Title: Repression of the F-box protein Skp2 is essential for actin damage-induced tetraploid G1 arrest.

Article Type: Article

Keywords: actin damage; cytokinesis; pectenotoxin-2; Skp2; tetraploid G1 arrest

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1 **Repression of the F-box protein Skp2 is essential for actin damage-**

2 **induced tetraploid G1 arrest.**

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7 Running title: A role of Skp2 in tetraploid G1 arrest

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1 **ABSTRACT**

2 We previously reported that p53 plays as a key regulator in tetraploid G1 checkpoint,

3 which is activated by actin damage-induced cytokinesis blockade and then prevents

4 uncoupled DNA replication and nuclear division without cytokinesis. In this study, we

5 investigated a role of Skp2, which targets CDK2 inhibitor p27/Kip1, in actin damage-

6 induced tetraploid G1 arrest. Expression of Skp2 was decreased, but p27/Kip1 was

7 increased after actin damage-induced cytokinesis blockade. The roles of Skp2

8 repression in tetraploid G1 arrest was investigated by analyzing the effects of ectopic

9 expression of Skp2. After actin damage, ectopic expression of Skp2 resulted in DNA

10 synthesis and accumulation of multinucleated cells, and eventually induced apoptosis.

11 These results suggest that Skp2 repression is important for sustaining the tetraploid G1

12 arrest after cytokinesis blockade and required to prevent uncoupled DNA replication

13 and nuclear division without cytokinesis.

14

1   **Introduction**

2   Cell cycle progression is regulated by checkpoint controls, which function to protect the

3   integrity of the genome. Such controls act to prevent cell cycle progression until after

4   completion of prior events (1). The G1 checkpoint permits repair prior to replication,

5   whereas arrest at the G2 checkpoint permits repair of the genome prior to its mitotic

6   segregation. The p53 tumor suppressor has been shown to be integral to both the G1 (2)

7   and G2 (3) checkpoint machinery. Cells lacking wild-type p53 are not prevented from

8   re-entering the cell cycle and can reduplicate their DNA unchecked, leading to

9   polyploidy and subsequent genomic instability (4-6).

10 Dihydrocytochalasin B (DCB), which induces cleavage failure by depolymerizing

11 actin filaments, and lead to tetraploid G1 arrest (7). Since DCB does not affect to

12 spindle function and chromatid segregation, but only inhibits cytokinesis, it is likely that

13 tetraploid G1 checkpoint seems to be a general checkpoint control acts in G1 to

14 recognize tetraploidy and induce cell cycle arrest before DNA replication, and thereby

15 prevents the propagation of errors of late mitosis and the generation of aneuploidy.

16 We previously reported that loss of p53 sensitizes tumor cells to actin damage,

17 which is induced by treatment with actin-depolymerizing or knotting agents (8). Upon

18 actin damage, Bim expression was induced in tumor cells lacking functional p53, but

19 not in cells with functional p53 (8). Previously, we also demonstrated a molecular

20 mechanism underlying Bim-mediated apoptosis of p53-deficient tumor cells following

21 actin damage. p53-deficient cells fail to induce p21 and hence kept both Cdk2 and Cdc2

22 kinases active even after treatment with actin inhibitor. Those cells become

3

1   multinucleate and polyploidy, and eventually dead by apoptosis, indicating that Bim-

2   mediated apoptosis following actin damaged-p53 deficient cells due to failure to

3   inactivate Cdk2 and to sustain tetraploid G1 arrest.

4   　　　This study examined a role of another cell cycle G1 regulator, Skp2, which is

5   associated with a SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex in actin

6   damage-induced tetraploid G1 arrest. The major target of the as SCF complex is the

7   CDK (cyclin-dependent kinase) inhibitor p27/Kip1 that inhibits CDK2 activity, which is

8   essential for G1-S transition (9). During S phase, p27/Kip1 is degraded by the E3-

9   ubiquitin ligase activity of SCF (10). Various types of cancer show elevated levels of

10   Skp2 and decreased levels of p27/Kip1 (11, 12). While Skp2 overexpression facilitates

11   cell cycle progression (13) and is associated with many human cancers, its depletion is

12   essential for cell cycle arrest and can promote senescence (14).

