

**Parkin induces G2/M  
cell cycle arrest in TNF- $\alpha$ -treated  
HeLa cells**

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arrest in TNF- $\alpha$ -treated HeLa cells.**

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**This certifies that the master's thesis  
of Min Ho Lee is approved.**

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## ABBREVIATIONS

APC	:	adenomatous polyposis coli
CDC2	:	cell division cycle 2
CDC25	:	cell division cycle 25
DMEM	:	Dulbecco's modified Eagle's medium
EDTA	:	ethylenediaminetetraacetic acid
FACS	:	fluorescence-activated cell sorting
FBS	:	fetal bovine serum
GAPDH	:	glyceraldehydes-3-phosphate dehydrogenase
MMLV-RT:		Moloney murine leukemia virus reverse transcriptase
M.O.I.	:	multiplicity of infection
PBS	:	phosphate buffered saline
SDS	:	sodium dodecyl sulfate
TNF- $\alpha$	:	tumor necrosis factor-alpha

# **Parkin induces G2/M cell cycle arrest in TNF- $\alpha$ -treated HeLa cells.**

## **ABSTRACT**

Parkin is known to be a tumor suppressor protein. It was reported that Parkin was mutated or its expression reduced in a variety of cancer cells and reintroduction of Parkin into cancer cells suppressed cancer cell growth. There are several reports proposed the mechanism of Parkin acting as a tumor suppressor, nevertheless, it still remains to be fully elucidated. Malignant tumors usually possesses resistance toward tumor necrosis factor-alpha (TNF- $\alpha$ ). Previously, our laboratory determined that parkin expression restores susceptibility to TNF- $\alpha$ -induced death of HeLa cells, a human cervical cancer cell line resistant to TNF- $\alpha$ -induced cell death. However, tumor suppressors often act in a multiple mechanisms. In this study, I investigated the role of Parkin in growth of cancer cells. I found that Parkin expression inhibits cell division cycle 2 (CDC2) activity via phosphorylation of CDC2 at Tyr15, thereby inducing G2/M cell cycle arrest. This

seems to be due to the Parkin induced phosphorylation of cell division cycle 25C (CDC25C) and increased Myt1 protein level both of which are associated with the CDC2 phosphorylation. Furthermore, Parkin expression resulted in the dephosphorylation of histone H3 which may hinder the chromatin condensation that is essential for cell division. These results suggest that Parkin acts as a tumor suppressor not only by inducing apoptotic cancer cell death but also by regulating cell cycle progression via novel molecular mechanism I proposed.

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Key Words: Parkin, TNF- $\alpha$ , G2/M arrest, Cell cycle, Tumor suppressor

# I. INTRODUCTION

The Parkin gene (*PARK2*) was first described in Parkinson's disease as a hereditary cause of familial parkinsonism <sup>1</sup>. The gene is located on chromosome 6q25.2-6q27 which is involved in a common fragile site <sup>2</sup>. Many reports described the lack of Parkin expression or mutations in Parkin. Loss of heterozygosity (LOH) on chromosome 6q26 was reported in hepatocellular carcinoma, ovarian, breast, lung, and squamous cell lung cancers <sup>3-5</sup>, and alternative transcripts of parkin gene were found in a variety of cancers including ovarian, brain, liver, colorectal, cervical, pancreas, kidney, and breast cancer due to deletion or duplication of the gene <sup>2, 6-10</sup>. Moreover, hypermethylation of the parkin promoter region resulted in diminished expression of parkin in acute lymphoblastic leukemia, chronic myeloid leukemia, and colorectal cancers <sup>7, 11</sup>. It was reported that parkin over-expression resulted in growth inhibition in hepatocarcinoma, glioblastoma, lung cancer, breast cancer, and colon cancer <sup>7-10, 12</sup>. These reports attracted interest in the role of Parkin as a putative tumor suppressor and several studies began to suggest the role of Parkin in cancers. In the breast cancer cell line MCF7, parkin stabilized microtubules, increased

sensitivity to anti-cancer agents, and induced growth arrest <sup>8</sup>. In a glioblastoma cell line, parkin ubiquitinates cyclin E resulting in cyclin E degradation and subsequent cell cycle arrest <sup>9</sup>. Mutation in both the parkin gene and adenomatous polyposis coli (APC) gene accelerates development of colorectal carcinoma <sup>7</sup>. Collectively, these reports highlight the idea that parkin acts as a tumor suppressor. However, the mechanism by which Parkin act as a tumor suppressor still remains to be fully elucidated.

Cell cycle is a complex process that is regulated by a variety of molecules. Cell division cycle 2 (CDC2) is one of these molecules involved in the regulation of G2/M cell cycle transition. In eukaryotic cells, entry into mitosis is regulated by activation of CDC2 kinase, a process controlled at several steps including cyclin binding and phosphorylation of CDC2 <sup>13</sup>. The critical regulatory step in activating CDC2 during progression into mitosis appears to be dephosphorylation of CDC2 at Tyr15 and Thr14 <sup>14</sup>. Phosphorylation of CDC2 at Thr14 and Tyr15 can be carried out by Myt1 protein kinase <sup>15</sup>. Cell division cycle 25C (CDC25C) is a protein phosphatase responsible for dephosphorylation and activation of CDC2 kinase, a crucial step in regulating the entry of all eukaryotic cells into mitosis <sup>16</sup>. When phosphorylated at Ser216, CDC25C binds to members of the 14-3-3 family

of proteins, sequestering CDC25C in the cytoplasm and preventing premature mitosis<sup>17</sup>.

The nucleosome, made up of four core histone proteins (H2A, H2B, H3 and H4), is the primary building block of chromatin<sup>18</sup>. The amino-terminal tails of core histones undergo various post-translational modifications, including acetylation phosphorylation, methylation and ubiquitination<sup>19,20</sup>. Phosphorylation at Ser10, Ser28 and Thr11 of histone H3 is tightly correlated with chromosome condensation and the phosphorylation is required for cell cycle progression<sup>21 22</sup>.

In this study, I investigated the molecules involved in Parkin induced G2/M phase cell cycle arrest in TNF- $\alpha$ -treated HeLa cells. I report that Parkin induces phosphorylation of CDC2 and its upstream molecule CDC25C, and it increases protein level of Myt1. Furthermore, Parkin expression results in dephosphorylation of Histone H3.

## II. MATERIALS AND METHODS

### 1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, and trypan blue stain solution were obtained from Gibco BRL (Grand Island, NY, USA). Recombinant adenoviral vector including parkin gene (Parkin virus) was produced as previously described by Kim *et al*<sup>23</sup>. Recombinant human TNF- $\alpha$  was purchased from R&D System (Minneapolis, MN, USA). Propidium iodide was purchased from BD biosciences (San Jose, CA, USA). Trizol reagent, random hexamer, and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) were purchased from Invitrogen (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) and RNase A were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for phospho-CDC2(Tyr15), phospho-CDC25C(Ser216), Myt1, phospho-Histone H3(Ser10), Cyclin A2, Cyclin B1, p21 and p53 were purchased from cell signaling technology (Danvers, MA, USA). Antibodies specific for parkin and  $\beta$ -actin were purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA).

## **2. Cell culture**

HeLa (Human cervical adenocarcinoma cells, ATCC) was grown in DMEM supplemented with 10% FBS and streptomycin-penicillin. Cells were maintained at 37 °C in humidified atmosphere with 5% CO<sub>2</sub>.

