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作品名稱　「噬」者生存—有絲分裂時，USP24 的下降誘發細胞自噬，有利於基因體的穩定

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關鍵詞　有絲分裂、細胞自噬、

泛素特異性胜肽酶二十四(USP24)

作者簡介

大家好，我是唐新惟，目前就讀於臺南一中，平時的興趣是攝影以及做實驗。升上高中後接觸了許多科學知識，最終決定以生物醫學作為研究方向。自高一以來有幸加入洪建中教授的實驗室，在大家的指導及協助下慢慢找到自己的研究方向。感謝洪教授及實驗室學長姐在研究路上的協助，也謝謝所有幫助過我的人，使本研究能更加完善。

研究摘要

抗藥性是癌症治療時的一大阻礙。如何解決癌症的抗藥問題是十分重要的議題。過去的研究指出在肺腺癌細胞株中 USP24 表現量在有絲分裂期間會明顯下降，但此現象之功能仍有待釐清。在本研究中，我們發現該現象會使 TRAF6 表現量下降，從而使 Beclin-1 表現量上升，進而誘發細胞自噬。另外，我們更發現了有絲分裂期的細胞自噬有助於減少 DNA 碎片，達到穩定基因體的功用。此外，在與抗藥細胞株比較時發現，上述誘發細胞自噬之現象在抗藥細胞株中明顯下降。因此，我們推斷在抗藥細胞株中有絲分裂期的 USP24 下降變少，細胞自噬被抑制，造成基因體不穩定，最後造成抗藥性的產生。綜上所述，我們的研究不只闡明了 USP24 的下降在有絲分裂期間是如何誘發細胞自噬，更說明了有絲分裂時的細胞自噬有助於基因體的穩定，從而在肺癌的治療中阻止癌症抗藥性的產生。

Abstract

Drug resistance has been one of the greatest obstacles during cancer treatment. It is urgent to solve this clinical unmet issue globally. Our previous study has indicated that USP24 is decreased during mitosis in A549 cells (human lung adenocarcinoma epithelial cell line), but the role of USP24 down-regulation still requires clarification. In this study, we found that down-regulation of USP24 during mitosis induces autophagy by destabilizing TRAF6, a ubiquitin E3 ligase of Beclin-1. Inhibiting autophagy by its inhibitor, bafilomycin A1, during mitosis not only blocks the progression of mitosis but also increases the DNA debris, implying that the role of autophagy during mitosis can be maintaining the genomic integrity, thereby prevents the development of heterogeneity in cancer cells, subsequently leading to drug resistance. We also found that the level of USP24 is higher and that of autophagy is lower in mitotic-drug-resistant cell line, A549-T24, compared to those in mitotic-drug-sensitive cell line, A549.To sum up, our results not only reveal how the down-regulation of USP24 induce autophagy during mitosis, but

also demonstrate that mitotic autophagy can facilitate the maintenance of genomic integrity, thereby prevent the development of drug resistance during lung cancer treatment.

壹、前言

一、研究背景及動機

Lung cancer is the second-most diagnosed cancer and the leading cause of cancer-related death in 2020 worldwide (Sung *et al.*, 2021). Since the symptoms of early-stage lung cancer are not obvious and also similar to other diseases, most cases of lung cancer are at an advanced stage when diagnosed, which leads to the poor prognosis (Cruz *et al.*, 2011). However, there are various treatment options that could be used as first-line treatment, including surgery, radiotherapy, chemotherapy, target therapy, and immunotherapy. The first-line chemotherapeutic drugs for non-small cell lung cancer (NSCLC) include cisplatin, paclitaxel, pemetrexed (only used in non-squamous NSCLC) … etc. Although the efficacy of initial chemotherapy treatment might be significant, the vast majority of lung cancer still develop drug resistance ultimately, which is the primary reason why conventional chemotherapy usually ends up failure. Apparently, it is drug resistance that is considered as one of the greatest obstacles in lung cancer treatment. Hence, to optimize the current treatment options and prevent drug resistance from developing, it is urgent that the mechanism of drug resistance should be fully understood.