13   　　　In this study, we report the Skp2 repression after cytokinesis blockade, which is

14   achieved by treatment with actin inhibitors, and its role in tetraploid G1 checkpoint

15   arrest of actin damaged cells.

16

1   **Results & Discussion**

2   Actin damage leads to cytokinesis blockade and results in cell cycle arrest at G1 with

3   tetraploidy. We previously reported that p53 tumor suppressor proteins play a key role

4   to induce and maintain tetraploid G1 arrest following actin damage-induced cytokinesis

5   blockade (15-17). In this study, we examined a role of Skp2 in actin damage-induced

6   G1 tetraploid checkpoint that arrests the tetraploid cells at G1. Skp2 is known as a key

7   regulator in ubiquitin-dependent degradation of cell cycle regulatory proteins (18). To

8   induce tetraploid G1 arrest by actin damage, HCT116 cells were treated with PTX-2,

9   cytochalasin D, or psychosine. After treatment with the actin inhibitors, p53-positive

10   HCT116 cells became binucleates by failure in cytokinesis (Fig. 1A). The protein levels

11   of Skp2 were decreased after PTX-2 treatment, whereas Skp1 expression was not

12   affected (Fig. 1B). We also tested the effects of other actin inhibitors, such as

13   psychosine and cytochalasin D, to generalize the effects that actin-cytoskeleton

14   perturbation results in Skp2 repression. Treatment with cytochalasin D and psychosine

15   also resulted in Skp2 repression (Fig. 1B). Therefore, these results indicate that actin

16   damage leads to Skp2 repression.

17   Skp2 proteins are degraded in cell cycle G1-arrested cells, but its stability is

18   increased when the cells re-enter into the cell cycle (19). Knockdown of Skp2

19   expression results in an inhibition of entry into cell cycle S phase from G1 phase (19),

20   indicating Skp2 is an important regulator of G1-S transition of the cell cycle. We next

21   examined the mRNA levels of Skp2 after treatment with actin inhibitor, PTX2 (Fig. 1D).

22   Skp2 mRNA levels were significantly decreased after PTX-2 treatment, indicating that

23   Skp2 transcription is inhibited in actin damaged cells (Fig. 1D). Therefore, these results

1   suggest that both protein degradation and transcriptional repression of Skp2 ensure to

2   inhibit cell cycle progression from tetraploid G1 arrested state after actin damage.

3   To clarify whether Skp2 repression due to perturbation of actin cytoskeleton or

4   cytokinesis blockade, HCT116 cells were synchronized at early S phase by double

5   thymidine treatment, and then treated with PTX-2 (Fig. 1C). Fig 1D showed that Skp2

6   expression was decreased in asynchronously growing cells after treatment with PTX2;

7   however, the early S-synchronized cells showed no alterations in Skp2 protein levels

8   after PTX2 treatment (Fig. 1C). Since the early S-synchronized cells did not travel

9   through the cell cycle when they were treated with PTX2, actin damage alone is not a

10   cause of Skp2 repression in PTX2-treated cells. Rather than this, actin damage-induced

11   cytokinesis blockade lead to repression of Skp2 in association with tetraploid G1 arrest.

12   Since it has been shown that p27/Kip1 plays as a cell cycle inhibitor by

13   inactivating CDK2, but is ubiquitinated and degraded by Skp2-SCF complex, it has

14   been considered to be critical for cell cycle progression from the G1 to S phase (10, 20).

15   To evaluate an inactivation of Skp2-SCF complex upon actin damage, we examined the

16   protein levels of target proteins of Skp2-SCF, such as p27/Kip1 and Cyclin E. After

17   treatment with PTX-2, the protein levels of p27/Kip1 and Cyclin E were increased (Fig.