## **3. Parkin gene expression**

HeLa cells ( $2 \times 10^5$ ) were seeded into each well of a 6-well plate and 24 h later infected with different concentrations of Parkin virus and Mock virus in serum-free DMEM. After an additional 90 min 10% FBS-DMEM was added to each well. In Parkin virus dose-dependent experiments, cells were infected with Parkin virus at different multiplicity of infection (M.O.I.; 0, 25, 50, 100, 200). To compensate for the effect of the viral vector Mock virus was added with the Parkin virus to maintain equal concentration of 200 M.O.I. A non-infected group was added as a negative control.

## **4. Trypan blue exclusion assay**

For counting viable cells, cultured medium was removed and cells

were incubated with Trypsin-EDTA (200ul per well) for 2 min. and then, 1.8 ml DMEM with 10% FBS was added. 10 ul of 0.4% trypan blue stain solution was mixed with 10 ul of cell suspension. Non-stained cells (viable) were counted from this mixture using hematocytometer (Marienfeld, German).

## **5. Cell cycle analysis by flow cytometry**

HeLa cells seeded into 6-well plate at  $2 \times 10^5$  cells /well were cultured in 10% FBS DMEM. After 24 h, cells were infected with Parkin virus or Mock virus for 24 h. Then, cells were treated with TNF- $\alpha$  (5 ng/ml). Next, trypsinized cells were fixed with 70% ethanol in PBS and incubated for 2 h at 4°C. The fixed cells were stained with a solution containing 0.1 mg/ml RNase A and 5 mg/ml propidium iodide in PBS. After incubation for 40 min at 37°C, the cell suspension was analyzed using FACS Calibur (BD biosciences, USA).

## **6. RT-PCR (Reverse transcriptase - polymerase chain reaction)**

Total RNA was extracted from cultured cells using Trizol reagent

(Invitrogen, New York, USA) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription with 2 µg total RNA, 0.25 µg of random hexamer (Invitrogen, New York, USA) and 200 U of Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT; Invitrogen, New York, USA) for 50 min at 37°C and 15 min at 70°C. Subsequent PCR amplification using 0.2 U of *Taq* polymerase (Genetbio, Nonsan, Chungnam, Korea) was performed in a thermocycler using specific primers. The sequences of the PCR primers are listed in Table 1. PCR products were electrophoresed on 1.5% agarose gels, stained for 10 min with ethidium bromide and destained for 20 min. Product size were determined by comparing to a 100 bp DNA ladder marker (Bioprince, Georgia, USA). Gel images were taken using Gel Doc (Bio-Rad, Hercules, CA, USA).

**Table 1.** Summary of PCR primers used for analyses of mRNA expression

Primers	Sequences(5'-3')		Product length (bp)	Annealing temperature (°C)	Cycles
	Forward	Reverse			
p53	ATTGATGCTGTCCCGGACGATATTGAAC	ACCCTTTTGGACTTCAGGTGGCTGGAGTG	998	69	30
p21	GTG AGC GAT GGA ACT TCG ACT T	GGC GIT TGG AGT GGT AGA AAT C	330	57	26
CyclinB1	TGGGTCGGCCTCTACCTTTGCACTTC	CGATGTGCATACTTGTCTTGACAGTCA	332	60	30
CDC25C	TTT ATG TCA TTG ATT GTC GC	AAG AAG TCT CTG TAG CCG CC	286	55	35
Myt1	AAGCTGGGTGACTTCGGACT	ACAGAACGCAGCTCGGAAGA	263	62	30
CDC2	GGT TCC TAG TAC TGC AAT TCG	TTT GCC AGA AAT TCG TTT GG	709	62	26
GAPDH	CGGAAAGCTTGTCATCAATGG	GGCAGTGATGGCATGGACTG	349	55	22

## 7. Western blot analysis

Cells were washed with PBS and then lysed at 4 °C with a lysis buffer containing 1% Triton X-100, protease inhibitor cocktail (Sigma, St. Louis, MO, USA), phosphatase inhibitor cocktail (Roche, Mannheim, Germany) and PBS. The lysate was centrifuged at 14000 rpm for 5 min at 4°C. The supernatant was collected and proteins were quantified with using Lowry Protein assay (Bio-rad, USA). Then, it was mixed with sample buffer. Protein samples (15 ug per lane, but 50 ug per lane in caspases analysis) were separated by SDS-polyacrylamide gel electrophoresis (10%, 12%) and transferred to nitrocellulose membrane (Pall, Mexico). The membrane was blocked with 5% (W/V) skim milk (BD biosciences, USA) for 30 min. Blocked membrane was incubated with primary antibody for overnight at 4°C. Each immunoblot was then incubated with appropriate horseradish peroxidase-labeled secondary antibody (anti-mouse, anti-rabbit). The immune-labeled proteins were visualized using ECL kit (Thermo, USA) and X-ray film (AGPA, Belgium).  $\beta$ -actin-antibody was used as an internal control.

### III. RESULTS

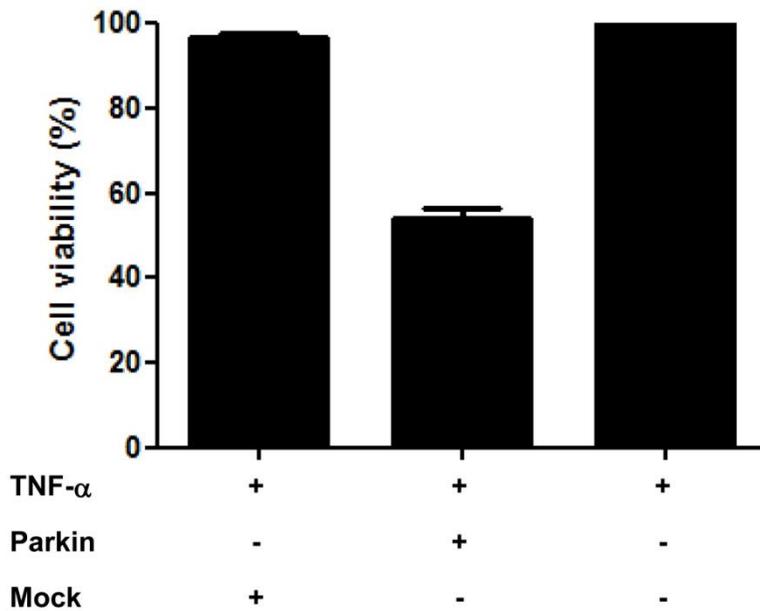
#### **Expression of parkin in TNF- $\alpha$ -stimulated HeLa cells induces G2/M phase cell cycle arrest.**

HeLa cells are resistant to TNF- $\alpha$ -induced cell death<sup>24</sup>. However, it was previously reported that parkin expression in HeLa cells sensitizes them to TNF- $\alpha$ -induced cell death by stimulating apoptotic signaling cascade<sup>25</sup>. In this study, I investigated if parkin has an effect on cell growth as well as cell death. First, I reconfirmed whether Parkin expression repressed the cell viability in TNF- $\alpha$ -treated HeLa cells. HeLa cells were infected with either the Parkin virus or Mock virus (200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for an additional 24 h. A non-infected group was added as a control (Con). The number of viable cells were counted by trypan blue exclusion assay. The results showed that the number of viable cell dramatically decreased to 54.3% which was similar to a previous report<sup>25</sup> (Figure 1). Next, to investigate if Parkin has an effect on cell cycle, HeLa cells were infected with varying doses of the Parkin virus (0, 25, 50, 100, 200 M.O.I.) and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. The cells were harvested and stained with propidium iodide and then cell cycle status was

analyzed by flow cytometry. Percentage of cells in each phase of the cell cycle was analyzed with CellQuestPro software and graphically presented. The resulted cell cycle status showed that there was a dramatic increase in G2/M phase of the cell cycle to 42.9% in Parkin virus infected HeLa cells with 5 ng/ml TNF- $\alpha$  treatment (Figure 2A, 2B). To reconfirm whether increase in G2/M phase was dependent on Parkin expression and TNF- $\alpha$  dose, HeLa cells were infected with either the Parkin virus or Mock virus (200 M.O.I.) for 24 h and then treated with indicated concentration of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for an additional 24 h. When TNF- $\alpha$  was administered, in Parkin virus infected HeLa cells there was an increase in G2/M phase and a decrease in G0/G1 phase peak. In contrast, HeLa cells infected with Mock virus and treated with 5 ng/ml of TNF- $\alpha$  showed no increase in G2/M phase of the cell cycle (Figure 3A, 3B). Furthermore, G0/G1 phase was decreased and G2/M phase was increased in a TNF- $\alpha$  dose dependent manner (Figure 3A, 3B).