Genomic integrity, known as genomic stability, is essential for organism survival and for the inheritance of traits to offspring (Papamichos-Chronakis & Peterson, 2013). When genomic integrity of cells is jeopardized, they usually have a higher rate of chromosome abnormality, which is referred to as genomic instability. Genomic instability is caused by DNA damage, aberrant DNA replication or uncoordinated cell division, which can lead to chromosomal aberrations, and gene mutations (Papamichos-Chronakis & Peterson, 2013). Recently, genomic instability has been considered as a characteristic of most cancer

cells (Yao & Dai, 2014) and has been shown to be associated with poor prognosis (Andor *et al.*, 2017). Furthermore, previous study stated that cancer genomic instability contributes to the phenomenon of intratumoral genetic heterogeneity, providing the genetic diversity required for natural selection, and enables the extensive phenotypic diversity that is frequently observed among patients (Andor *et al.*, 2017). Moreover, another study suggested that intratumoural genetic heterogeneity serve as a driver of chemotherapy resistance in cancer patients (Saunders *et al.*, 2012). To sum up, genomic instability contributes to the development of intratumoral genetic heterogeneity, ultimately leading to the development of drug resistance in cancer. Hence, to overcome the treatment obstacle which is caused by drug resistance in cancer, it is crucial to understand the factors which maintain genomic integrity in cells.

Autophagy is an intracellular degradative process *via* lysosome, which is conserved in virtually all eukaryotic cells (Levine & Klionsky, 2004). It involves the selective degradation of cellular components, including long-lived proteins, protein aggregates, damaged cytoplasmic organelles, and intracellular pathogens, resulting in the recycling of nutrients and the generation of energy (Su *et al.*, 2013). Therefore, autophagy serves as an essential pathway to maintain cellular homeostasis (Chun & Kim, 2018). Recently, emerging studies have reported several correlations between autophagy and mitotic progression. A study revealed that autophagy plays an essential role in the degradation of cyclin A2, which makes autophagy indispensable during mitosis (Loukil *et al.*, 2014). Not only mitotic progression, autophagy has also been shown to maintain genomic integrity. Another study stated that specialized regulatory functions for autophagy or autophagy-related factors in the cell division progress, such as correcting segregation of the duplicated genome during cell division, is a prerequisite for preventing chromosome instability and aneuploidy (Mathiassen *et al.*, 2017). Still another study also revealed that autophagy regulates cell fate after DNA damage and also has a pivotal role in the maintenance of nuclear and mitochondrial genomic integrity (Vessoni *et al.*, 2013). To sum up, autophagy has been reported to

play a regulatory role in cell cycle progression, correcting segregation of the duplicated genome during cell division, and DNA damage regulation. Hence, autophagy seems to play an important role in maintaining genomic integrity.

Ubiquitin-specific peptidase 24, or USP24 in short, is a 2620-amino acid protein, which is one of the deubiquitinases under the ubiquitin-specific peptidase superfamily. It contains a ubiquitin-associated domain (UBA), which binds to the ubiquitin molecules on substrate proteins, a ubiquitin-like domain (Ub-like), and a ubiquitin C-terminal hydrolase domain, which serves as a catalytic domain (Komander *et al.*, 2009). The function of USP24 is to identify and disassociate the ubiquitin tag attached on the target protein, thereby preventing the target protein form being degraded by the 26S proteasome. One of our previous studies indicated that higher level of USP24 results in metastasis and is associated with poor prognosis in lung cancer (Wang *et al.*, 2016). In another of our previous studies, USP24 had been demonstrated to increase the levels of the ATP-binding cassette (ABC) transporters P-glycoprotein, ABCG2, and ezrin to enhance the pumping out of Taxol from cancer cells, thus resulted in drug resistance during cancer therapy (Wang *et al.*, 2021). Further, in the same study, we had also found that USP24 represses DNA-damage repair (DDR) activity by decreasing Rad51 expression to cause the tumor genomic instability and cancer stemness, implying that USP24 might facilitate the development of genomic instability (Wang *et al.*, 2021). Moreover, in others of our studies, USP24 was shown to stabilize securin to block the cell cycle progression from metaphase to anaphase, leading to cell cycle arrest (Wang *et al.*, 2017; Young *et al.*, 2019). As a whole, our previous studies suggested that USP24 not only promotes cancer metastasis and drug resistance, but also facilitates the development of genomic instability and blocks mitotic progression.