18   2). Treatment with other actin inhibitors, psychosine and cytochalasin D, also led to

19   increase in p27/Kip1 and Cyclin E (Fig. 2), indicating that actin damage leads to

20   inhibition of Skp2-mediated p27/Kip1 degradation.

21   We further addressed whether Skp2 repression is critical for actin damage-

22   induced tetraploid G1 arrest by examining the effects of ectopic expression of Skp2. We

6

1 first examined whether the Skp2-expressing cells undergo DNA synthesis after

2 treatment with PTX-2 (Fig. 3A). BrdU incorporation was significantly decreased after

3 PTX-2 treatment in parental HCT116 cells, but not in Skp2-expressing cells, implying

4 that ectopic expression of skp2 bothers actin damage-induced tetraploid G1 arrest and

5 results in DNA replication without cytokinesis (Fig. 3A). Since uncoupled DNA

6 replication and nuclear division without cytokinesis results in accumulation of

7 multinucleated cells (16), We next examined the numbers of nuclei in actin damaged

8 cells. While staining with DAPI showed that the majority of parental HCT116 cells

9 arrested with two nuclei after treatment with PTX2, Skp2-expressing HCT116 cells

10 became multinucleate, containing four or more nuclei in the cells after treatment with

11 PTX-2 (Fig. 3B), indicating that ectopic expression of Skp2 results in failure to sustain

12 tetraploid G1 arrest. Expression of p27 was induced in paralleled with Skp2 repression

13 after actin damage; however, Fig 3C showed that p27/Kip1 was not induced in cells

14 with ectopic expression of Skp2 (Fig. 3C), implying that p27 induction in actin

15 damaged cells due to Skp2 repression.

16   It was previously reported that DNA replication and nuclear division of actin

17 damaged cells trigger apoptotic death (8, 16). Flow cytometric analysis showed that

18 HCT116 cells treated with PTX2 became to be tetraploidy (4N) and polyploidy (8N and

19 16N) (Fig. 4A); however, in Skp2-expressing cells, apoptosis was significantly

20 increased instead of accumulation of tetraploidy and polyploidy (Fig. 4A). DAPI

21 staining also showed apoptotic body formation after PTX2 treatment (Fig. 4B),

22 indicating that apoptosis was induced in HCT116 cells expressing Skp2. Therefore,

23 these results suggest that repression of Skp2, which results in inactivation of SCF-Skp2

1 complex, contributes to tetraploid G1 arrest of actin-damaged cells through induction of

2 p27/Kip1 in an independent manner with the p53-p21 signaling pathway (Fig 4C).

3   In this study, we demonstrated actin damage-mediated SKP2 repression. In

4 consistent with SKP2 repression (Fig. 1), its target proteins such as p27/Kip1 and cyclin

5 E were increased in actin damaged, tetraploid G1 arrested cells (Fig. 2), which indicates

6 inactivation of Skp1-SCF complex. We previously reported that p53 is essential for

7 actin damage-induced tetraploid G1 arrest (8). In the absence of p53, actin damaged cell

8 cannot sustain the tetraploid G1 arrest because of failure in p21-mediated inactivation of

9 CDK2 and result in accumulation of multinucleated and polyploid cells, which are

10 finally dead by apoptosis (8, 16). Therefore, the ectopic expression of Skp2, as like a

11 loss of p53 functions, causes DNA replication and nuclear division without cytokinesis

12 after actin damage (Fig. 3). Therefore, apoptosis of actin damaged cells by ectopic

13 expression of SKP2 implicates the role of SKP2 to sustain tetraploid G1 arrest and

14 prevent uncoupled cell cycle progression following cytokinesis blockade. Therefore, the

15 Skp2-p27 pathway, independently with p53-p21 signaling pathway, leads the actin

16 damaged cells to tetraploid G1 arrest (Fig. 4C). Since it has been reported that Skp2

17 overexpression has been found in many cancer tissues (11, 12), therefore, these results

18 also suggest a new strategy for anti-cancer therapy; actin inhibitors selectively induce

19 apoptosis in Skp2-overexpressing cancers as well as p53-deficient cells.