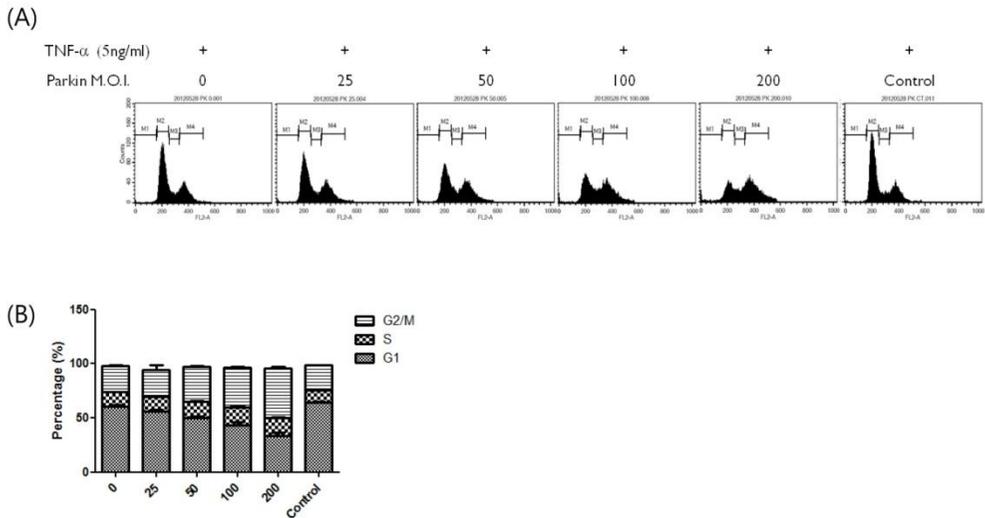
To investigate the time period that Parkin+TNF- $\alpha$  induces the G2/M phase increase the most, I conducted a time course experiment. HeLa cells were infected with the Parkin virus for 24 h and then treated with TNF- $\alpha$  for various times. In Parkin virus infected HeLa cells, G2/M phase remained

highly increased after 16 h of TNF- $\alpha$  treatment (Figure 4A, 4B). These results demonstrate that parkin expression in TNF- $\alpha$ -treated HeLa cells results in the G2/M phase cell cycle arrest.



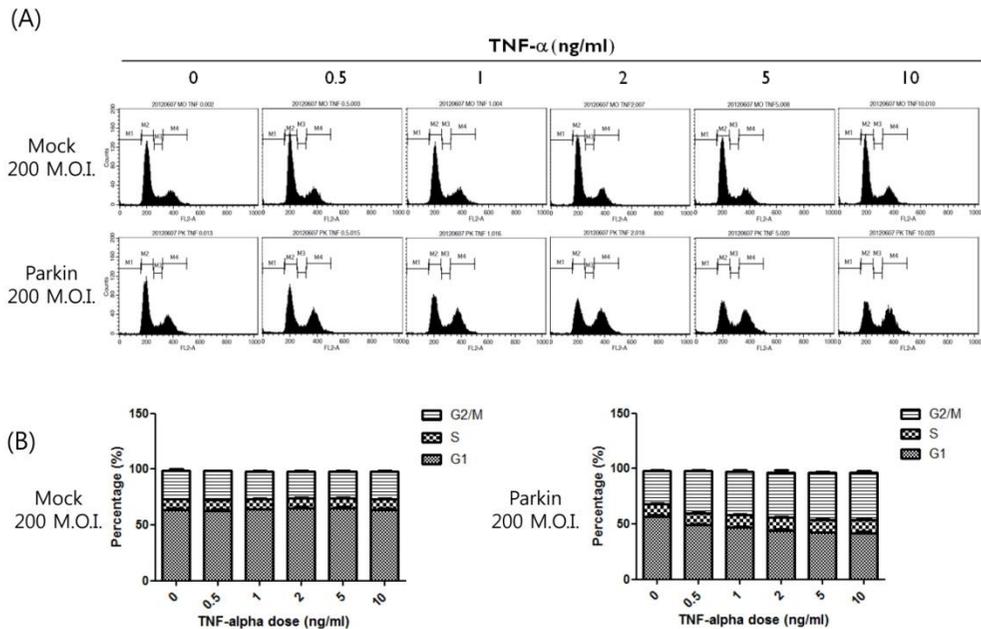
**Figure 1. Parkin expression results in decreased cell viability in TNF- $\alpha$ -induced HeLa cell death.**

HeLa cells were infected with either Parkin virus or Mock virus (200 M.O.I.) for 24 h followed by treatment of TNF- $\alpha$  (5 ng/ml) for 24 h. A non-infected group was added as a control (Con). Viable cells were counted by trypan blue exclusion assay (3 independent experiments). Number of viable cells in non-infected control group was set as 100%.



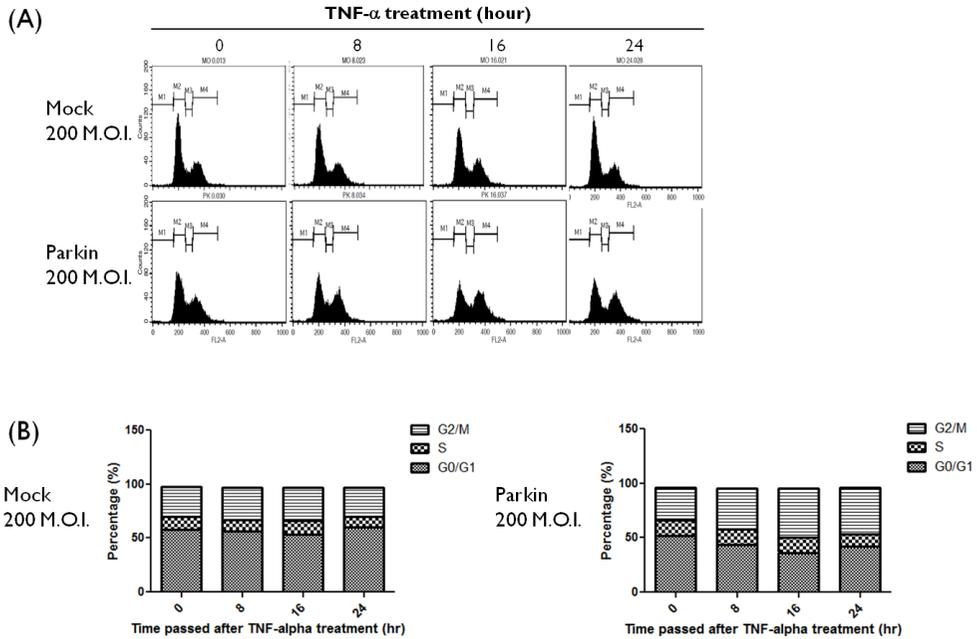
**Figure 2. Parkin expression induces G2/M cell cycle arrest in TNF- $\alpha$ -treated HeLa cells in a parkin dose dependent manner.**

HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to 200 M.O.I. per well. A non-infected group was added as a control (Con). (A) Cells were harvested and stained with propidium iodide and cell cycle analyzed by flow cytometry. (B) Percentage of cells in each phase of the cell cycle was analyzed with CellQuestPro software and graphically presented.