Previous studies had revealed the correlation between autophagy, mitotic progression and genomic

integrity, which contributes to the development of drug resistance. Also, USP24 not only has been shown to promote cancer metastasis and drug resistance, but also facilitates the development of genomic instability and blocks mitotic progression. Further, our previous study indicates that the level of USP24 will decrease during mitosis (Wang *et al.*, 2017). Moreover, our preliminary result revealed that when A549 cells (human lung adenocarcinoma epithelial cell line) are treated with USP24 inhibitor, the level of autophagy will soar dramatically (data not shown), implying that USP24 might negatively regulates autophagy. Hence, in this study, our primary goal is to clarify the correlation between USP24 and autophagy and to clarify the role which USP24 and autophagy play during mitosis. After understanding the correlation between USP24 and autophagy, our next goal is to reveal the possible mechanism *via* which USP24 negatively regulates mitotic autophagy. Moreover, we are also desired to understand how this mechanism affects genomic integrity, which ultimately leads to the development of drug resistance in lung cancer treatment.

二、研究目的

1. To study the role which autophagy plays during mitosis, and how USP24 mediates this mechanism.
2. To clarify the possible molecular mechanism *via* which USP24 negatively regulates mitotic autophagy.
3. To study how induction of autophagy during mitosis affects genomic integrity and the development of drug resistance.

貳、研究方法及過程

一、研究方法

1. Cell Culture

Human lung adenocarcinoma epithelial cell line A549, obtained from the American Type Culture

Collection (ATCC), was cultured with RPMI 1640 medium (Life Technologies, NY, USA), which contains 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Grand Island, NY, USA), 100 μg/ml streptomycin sulfate, and 100 μg/ml penicillin G sodium (P/S) (Invitrogen Life Technologies, Grand Island, NY, USA). A549 cell line is maintained at 37 ˚C and 5% CO_2. Taxol-resistant A549 cell line, A549-T24, is maintained in the same culture medium as A549 cells but containing Taxol (Sigma-Aldrich, St. Louis, MO, USA).

2. Cell Synchronization

Mitotic cells were collected by incubating A549 cells in complete medium with 50 ng/ml of nocodazole (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C for 18 hours. After 18 hours of incubation, the cells were released from G2/M phase by washed with PBS (phosphate-buffered saline, containing 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 1.8mM KH_2PO_4) for three times and fresh medium was added subsequently. After released, the cells were harvested at different time intervals and then prepared for immunoprecipitation, immunoblotting or western blotting. In the western blotting experiments, the released cells were lysed in sample buffer (62.5mM of Tris-HCl pH6.8, 2.5% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, 2mM EDTA, 0.002% bromophenol blue). In immunoprecipitation, sample buffer was changed to RIPA lysis buffer (radioimmunoprecipitation assay buffer) (25mM Tris-Cl pH=7.4, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% TritonX-100) instead.

3. Western Blot Analysis

Protein samples were collected by sample buffer and analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Proteins were then transferred to PVDF (polyvinylidene difluoride) membrane (Merck Millipore, Bedford, MA, USA) and TBST buffer (Tris-buffered saline with Tween 20)

(10mM Tris-HCl, Ph 8.0, 150mM NaCl and 0.05% Tween 20) containing 5% nonfat milk was used for blocking. After blocking was done, PVDF membrane was then incubated with primary antibodies (diluted in TBST buffer containing 0.5% bovine serum albumin). The list of primary antibodies used in the following experiments and its dilution ratios were listed below at table 1. After incubated with primary antibodies, PVDF membrane was then incubated with secondary immunoglobulin antibodies linked with horseradish peroxidase (diluted in TBST buffer containing 2.5% nonfat milk). The list of secondary antibodies used in the following experiments and its dilution ratios were listed below at table 2. ECL Western blotting detection system (Merck Millipore, Bedford, MA, USA) and ChemiDoc-it imager (UVP) were used to detect signals. ImageJ (Bethesda, Maryland, USA) was used to perform the statistical analysis of signal bands.