8

1 **Methods and Materials**

2 **Cell culture and DNA transfection**

3 Human colorectal cancer cell line (HCT116) and hepatocarcinoma cell line (HepG2)

4 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal

5 bovine serum (Life Technologies Inc., Grand Island, NY, USA) and penicillin–

6 streptomycin (50U/ml). DNA transfections were performed using the $CaPO_4$ co-

7 precipitation procedure (Graham and van der Eb, 1973).

8 **Cell cycle analysis**

9 To assess nuclear morphology, we seeded HCT116 cells on cover glasses, and after

10 allowing them to grow for 36 h, treated them with PTX-2 (10 µM), cytochalasin D (10

11 µM) or psychosine (50 µM) and then fixed them in Cannoid fixative (methanol/acetic

12 acid (3:1)). After staining the nuclei with DAPI (0.2 mg/ml, Sigma, St Louis, MO,

13 USA), the distribution of binucleated cells was observed under the microscopy.

14 For DNA content analysis, 1×10^6 cells were harvested by trypsinization and fixed

15 by rapid submersion in 1 ml cold 70% ethanol. After fixation at -20°C for at least 1 h,

16 cells were pelleted and subsequently resuspended in 1 ml staining solution (50 µg/ml

17 propidium iodide, 50 µg/ml RNase, 0.1% Triton X-100 in citrate buffer, pH 7.8), and

18 analyzed with FACScan (Becton Dickinson, USA) using Lysys software (21, 22). The

19 presented data are representative of more than three experiments.

20 Proliferation assays were performed using a 5-Bromo-2'-deoxy-uridine Labeling

9

1 and Detection Kit II (Roche, Mannheim, Germany). HCT116, p53-/-, and p53281 cells

2 were seeded on cover glasses, grown for 36 h before PTX-2 treatment (100 ng/ml).

3 After 1, 2, 3 days, cells were labeled for 1 h with 10 μM BrdU and BrdU-incorporated

4 cells were detected with the manufacturer's instructions.

5 **Cell cycle synchronization**

6 HCT116 cells were synchronized at G1/S with a modified double thymidine block

7 protocol as previously described (23). Cells were plated at 0.3×10^6 cells /100-mm dish

8 and 0.1×10^6 cells / 60-mm dish. After 1 day, cells were arrested by treatment with

9 thymidine (5 mM, Sigma Corp.) for 17 h, and were then released from the arrest by

10 washing in Dulbecco's phosphate buffered saline (8 mg/ml NaCl, 0.2 mg/ml KCl, 0.1

11 mg/ml $CaCl_2$, 0.1 mg/ml $MgCl_2$, 2.31 mg/ml $Na_2HPO_4 \cdot 12H_2O$, 0.2 mg/ml KH_2PO_4) and

12 replenishing with thymidine-free medium. After 9 h, cells were subjected to a second

13 thymidine treatment to completely block the cell cycle at early S phase.

14 **Western blot analyses**

15 Protein (20 μg) was subjected to SDS–polyacrylamide gel electrophoresis and

16 transferred to PolyScreen membranes (NEN, Boston, MA, USA).The membranes were

17 subsequently blocked with 5% nonfat dry milk in Tris-buffered saline Tween-20 and

18 probed with antibodies. The following antibodies were used in this study: antibodies

19 against Skp1 and Skp2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); p27

20 (Oncogene, Boston, MA, USA); cyclin E and actin (Santa Cruz Biotechnology, Santa

21 Cruz, CA, USA). Primary antibodies were detected with a horseradish peroxidase-

1   conjugated goat anti-mouse, goat anti-rabbit or donkey anti-goat secondary antibody

2   with enhanced chemiluminescence detection (Amersham, Buckinghamshire, UK).