**Figure 3. Parkin expression induces G2/M cell cycle arrest in HeLa cells in a TNF- $\alpha$  dose dependent manner.**

HeLa cells were infected with either Parkin virus or Mock virus (200 M.O.I.) for 24 h and then treated with indicated concentrations of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for 24 h. (A) Cells were harvested and stained with propidium iodide and cell cycle analyzed by flow cytometry. (B) Percentage of cells in each phase of the cell cycle was analyzed with CellQuestPro software and graphically presented.

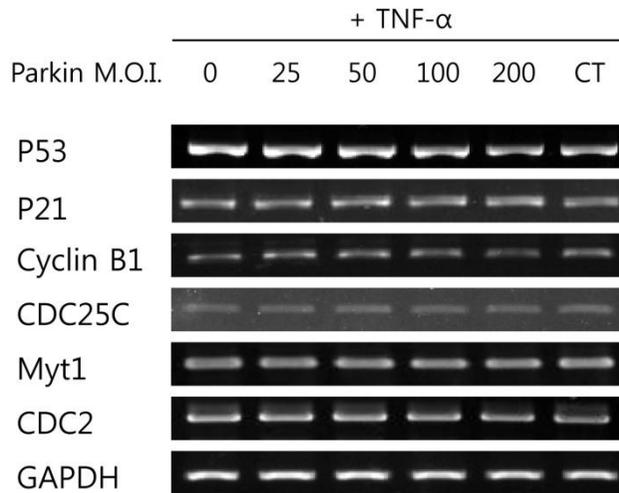


**Figure 4. Parkin expression induces G2/M cell cycle arrest in HeLa cells in a TNF- $\alpha$  time dependent manner.**

HeLa cells were infected with either Parkin virus or Mock virus (200 M.O.I. each) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for each indicated time period. (A) Cells were harvested and stained with propidium iodide and cell cycle analyzed by flow cytometry. (B) Percentage of cells in each phase of the cell cycle was analyzed with CellQuestPro software and graphically presented.

## **Screening for molecules associated with G2/M phase cell cycle arrest in TNF- $\alpha$ -treated HeLa cells.**

There are many molecules involved in the G2/M phase cell cycle regulation. I conducted an experiment to identify the molecules associated with parkin induced G2/M phase cell cycle arrest in TNF- $\alpha$ -treated HeLa cells. First, HeLa cells were infected with varying doses of the Parkin virus (0, 25, 50, 100, 200 M.O.I.) and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. mRNA was extracted and cDNA was made. I performed PCR for the cell cycle molecules, p53, p21, Cyclin B1, CDC25C, CDC2 and Myt1. However, there were no significant changes in the mRNA levels between the Parkin and Mock group (Figure 5).



**Figure 5. Parkin expression did not change mRNA expression of various molecules associated with G2/M in TNF- $\alpha$ -treated HeLa cells.**

HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to 200 M.O.I. per well. A non-infected group was added as a control (Con). PCR analysis of cDNA was performed using specific primers for each molecule. The PCR products were resolved on a 1.5% agarose gel. GAPDH was used as an internal control.

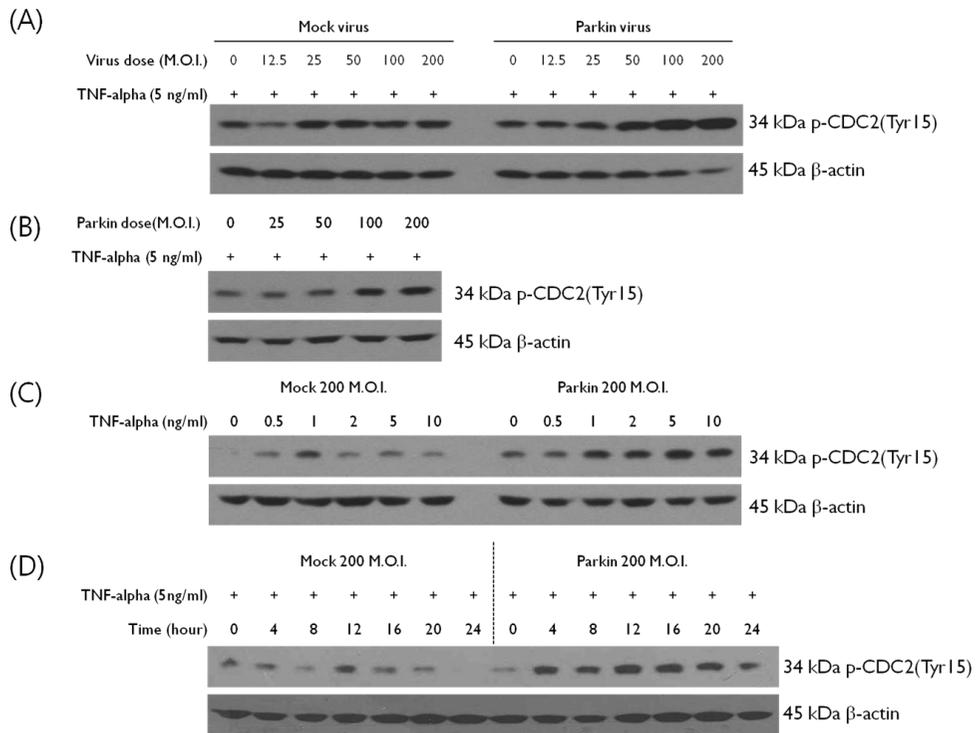
### **Parkin inhibits CDC2 by phosphorylation.**

Although the mRNA expression of the cell cycle molecules was unchanged, they still need to be examined by its phosphorylation level and protein level because these cell cycle molecules are activated or inactivated by upstream kinases or phosphatases. Therefore I investigated the phosphorylation status and protein level of these molecules by western blot.

The entry of eukaryotic cells into mitosis is regulated by CDC2 kinase activation<sup>13</sup>. Several conditions are required for activation of CDC2 and dephosphorylation at Tyr15 is one of those critical steps for CDC2 kinase activation<sup>14</sup>. On the basis of these reports, I investigated whether parkin expression influences CDC2 phosphorylation at Tyr15. HeLa cells were infected with indicated concentration of either the Parkin virus or Mock virus (0, 12.5, 25, 50, 100, 200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for an additional 24 h. The result indicated that Parkin expression increased the level of phospho-CDC2(Tyr15) (Figure 6A). For an additional experiment, HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers

of virus particles to 200 M.O.I. per well. In this experiment, phospho-CDC2 was also increased in a Parkin dose dependent manner (Figure 6B). In the next experiment, HeLa cells were infected with either the Parkin virus or Mock virus (200 M.O.I.) for 24 h and then treated with indicated concentration of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for an additional 24 h. CDC2 phosphorylation was increased by more than 2 ng/ml of TNF- $\alpha$  treatment in Parkin virus infected group as compare to the Mock virus infected group (Figure 6C).

I further conducted time course experiment. HeLa cells were infected with Mock or Parkin virus for 24 h and then treated with TNF- $\alpha$  for various times. In the Parkin expressed group, phosphorylation of CDC2 was increase after 4h of TNF- $\alpha$  treatment in contrast to the Mock infected group (Figure 6D). These results suggest that Parkin expression in TNF- $\alpha$  treated HeLa cells increases the phosphorylation of CDC2 at Tyr15, thus inhibiting activity of CDC2. Phosphorylation at Tyr15 may be one of the reasons for cell cycle arrest of HeLa at G2/M phase.