Table 1: List of primary antibodies used in the following experiments and its dilution ratios

Antibodies	Name of the target protein (manufacturer)	Dilution ratio (antibody: TBST)
Primary antibody	Actin (GeneTex, Irvine, CA, USA)	1:5000
	Beclin-1 (Cell Signaling Technology, Danvers, MA, USA)	1:1000
	Cullin-3 (Abcam, Cambridge, UK)	1:10000
	Cyclin B1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)	1:1000
	Normal Rabbit IgG (Cell Signaling Technology, Danvers, MA, USA)	1:3000
	KLHL20 (Novus Biologicals, Littleton, CO, USA)	1:1000

	LC3B A/B (Cell Signaling Technology, Danvers, MA, USA)	1:3000
	NEDD4 (Cell Signaling Technology, Danvers, MA, USA)	1:3000
	RNF216 (Abcam, Cambridge, UK)	1:3000
	TRAF6 (Cell Signaling Technology, Danvers, MA, USA)	1:1000
	USP24 (Proteintech Group, Rosemont, IL, USA)	1:3000

Table 2: List of secondary antibodies used in the following experiments and its dilution ratios

Antibodies	Name of the antibodies (manufacturer)	Dilution ratio (antibody: TBST)
Secondary antibody	HRP-linked anti-rabbit IgG (Merck Millipore, Bedford, MA, USA)	1:3000
	HRP-linked anti-mouse IgG (Merck Millipore, Bedford, MA, USA)	1:3000
	HRP-linked anti-rabbit IgG light chain	1:3000

4. Immunofluorescent analysis

A549 cells were seeded in a 6-well plate with cover slips inside for 48 hours. Then, the coverslips on which the cells grew were removed from the 6-well plate and were fixed with 4% paraformaldehyde at 4℃ for 15 minutes. After fixation, cover slips were washed with PBS, and were incubated with 0.5%

Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 5 minutes at room temperature. PBS containing 1% BSA (bovine serum albumin) was used for blocking the coverslips for 1 hour. The coverslips were then stained with primary antibodies for 16 hours at 4℃. After washed with PBS with 0.5% of Tween 20, cells were stained with secondary antibodies for 2 hours at room temperature. After washed with PBS with 0.5% of Tween 20, cells were stained with DAPI (Invitrogen Life Technologies, Grand Island, NY, USA) for 15 minutes at room temperature. Then the coverslips were fixed in glycerol with nail polish. To quantify the number of stained cells, foci were examined by fluorescent microscopy (Olympus, Tokyo, Japan). ImageJ (Bethesda, Maryland, USA) was used to perform the statistical analysis of foci.

5. Immunoprecipitation

Cell lysate was harvested with RIPA buffer containing protease inhibitor cocktail (Merck Millipore, Bedford, MA, USA) and phosphatase inhibitor (BioVision, Milpitas, CA, USA). 1000μl of cell lysate was incubated with 4μl of the anti-USP24 antibody (Proteintech Group, Rosemont, IL, USA) or anti-Rabbit IgG antibody (Cell Signaling Technology, Danvers, MA, USA) respectively, followed by the precipitation using protein A/G agarose (Merck Millipore, Bedford, MA, USA). After washed four times with RIPA buffer, the precipitant was lysed with sample buffer, and was subjected to western blot analysis subsequently.