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1 **Figure Legends**

2 **Fig 1. Expression of Skp2 upon actin damage-induced tetraploid G1 arrest.**

3 (A) Actin immunofluorescence and DAPI staining of HCT116 cells treated with PTX-2

4 (10μM), cytochalasin D (10μM) or psychosine (50μM) for 24 h.

5 (B) Western blot analysis for Skp1 and Skp2 proteins in HCT116 cells after treatment

6 with the indicated actin inhibitors for the indicated times.

7 (C) Skp2 expression in cell cycle-synchronized and asynchronously growing HCT116

8 cells. The cells were synchronized at early S phase by double thymidine treatment as

9 described in Materials and Methods. The cells were treated with an actin inhibitor, PTX-

10 2 for the indicated times without release from early S phase synchronization. The

11 protein levels of Skp2 were measured by Western blotting.

12 (D) Skp2 mRNA expression. Total RNA was obtained from HCT116 cells treated with

13 PTX-2 for the indicated times and applied to Northern blot analysis for detection of

14 Skp2 mRNA. 28S and 18S rRNAs were used for evaluating the quality and quantity of

15 RNA loading.

16 **Fig 2. Expression of Skp2 target proteins, p27/Kip1 and Cyclin E after treatment**

17 **with the actin inhibitors**. HCT116 cells treated with PTX-2 (10μM), cytochalasin D

18 (10μM) or psychosine (50μM) for the indicated times and their protein extracts were

19 applied to Western blotting with the indicated antibodies.

20 **Fig. 3. The effects of Skp2 ectopic expression in tetraploid G1 arrest.**

15

1   (A) BrdU incorporation assay. Parental HCT116 cells and Skp2-exressing cells were

2   treated with PTX-2 for the indicated periods and were then subjected to BrdU

3   incorporation assay as described in Materials and methods.

4   (B) HCT116 and Skp2-expressing cells were treated with PTX-2 for 48 h and were then

5   DAPI-stained. The number of nuclei in each cell was counted as described in Materials

6   and methods.

7   (C) Protein analysis of Skp2 and p27/KIP1. Expression of Flag-Skp2 and p27/kip1 were

8   determined in HCT116 and Skp2-expressing cells treated with PTX2 for 48 h by

9   Western blotting analysis with the indicated antibodies.

10   **Figure 4. Actin damage induced apoptotic death by ectopic expression of Skp2**

11   (A) Flow cytometric analysis of HCT116 cells and its derivative expressing flag-tagged

12   Skp2 after treatment with PTX2 for 72 h. Cells were labeled with propidium iodide (PI)

13   as described in Materials and Methods. A set of data representative of three independent

14   experiments is shown.

15   (B) Apoptotic body formation in PTX2-treated cells. HCT116 cells and its derivative

16   expressing Skp2 were treated with PTX2 for 72 h and apoptotic body formation (arrows)

17   as an indicator of apoptosis was determined by DAPI staining then photographing cells

18   under fluorescence microscopy (×200).

19   (C) The roles of Skp2 and p53 in actin inhibitor-induced tetraploid G1 arrest. Actin

20   inhibitors that block cytokinesis lead to Skp2 repression as well as p53 activation. While

21   p53 induces p21 expression, Skp2 repression does p27 expression. Therefore, actin

16

1   damage induced tetraploid G1 arrested cells have increased amounts of CDK inhibitors,