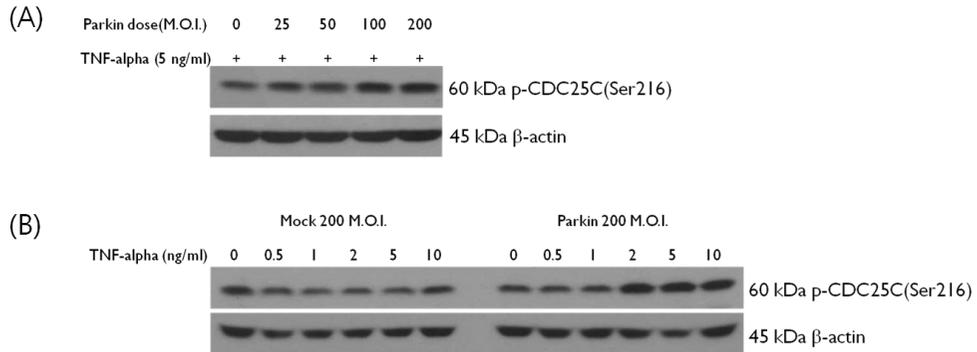


**Figure 6. Parkin expression induces phosphorylation of CDC2 in TNF- $\alpha$ -treated HeLa cells.** (A) HeLa cells were infected with indicated concentration of mock or parkin virus (0, 12.5, 25, 50, 100, 200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. (B) HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to

200 M.O.I. per well. (C) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with indicated concentration of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for 24 h. (D) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for indicated time period. Cell lysates were separated on SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Phospho-CDC2(Tyr15) was detected by Western blotting using a specific antibody. Level of  $\beta$ -actin was used as an internal control.

### **Parkin induces phosphorylation of CDC25C.**

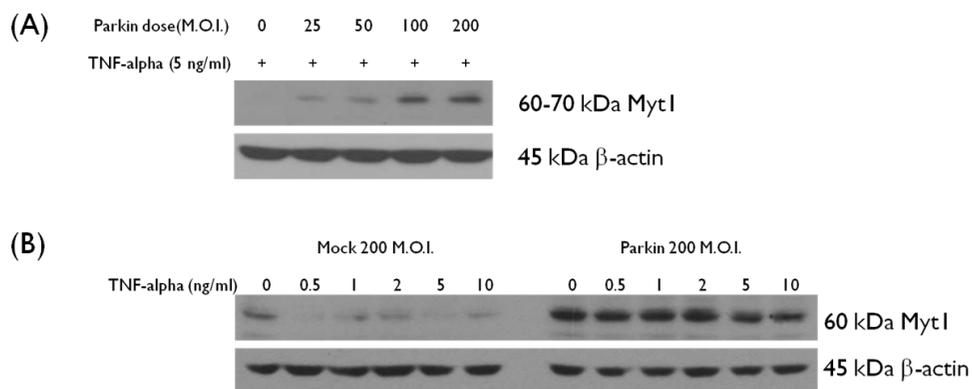
CDC25C is a protein phosphatase responsible for dephosphorylating and activating CDC2, a crucial step in regulating the entry of all eukaryotic cells into mitosis <sup>16</sup>. Phosphorylation at Ser216 leads CDC25C to bind to members of the 14-3-3 family of proteins exporting CDC25C to the cytoplasm and destruction <sup>17, 26</sup>. Therefore, CDC25C inhibition by phosphorylation at Ser216 results in increase of inactivated phospho-CDC2 (Tyr15). I investigated the level of phospho-CDC25C (Ser216) and found that CDC25C was phosphorylated by parkin expression (Figure 7A). In addition, phospho-CDC25C (Ser216) was increased when more than 2 ng/ml of TNF- $\alpha$  was administered (Figure 7B).



**Figure 7. Parkin expression induces phosphorylation of CDC25C in TNF- $\alpha$ -treated HeLa cells.** (A) HeLa cells were infected with indicated concentration of mock or parkin virus (0, 12.5, 25, 50, 100, 200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. (B) HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to 200 M.O.I. per well. A non-infected group was added as a control (Con). (C) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with indicated concentration of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for 24 h. (D) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for indicated time period. Cell lysates were separated on SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Phospho-CDC25C(Ser216) was detected by Western blotting using a specific antibody. Level of  $\beta$ -actin was used as an internal control.

### **Parkin induces increase in protein level of Myt1.**

Myt1 is a kinase responsible for inhibition of CDC2 activity by phosphorylating on Tyr15<sup>27</sup>. I investigated whether Myt1 is involved in the inhibition of CDC2 in TNF- $\alpha$ -treated HeLa cells expressing Parkin. The results showed that protein level of Myt1 was increased in a Parkin dose dependent manner (Figure 8A) but not influenced by TNF- $\alpha$  treatment (Figure 8B). These results suggest that Parkin induced inhibition of CDC2 was associated with increase of Myt1 protein level.

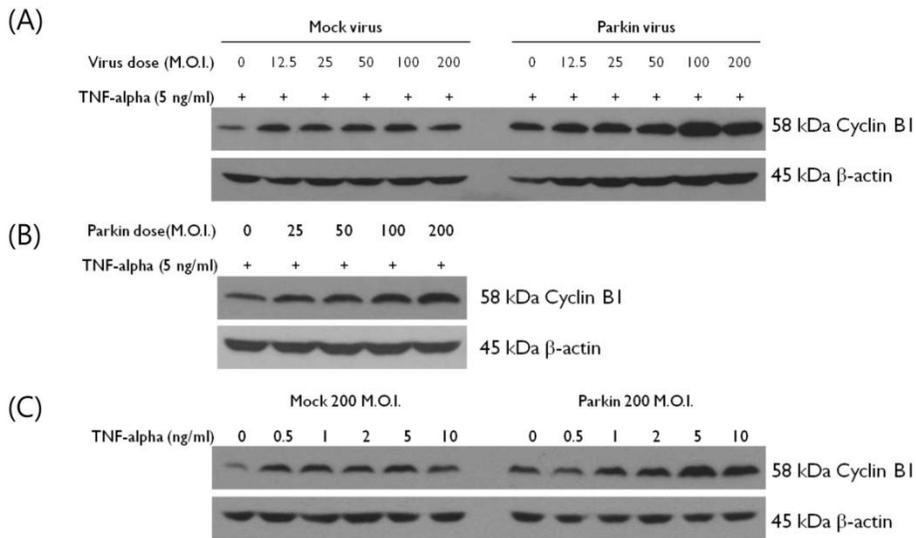


**Figure 8. Parkin expression increases Myt1 at protein level in TNF- $\alpha$ -treated HeLa cells.**

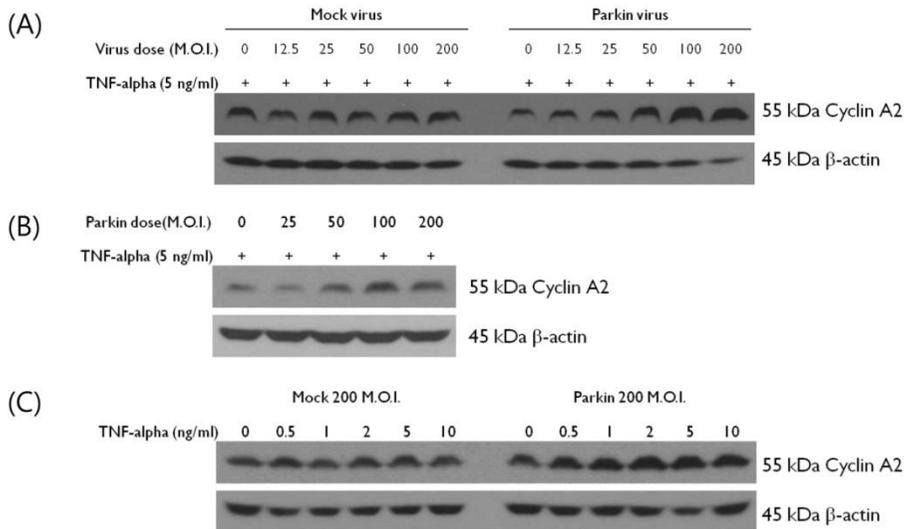
(A) HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to 200 M.O.I. per well. (B) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with indicated concentration of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for 24 h. Cell lysates were separated on SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Myt1 was detected by Western blotting using a specific antibody. Level of  $\beta$ -actin was used as an internal control.