6. Fluorescence-activated cell sorting (FACS)

The cells were seeded in a 6-well plate. Culturing time depends on the different experiments. After achieving proper cell density, cells were washed with PBS and fixed with 75% ethanol at 4 ˚C overnight. Cells were then incubated in cold PBS with 0.1% Triton X-100 for 10 min for permeabilization. Finally, Permeabilized cells were treated with 50 μg/ml of propidium iodide in PBS, which contained RNase A

at room temperature for 1 h. Stained cells were then analyzed by Attune NxT Flow Cytometer (Thermo Fisher, Waltham, MA, USA). For mitotic progression analysis, A549 cells were synchronized with nocodazole and released with fresh medium at different time intervals in advance.

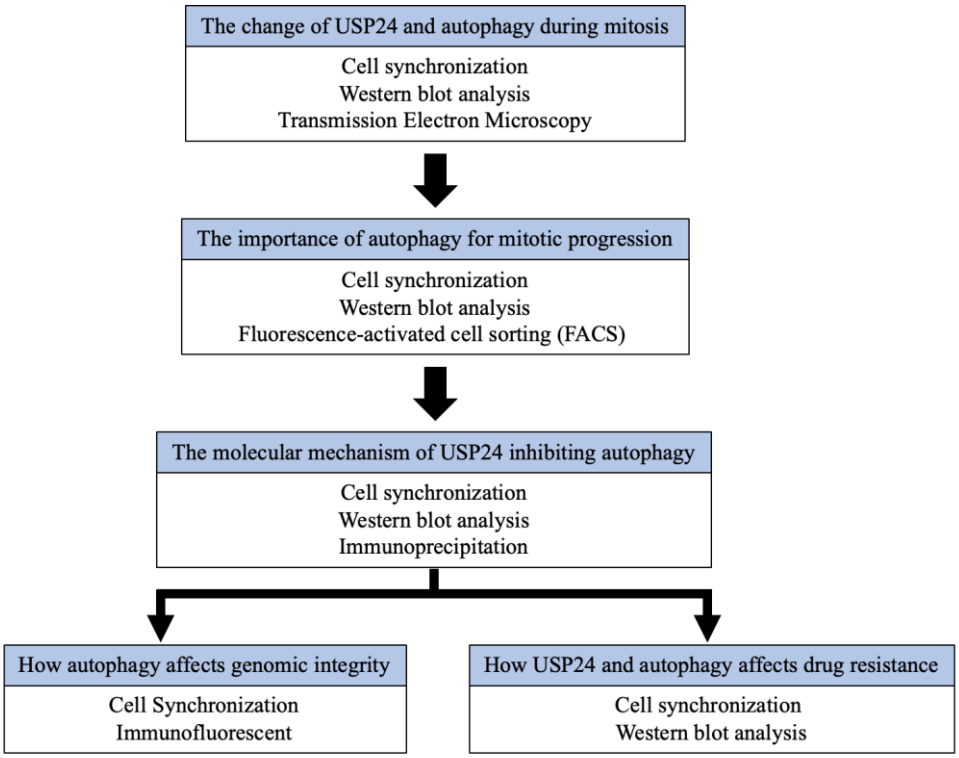
7.Transmission electron microscopy (TEM)

A549 cells were seeded in a 10cm plate containing complete medium. After reaching proper density, the cells underwent the procedure of cell synchronization. After synchronization, the cells were released from G2/M phase at different time intervals, and the cells were fixed subsequently. After fixation, the cell samples were observed with H-7650 transmission electron microscope (Hitachi, Tokyo, Japan)

8.Statistics

All samples were used for statistical analysis. For all experiments, at least three independent biological replicates of each condition were analyzed. Estimated variation within each experiment group is similar. The difference between two groups was analyzed by two-tailed unpaired Student' s *t* test. The *p* value, which is <0.05, was considered as statistically significant. Center value is defined as mean value, and S.E.M (standard error of the mean) is used to calculate and plot error bars from raw data.

二、研究過程及流程圖



參、研究結果及討論

一、研究結果

1. The level of USP24 is decreased and that of LC3B is increased during mitosis, which facilitates the mitotic progression.

