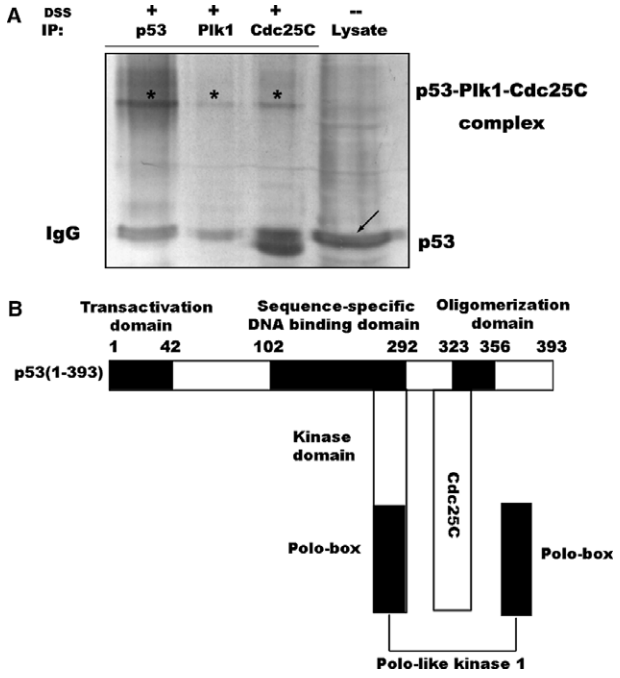


Fig. 5. Plk1, p53 and Cdc25C formed a large complex in UV-induced DNA damage arrest cells. (A) Synchronized HEK293 cells with Nocodazole (150 ng/ml) for 24 h to arrest the cells at $G_2$/M phase. After treat with UV for another 2 h, cells were washed with PBS treated with or without DSS. Freshly prepared DSS was then added to a final concentration of 0.25 mM to the cells in PBS. IP with indicated antibody and western blotting with p53 antibody shows that endogenous Plk1, p53 and Cdc25C can form a large complex (*: Plk1, p53 and Cdc25C complex; arrow: p53). (B) Schematic representation of interaction among Plk1, p53 and Cdc25C. Plk1 and Cdc25C might bind to the different domain of p53. Plk1 could physically binds to the sequence-specific DNA-binding region of the tumor suppressor p53 on amino acids 72–292 through kinase domain, Cdc25C direct binding to p53 on amino acids 287–340.

promote p53 degradation through Mdm2 pathway. Our results suggested that Plk1 might modulate p53 activity through another pathway, by which Plk1 could inhibit UV-induced p53 phosphorylation at Ser15. Thus Plk1 can induce p53 degradation and the resumption of mitosis arrest.

As we all know, Plk1 is a protein kinase and can make its substrate phosphorylation. How could Plk1 make p53 dephosphorylation? Although Plk1 and P53 could interact directly with each other, there must be another mediator who mediates p53 dephosphorylation. Plk1 can activate Cdc25C phosphatase activity by direct phosphorylation and thereby contribute to activate Cdk1/cyclin B complex [30,31]. It was previously shown that the phosphatase Cdc25C suppresses the p53 induced growth arrest in fission yeast. Moreover, expression of Cdc25C leads to a reduction of the p53-dependent UV-sensitivity [32]. In Rief's study, they demonstrated a direct binding of p53 to Cdc25C. By using different deletion mutants, the binding region was narrowed down on amino acids 287–340 of p53 and this binding of Cdc25C to p53 does not modify the DNA binding activity of p53 [33]. Ando has reported that overexpressed Plk1 could physically binds to the DNA-binding region of the tumor suppressor p53 in mammalian cultured cell. Our co-immunoprecipitation data suggested that Plk1, p53 and Cdc25C formed a large complex. Plk1 and Cdc25C might bind to the different domain of p53 (a working diagram was shown as Fig. 5B). So we hypothesized that Plk1 may first hyperphosphorylate Cdc25C and active it. Then activated Cdc25C may dephosphorylate p53 at Ser15 and cause it degradation.

In summary, our results demonstrate that endogenous Plk1, p53 and Cdc25C interacted with each other. Plk1 can regulated p53 phosphorylation at Ser15 and cause its degradation through Mdm2 pathway. These results suggested that Plk1-mediated negative regulation of p53 might be a fundamental mechanism to rescue cell from mitotic arrest due to DNA damage by UV irradiation.

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