## **Parkin induces accumulation of Cyclin B1 and Cyclin A2**

Cyclin B1 is degraded by the ubiquitin pathway in the exit from mitosis<sup>28</sup>. Therefore, when cells are arrested in G2 phase, Cyclin B1 accumulation is observed<sup>29</sup>. In the experiment I performed, I have found that protein level of Cyclin B1 was increased both in a Parkin dose dependent (Figure 9A and 9B) and TNF- $\alpha$  dose dependent manner (Figure 9C). In addition, Cyclin A2 degradation is required for the metaphase-to-anaphase transition<sup>30</sup>. On this basis of the report, I also investigated the protein level of Cyclin A2. The protein level of Cyclin A2 was also increased both in a Parkin dose dependent (Figure 10A, 10B) and TNF- $\alpha$  dose dependent manner (Figure 10C). Based on these results, I presume that Cyclin A2 and Cyclin B1 is accumulated by inhibited mitotic progression and reconfirmed the G2/M cell cycle arrest induced by Parkin expression.



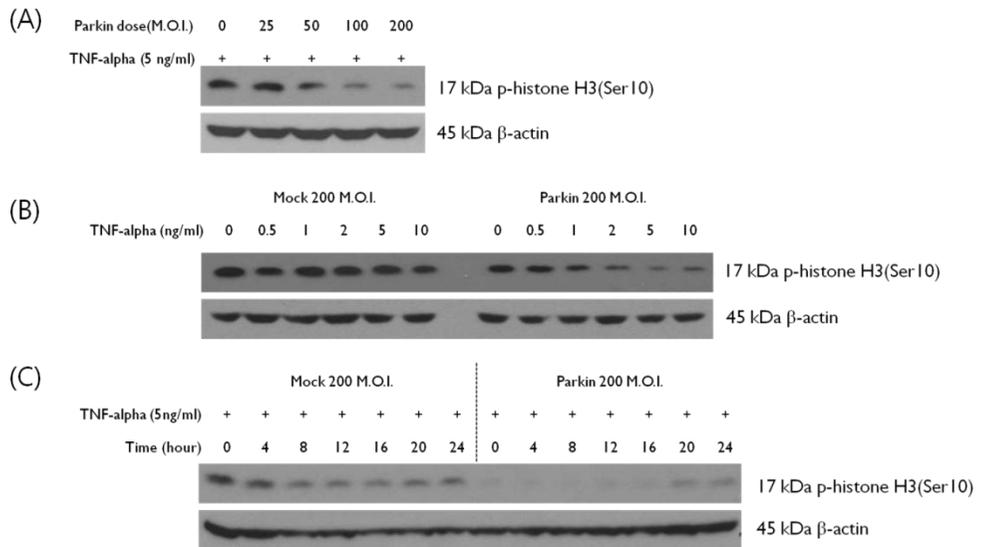
**Figure 9. Parkin induces accumulation of Cyclin B1 in TNF- $\alpha$ -treated HeLa cells.** (A) HeLa cells were infected with indicated concentration of mock or parkin virus (0, 12.5, 25, 50, 100, 200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. (B) HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to 200 M.O.I. per well. (C) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with indicated concentration of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for 24 h. Cell lysates were separated on SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Cyclin B1 was detected by Western blotting using a specific antibody. Level of  $\beta$ -actin was used as an internal control.



**Figure 10. Parkin induces accumulation of Cyclin A2 in TNF- $\alpha$ -treated HeLa cells.** (A) HeLa cells were infected with indicated concentration of mock or parkin virus (0, 12.5, 25, 50, 100, 200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. (B) HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to 200 M.O.I. per well. (C) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with indicated concentration of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for 24 h. Cell lysates were separated on SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Cyclin A2 was detected by Western blotting using a specific antibody. Level of  $\beta$ -actin was used as an internal control.

### **Parkin induces dephosphorylation of Histone H3.**

The nucleosome, made up of four core histone proteins, is the primary building block of chromatin <sup>18</sup>. Phosphorylation at Ser10 of histone H3 is tightly correlated with chromosome condensation which is essential for chromosome segregation <sup>22</sup>. I investigated phosphorylation state of Histone H3 at Ser10 and found that Histone H3 is dephosphorylated in a Parkin dose dependent manner (Figure 11A) and in a TNF- $\alpha$  dose dependent manner (Figure 11B). In the time course experiment, phospho-Histone H3 (Ser10) was also decreased in Parkin expressed group and it was decreased more after 8 h of TNF- $\alpha$  treatment (Figure 11C). These results suggest that Parkin is involved in the dephosphorylation of Histone H3 and the dephosphorylation of Histone H3 is possibly associated with the arrest of the cell cycle before the cell division.

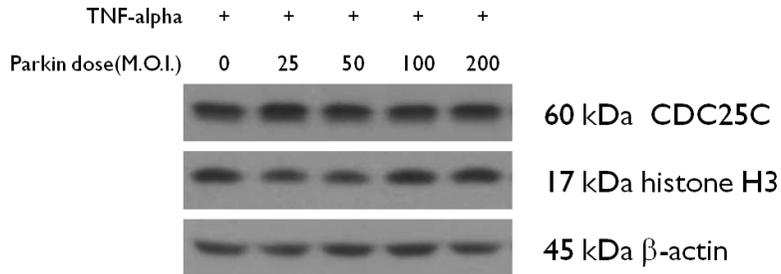


**Figure 11. Parkin expression reduces phosphorylation of Histone H3 in TNF- $\alpha$ -treated HeLa cells.**

(A) HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to 200 M.O.I. per well. (B) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with indicated concentration of TNF- $\alpha$  (0, 0.5, 1, 2, 5, 10 ng/ml) for 24 h. (C) HeLa cells were infected with mock or parkin virus (200 M.O.I.) for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for indicated time period. Cell lysates were separated on SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Phospho-Histone H3(Ser10) was detected by Western blotting using a specific antibody. Level of  $\beta$ -actin was used as an internal control.