Our previous study indicates that the level of USP24 will decrease during mitosis (Wang *et al.*, 2017). Also, in our preliminary study, we found that when A549 cells were treated with USP24 inhibitor, the level of LC3B, an autophagy marker, will increase. Accordingly, we suspected that the down-regulation of USP24 during mitosis might induce autophagy. Hence, we detected the level of LC3B during mitosis. During the experiment, A549 cells were synchronized to G2/M phase with nocodazole. Then, they were released with fresh medium and were harvested at different time intervals (0, 20, 40, 60, 80 minutes). These cell samples were examined by western blot analysis (Figure 1-A, B, C). The data indicated that

the level of LC3B increases and the level of USP24 decreases during mitosis. Since our findings suggested that the level of LC3B continues increasing, and reaches maximum level at 60 minutes after released from G2/M phase. Therefore, we quantified the amount of autophagosomes in these two samples by transmission electron microscopy (TEM) to confirm that the amount of autophagosomes does increase during mitosis (Figure 1-D, E). The results indicated that the amount of autophagosomes dramatically increases during mitosis, which met our previous result from western blot analysis. Next, to determine whether the up-regulation of autophagy is necessary for mitotic progression, we then inhibited autophagy during mitosis with bafilomycin A1, an autophagy inhibitor. After A549 cells were synchronized to G2/M phase, they were treated with/without bafilomycin A1 respectively. The two different groups of cells were examined morphologically and western blot analysis was also performed to detect the protein level. Morphologically, cells arrested at G2/M phase have a relatively round appearance, whereas cells at interphase have a relatively flat appearance. During the morphology test, we discovered that the group of cells treated with bafilomycin A1 and nocodazole had a higher ratio of "round" cells than the group treated with nocodazole alone after the same period of time (Figure 2-A). Also, the western blot analysis (figure 2-B) revealed that the level of cyclin B1 in A549 cells treated with bafilomycin A1 and nocodazole remains high after 80 minutes, whereas that in A549 cells treated with nocodazole only drops after 60 minutes (figure 1-A). Since we have known that the level of cyclin B1 soars in prophase and metaphase and falls at anaphase, our data indicated that the A549 cells treated with bafilomycin A1 left mitosis slower than A549 cells which did not receive it. These results implied that autophagy might facilitate the progression of mitosis. Further, in order to confirm the morphological finding, we examine the mitotic cells by flow cytometer. As our last experiment, we synchronized A549 cells to G2/M phase with nocodazole, and treated two groups of cells with/without bafilomycin A1 respectively. After A549 cells were released from G2/M phase, they were harvested at different time intervals (80, 90, 100, 110, 120 minutes). These samples were examined by flow cytometer (figure 2-C, D). The result indicated that

when mitotic cells were treated with bafilomycin A1, they left mitosis slower than cells which did not receive bafilomycin A1. As a whole, these data met our previous hypothesis, suggesting that autophagy is up-regulated during mitosis. Further, mitotic autophagy facilitates mitotic progression. Were it not for autophagy, the mitotic progression would be delayed. Since we have known that when A549 cells were treated with USP24 inhibitor, the level of LC3B will increase, and our results confirmed that the level of USP24 does decrease during mitosis. We presumed that the down-regulation of USP24 might induce mitotic autophagy in a subtle way.

2. USP24 regulates autophagy *via* TRAF6 and Beclin-1 during mitosis

After confirming the role that USP24 and autophagy plays during mitosis, we still desired to understand the molecular mechanism behind it. First, we searched for possible pro-autophagy proteins which may increase during mitosis to induce autophagy. Hence, we examined the level of pro-autophagy protein, Beclin-1, in mitotic A549 cells. The cell samples used in this experiment were harvested in the same condition as the experiment in figure 1-A. These samples were examined by western blot analysis (figure3-A, B). The data suggested that the level of Beclin-1 increases during mitosis. Hence, we supposed that the up-regulation of autophagy might be caused by the increasing level of Beclin-1. Several previous studies indicated that Beclin-1 is the substrate of several ubiquitin E3 ligases, such as TRAF6, RNF216, NEDD4, HERC5, AMBRA-1-containing CUL4-ligase complex, and KLHL20-contianing CUL3-ligase complex (Boutouja *et al.*, 2017). Therefore, we hypothesized that USP24 might regulate autophagy by stabilizing these E3 ligases. To confirm our hypothesis, we first detect the level of NEDD4, RNF216, and TRAF6 in mitotic A549 cells. The samples used in this experiment were harvested in the same condition as the experiment in figure 1-A. These samples were examined by western blot analysis (figure 3-C~F). The result indicated that the level of NEDD4, RNF216, and TRAF6 decrease during mitosis. To further confirm our hypothesis, we then examined the interaction of USP24