2   p21 and p27, which ensure to inhibit uncoupled DNA replication and nuclear division
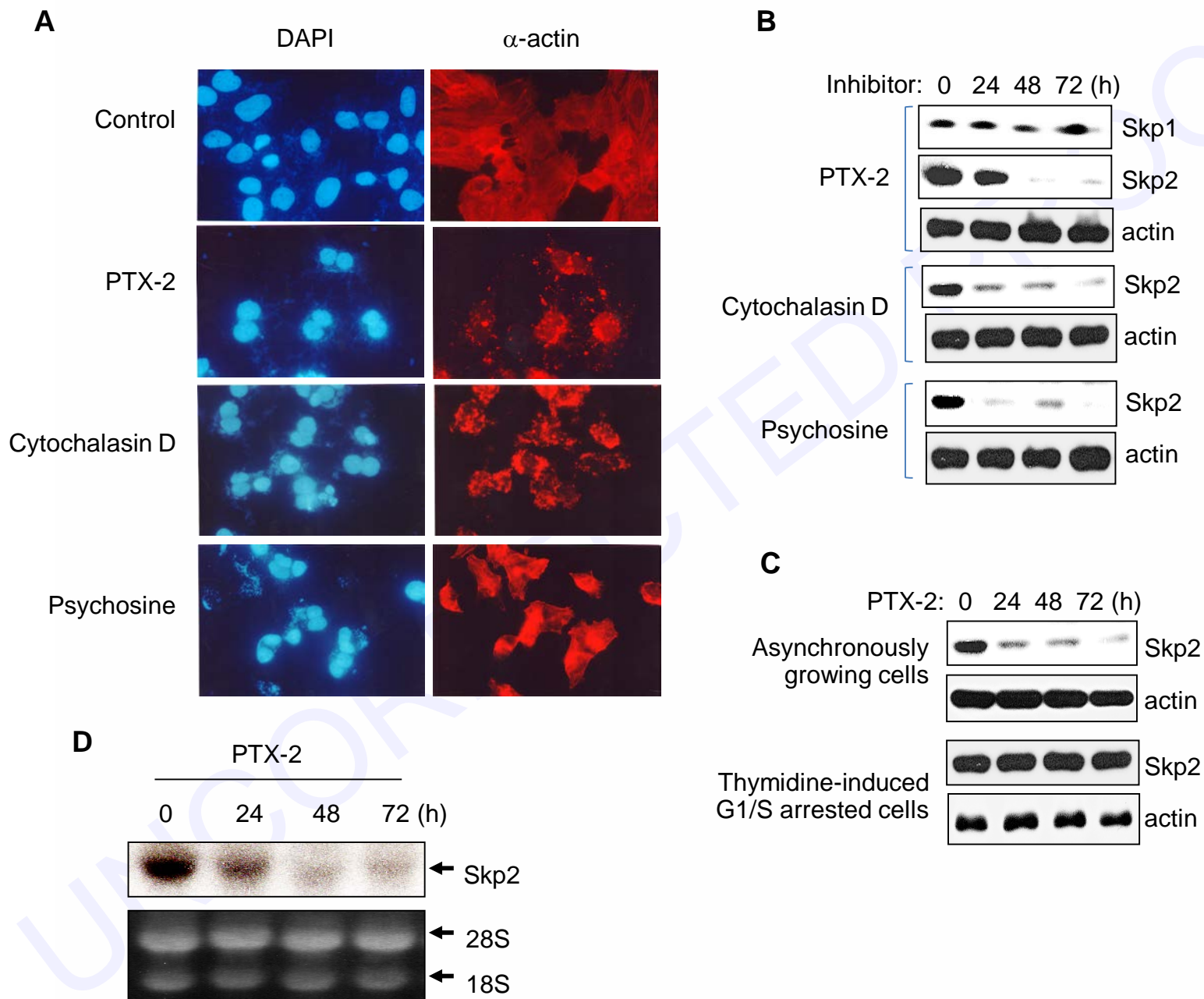
3   without cytokinesis.