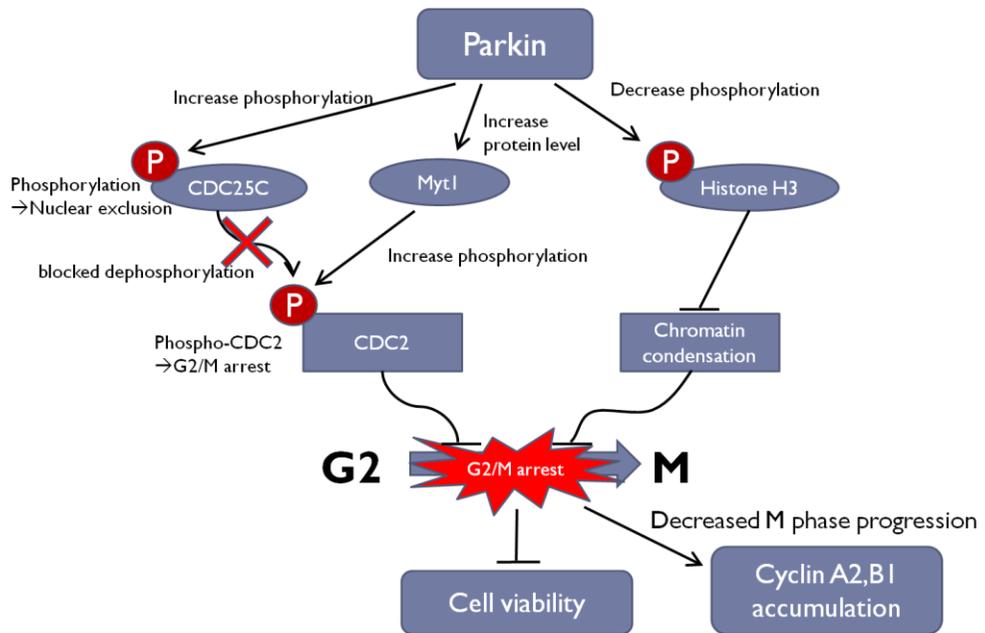
### **Parkin does not affect the protein level of CDC25C and histone H3**

In the western blot results, I found Parkin increased phospho-CDC25C and decreased phospho-histone H3. However, I wondered if these changes in phospho-forms are actually by regulation of phosphorylation or by regulation of protein level. Therefore, I investigated the protein level of CDC25C and histone H3 after Parkin expression. The results showed that protein level of CDC25C and histone H3 was not associated with Parkin expression level (Figure 12).



**Figure 12. Parkin expression does not affect the protein level of CDC25C and histone H3 in TNF- $\alpha$ -treated HeLa cells.**

(A) HeLa cells were infected with indicated concentrations (0, 25, 50, 100, 200 M.O.I.) of Parkin virus for 24 h and then treated with TNF- $\alpha$  (5 ng/ml) for 24 h. To compensate for the effect of the viral vector, appropriate concentration of the Mock virus was added to maintain equivalent numbers of virus particles to 200 M.O.I. per well. Cell lysates were separated on SDS-polyacrylamide gel and transferred to nitrocellulose membranes. CDC25C and histone H3 was detected by Western blotting using a specific antibody. Level of  $\beta$ -actin was used as an internal control.



**Figure 13. Schematic diagram showing putative points of Parkin involvement in G2/M arrest of TNF- $\alpha$ -treated HeLa cells.**

## IV. DISCUSSION

Parkin gene (*PARK2*) was first identified as a gene implicated in familial parkinsonism <sup>1</sup>. Currently, many reports suggest that Parkin performs a tumor suppressive role. Lack or mutation of Parkin expression was found in a variety of cancers <sup>2-5</sup>, and reintroduction of Parkin suppressed the cancer cell viability <sup>7-9</sup>. However, the mechanism by which parkin act as a tumor suppressor is yet to be fully uncovered. In the previous study, Parkin expression restored TNF- $\alpha$ -induced apoptotic cell death in HeLa cells which is naturally resistant to TNF- $\alpha$ -induced cell death <sup>25</sup>. In this study, I investigated the role of Parkin in cancer cell growth. I found that (1) Parkin expression arrested the cancer cells in G2/M phase of the cell cycle, (2) the G2/M arrest was due to the phosphorylation mediated inactivation of CDC2, (3) the CDC2 regulating molecules CDC25C and Myt1 was phosphorylated and increased respectively by Parkin expression, and (4) histone H3 was dephosphorylated by Parkin expression in TNF- $\alpha$ -treated HeLa cells.

Cell cycle is the series of events that take place in a cell leading to its division and duplication, and it is a complicated process which is regulated by a variety of molecules. The cell cycle consists of four distinct phases that

are G1, S, G2 and M phase. Activation of each phase is dependent on the proper progression and completion of the previous one. Cell cycle is tightly regulated because there are several processes crucial to the survival of a cell. For example, genetic damage should be detected and repaired before cell division, and uncontrolled cell division must be prevented. Cell cycle checkpoints are used by the cell to monitor and regulate the progress of the cell cycle<sup>31</sup>. Cell cycle progression is prevented at specific point until the processes necessary for each phase is achieved and DNA damage is properly repaired. There are two main checkpoints exist: the G1/S checkpoint and the G2/M checkpoint.

CDC2 is an important molecule involved in the regulation of G2/M check point. CDC2 kinase activation is a key process<sup>13</sup> and dephosphorylation at Tyr15 is the critical regulatory step in activating CDC2 during progression into mitosis<sup>14</sup>. I investigated the phosphorylation of CDC2 after I found the G2/M arrest. Phospho-CDC2 was increased both in a TNF- $\alpha$  and Parkin dose dependent manner. Therefore I could understand that Parkin expression inactivated CDC2 by increasing phosphorylation there by inducing G2/M cell cycle arrest. In addition, our result suggested that the cause of the CDC2 phosphorylation was increase of Myt1 protein kinase and

phosphorylation of CDC25C phosphatase by Parkin expression. Phosphorylation at Thr14 and Tyr15 resulting in inhibition of CDC2 can be carried out by Myt1 protein kinase<sup>15</sup>, and CDC25C, a protein phosphatase activating CDC2, binds to 14-3-3 protein and excluded from nucleus when phosphorylated at Ser216<sup>17</sup>.

In the result, Parkin induced dephosphorylation of histone H3. Histone H3 is one of four core histone proteins consisting of the nucleosome which is structural component of chromatin<sup>18</sup>. Phosphorylation at Ser10 of histone H3 is tightly correlated with chromosome condensation and the phosphorylation is required for cell cycle progression<sup>21 22</sup>. Therefore it is suspected that Parkin induced dephosphorylation of histone H3 may hinder the condensation of chromatin thereby preventing the cell division. However, it is difficult to affirm before I examine the direct or indirect evidence of suppressed chromatin condensation. Moreover, the upstream molecules involved in the histone H3 phosphorylation by Parkin should be determined. There are still more things to be determined. For example, Parkin expression seems to indirectly increase phosphorylation of CDC25C and protein level of Myt1. The upstream molecules associated with phosphorylation of CDC25C and increase of Myt1 should be determined and the molecules that

Parkin directly interact with is yet to be discovered.

p53, a potent tumor suppressor, plays a central role in tumor prevention. As a transcription factor, in response to stress, p53 transcribes its target genes to start various cellular responses, including cell-cycle arrest and apoptosis to prevent tumor formation<sup>32, 33</sup>. The association between Parkin and p53 was reported in several reports. Parkin was reported to transcriptionally repress p53 in neuroblastoma SH-SY5Y cells<sup>34</sup>. On the contrary, another report suggested that Parkin does not repress p53 expression but p53 regulates Parkin expression in H460 or HCT116 cells<sup>35</sup>. These reports suggest that Parkin potentially suppress tumor growth by regulating p53. In this study I could not find the Parkin induced changes in p53. Probably, this is because p53 is suppressed in human cervical cancer HeLa<sup>36</sup>. However, it seems necessary to investigate the association between Parkin and p53 in suppression of cancer cell growth in other type of cancer cells.

Further studies seems to be required to fully elucidate mechanism by which Parkin plays a role in suppressing cancer cell growth and it would also be interesting to determine if the other cell cycle molecules are also regulated by Parkin. In addition, cell cycle arrest is closely associated with apoptosis,

because prolonged cell cycle arrest leads cells to apoptosis. Therefore, it needs to be investigated whether Parkin induced cell cycle arrest of HeLa is followed by apoptosis or they are separated mechanism.