with these ubiquitin E3 ligases which decrease during mitosis (including NEDD4, RNF216, and TRAF6). The A549 cells were first synchronized to G2/M phase by nocodazole, then the cell lysate was harvested with RIPA buffer. Next, the cell lysate underwent the co-immunoprecipitation process, in which anti-USP24 antibody, anti-rabbit IgG antibody, and protein A/G resin were used. After the co-immunoprecipitation was done, the resin which conjugated with anti-USP24 antibody or anti-rabbit IgG antibody was lysed with sampling buffer respectively, and were subjected to western blot analysis (figure 3-G). The result indicated that TRAF6 interacts with USP24 during mitosis, meaning that USP24 might regulate the level of Beclin-1 by manipulating the level of TRAF6. To sum up, we proposed that USP24 might reduce the level of autophagy during mitosis by stabilizing the level of TRAF6, further destabilizing Beclin-1.

3. Induction of autophagy during mitosis reduces segregation defects to maintain genomic integrity, further preventing the development of drug resistance.

Last, we desired to reveal the correlation between mitotic autophagy, genomic integrity, and drug resistance. To achieve this, we divided our experiments into two sections. First, to find how mitotic autophagy affects genomic integrity, we examined the segregation defects when autophagy is inhibited. During our experiment, A549 cells were synchronized to G2/M with nocodazole, and two groups of cells were treated with/without bafilomycin A1 respectively. After synchronization and inhibition (or not), the cells were released from G2/M with fresh medium, and they were harvested 60 minutes after released. These samples were then subjected to immunofluorescent analysis to quantify the segregation defects. The result (figure 4-A, B) revealed that more micronucleus and segregation defects exist in A549 cells treated with bafilomycin A1. These results suggested that without autophagy, more segregation defects will occur during mitosis, which contribute to the development of genomic instability. Next, to further examine how mitotic autophagy affects drug resistance among cancer cells and how USP24 mediates this

mechanism, we invested the difference of mitotic autophagy and the level of USP24 between A549 cells and its drug-resistant cells, A549-T24. We detected the level of USP24 and LC3B in synchronized A549-T24 cells. After A549-T24 cells were synchronized by nocodazole, they were released from G2/M phase and were harvested at different time intervals (0, 20, 40, 60, 80 minutes). These samples were examined by western blot analysis (figure 4-C~G). The result indicated that the level of USP24 were slightly decreased and the level of LC3B were slightly increased, but both of them were merely statistically significant. When comparing the data of A549-T24 cells to those of A549 cells, we discovered that although the change of level of USP24 and LC3B are both statistically significant, it is obvious that the change of USP24 and LC3B among the A549-T24 cells were minor than that among the A549 cells.