Figure 1. Jo et al.
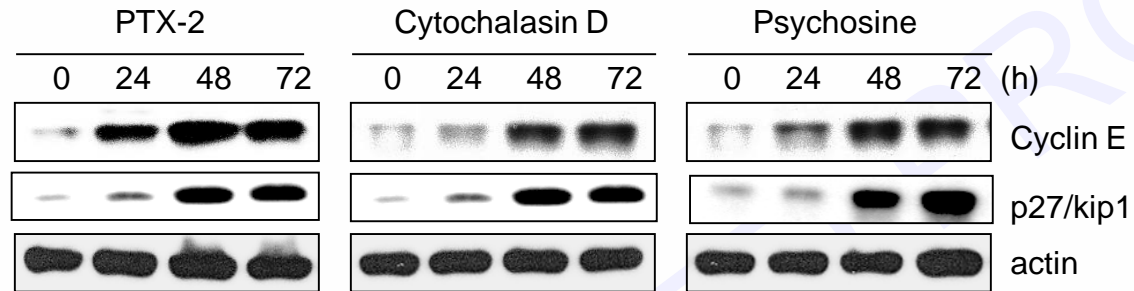
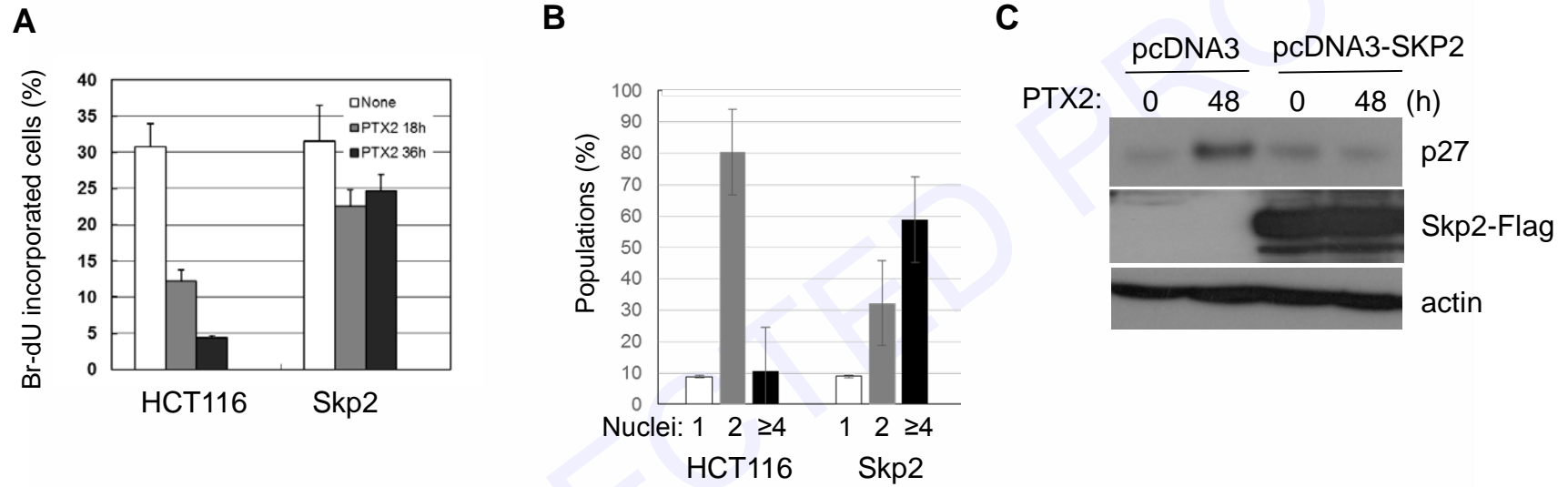**Figure 2. Jo et al.**

Figure 3. Jo et al.

Figure 4. Jo et al.

A

CONTROL   PTX-2

pcDNA3

2N
J
4.3%
4N

J 2N 4N 8N
26.8%
16N

pcDNA3
-SKP2-Flag

FL3 LOG
J
26.8%

J
85.8%

B

pcDNA3   pcDNA3-SKP2

C

Actin damage
↓
Cytokinesis blockade
↙   ↘
p53 Induction   Skp2 repression
↓   ↓
p21/Cip1 induction   p27/Kip1 induction
↘   ↙
Tetraploid G1 arrest