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## ABSTRACT IN KOREAN

### 종양괴사인자-알파(Tumor necrosis factor-alpha)를 처리한 자궁경부암 세포의 파킨단백질 발현에 의한 G2/M 세포주기 정체에 관한 연구

파킨은 암억제인자의 기능을 한다고 알려진 단백질로서, 다양한 암세포들에서 파킨단백질의 발현감소나 돌연변이가 보고되었고, 암세포들에 파킨을 발현시켰을 경우 암세포들의 생존률이 감소한다고 보고되었다. 또한 암억제인자로서 파킨단백질이 어떠한 기전으로 기능을하는지에 대해 일부 보고되고 있다. 하지만 이러한 파킨단백질의 암억제인자로서의 기능에 대해서는 여전히 밝혀야할 부분들이 많이 남아있다. 암세포들이 암 발달과정중에서 종양괴사인자-알파에 대한 저항성을 가지게 되며 그 결과 대부분의 암세포들이 종양괴사인자-알파에 의해 유도되는 세포사멸을 일으키지 않는다. 앞선 연구에서, 우리는 종양괴사인자-알파에 저항성을 가진 자궁경부암 세포주인 HeLa 세포에 파킨을 발현 시켰을 때 종양괴사인자-알파에 의해 세포자가사멸이 유도된다는 사실을 보고한바 있다. 하지만 암억제인자들이 한가지 기전을

통해서만 암세포를 억제하는 것이 아니라 다양한 기전을 통해 암세포를 억제하는 경우가 많다. 따라서, 본 연구에서는 파킨이 암세포의 세포사멸이 아닌 암세포의 성장에는 어떠한 영향을 미치는지 그리고 그 기전에 대해서 조사하였다. 그 결과, 파킨단백질의 발현에 의해 HeLa 세포에서 G2/M 세포주기 정체가 일어났다. 그 원인중 한가지는 파킨단백질의 발현에 따른 CDC2의 인산화 증가였는데, Tyr15 부분이 인산화된 CDC2는 활성이 억제되고 이로인해 G2/M 세포주기정체를 유발할 수 있다. 또한 파킨단백질의 발현은 CDC25C의 인산화를 증가시켰고 Myt1 kinase의 양을 증가시켰는데, 이는 CDC2 인산화의 원인으로 알려져 있다. 또한 파킨의 발현은 histone H3의 탈인산화를 일으켰다. Histone H3의 탈인산화는 세포분열을 위해 선행되어야 하는 염색질 응축을 방해하게 되는데, 파킨에 의한 histone H3의 탈인산화도 세포성장을 억제하는 원인들 중 하나일 것으로 추측한다. 이러한 결과들을 바탕으로 파킨이 암세포사멸을 통해서 뿐만 아니라 본 연구에서 제시한 세포주기 정체를 통해서 암세포 성장을 억제하여 암억제인자로서 기능을 함을 확인하였다. 본 연구를 통해 앞으로 파킨의 암억제인자로서의 기능을 이해하는데 도움이 될것이라고 사료된다.

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핵심되는 말 : 파킨, 암억제인자, 종양괴사인자-알파, 세포주기, G2/M 정체

## 감사의 글

공부를 하겠다고 마음을 먹고 처음 교수님 방문을 두드렸던게 벌써 2년이 지났습니다. 갑작스럽게 마음먹었기에 준비도 없이 무작정 시작하게된 대학원 생활이었지만, 누구보다 잘하고 싶은 욕심으로 부단히 공부하고 실험하여 이제 무사히 2년간의 여정을 마치려고 합니다. 존경하는 저의 스승님이신 김윤석 교수님께는 누구보다 먼저 인사를 드리고 싶습니다. 항상 존경하고있고, 감사하고, 사랑합니다 교수님. 저의 지도교수님은 아니시지만 자신의 학생처럼 돌보아 주셨던 이기종 교수님이 계셨기에 저는 마치 두분의 교수님께 가르침을 받은것처럼 느낍니다. 감사합니다 이기종 교수님. 마지막까지 작은 것 하나 놓치지 않고 저의 논문을 살펴 주셨던 김태우 교수님 감사합니다. 오옥두 교수님께서 종종 저희를 발으로 불러 주시던 추억은 잊을 수 없을 것 같습니다. 항상 나눠 주시고 싶은 마음이 전해져 오는 것 같았습니다. 감사합니다. 김종배 교수님께 배운 미생물은 지금도 머릿속에 남아서 항상 도움받고 있습니다. 감사합니다 교수님. 박용석 교수님께 세포생물학을 배웠기에 암과 관련된 공부를 하는 체계는 큰힘이 되었습니다 감사합니다. 이해영 교수님의 강의는 개인적으로 너무 좋아했습니다. 매시간 가득차도록 강의를 준비해오시지만 항상 마술처럼 빠져들게 되어버려서 지루한줄 모르고 재미있게 들었습니다. 학부때부터 석사때까지 교수님들의 가르침 너무 감사합니다. 또한 조원길 교수님, 김택중 교수님, 오제민 교수님 저희 과에 계시지는 않았지만 제가 석사 공부하는 동안 조언과 도움 아끼지 않고 주셔서 감사합니다. 지금은 교수님이 되신 이동섭 선생님, 인수형님, 사현형님 응원해 주셔서 너무 감사합니다. 우리방의 희망이자 대들보인 이동현 선생님, 함께 이겨냈던 시간동안 의지가 되어주셔서 감사합니다. 남아있는 우리 혈액방 가족들

병출, 여울, 주연, 성훈이형, 그리고 엄마역할을 해주시는 김현경쌤~!  
힘내세요 사랑합니다! 나의 힘들었던 시간동안 가장 오랜시간을 함께하고  
옆에서 돕고, 응원해주었던 친구 병출아 고맙다. 실험을 함께하면서 많이  
가르쳐주고 힘을 북돋아주었던 재원이형 고마워요. RT-PCR 고수 윤정아  
선배인데도 내가 오빠라고 많이 도와주고 가르쳐줘서 고마워. 내동생  
현준이, 형이 바빠서 잘해주지도 못했는데 항상 따라서줘 고맙다. 대학원  
생활동안 함께하고 많이 도움받고 가르침 받아왔던 상정이형, 민이형,  
성현이형, 연경이누나 너무 고마워요. 화연이, 성재, 민우야 함께먹던  
돈까스 잊지 못할것 같구나. 다시 만나서 더 좋았던 현정이, 앞으로  
분자방의 기둥이될 지혁이 고맙고 항상 건강하길 바란다.

8년을 타지에 아들을 내어놓으신채 이제나 올까 저제나 올까 애타는  
마음가득 기다려오셨습니다. 어제도 오늘도 내일도 항상 곁에 있어드리고  
싶습니다. 멀리서 응원해주시는 마음 언제나 제 가슴에 닿아있습니다.  
그누구보다도 감사하고 사랑합니다 어머니, 그리고 아버지. 또 함께  
응원해준 형, 진양이형, 멀리 독일의 고모와 선희누나 가족들 모두  
사랑합니다.

아름다운 캠퍼스를 떠나려고 하니, 봄의 키스로드에 흐드러진 벚꽃  
나무들,여름의 푸른 노천극장, 가을내 가득한 노오란 은행잎, 새하얀  
눈빛으로 뒤덮인 캠퍼스의 겨울이 제 마음을 쥐고 놓지 않으려 합니다.  
잊지 못할 아름다운 추억 가득한 이곳, 나의 두번째 고향, 나의 모교  
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