二、圖表

1. Figure 1:

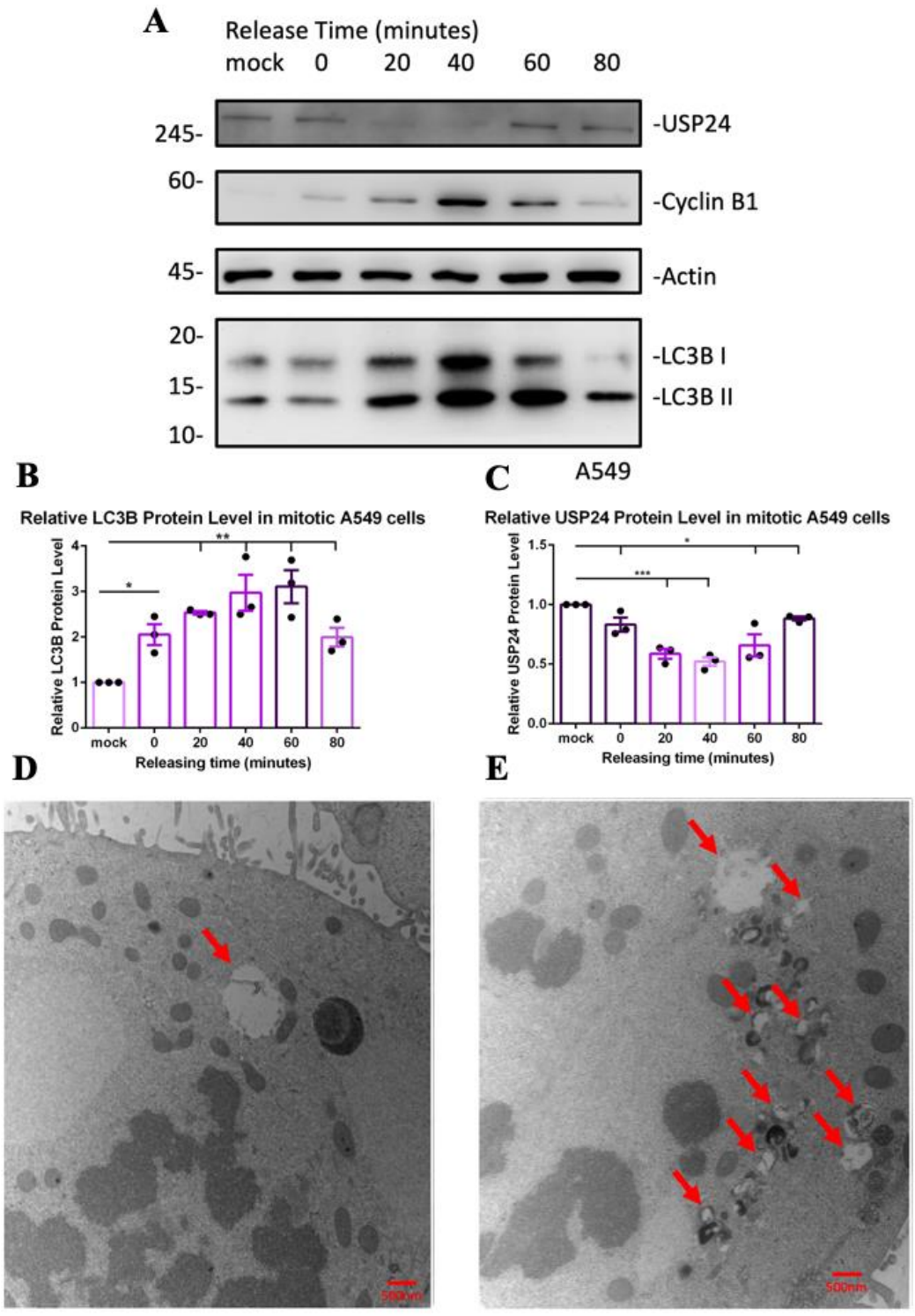


Figure 1:

(A) The A549 cells were released with fresh medium after they were synchronized by nocodazole (50ng/ml). After released, the cells were harvested at different time intervals (0, 20, 40, 60, 80 minutes). Western blot analysis was performed to detect the level of USP24, cyclin B1, actin, and LC3B. (B) The relative level of LC3B was quantified after three independent experiments were done, and the data were analyzed by t-test. (C) The relative level of USP24 was quantified after three independent experiments were done, and the data were analyzed by t-test. (D), (E) The autophagosomes in A549 cells treated with nocodazole (50ng/ml) and released at different time intervals ((D): harvested 0 minute after released, (E): harvested 60 minutes after released) were observed with transmission electron microscope. The autophagosomes were indicated with red arrows, and the proportional scale was shown below. (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$)

2. Figure 2:

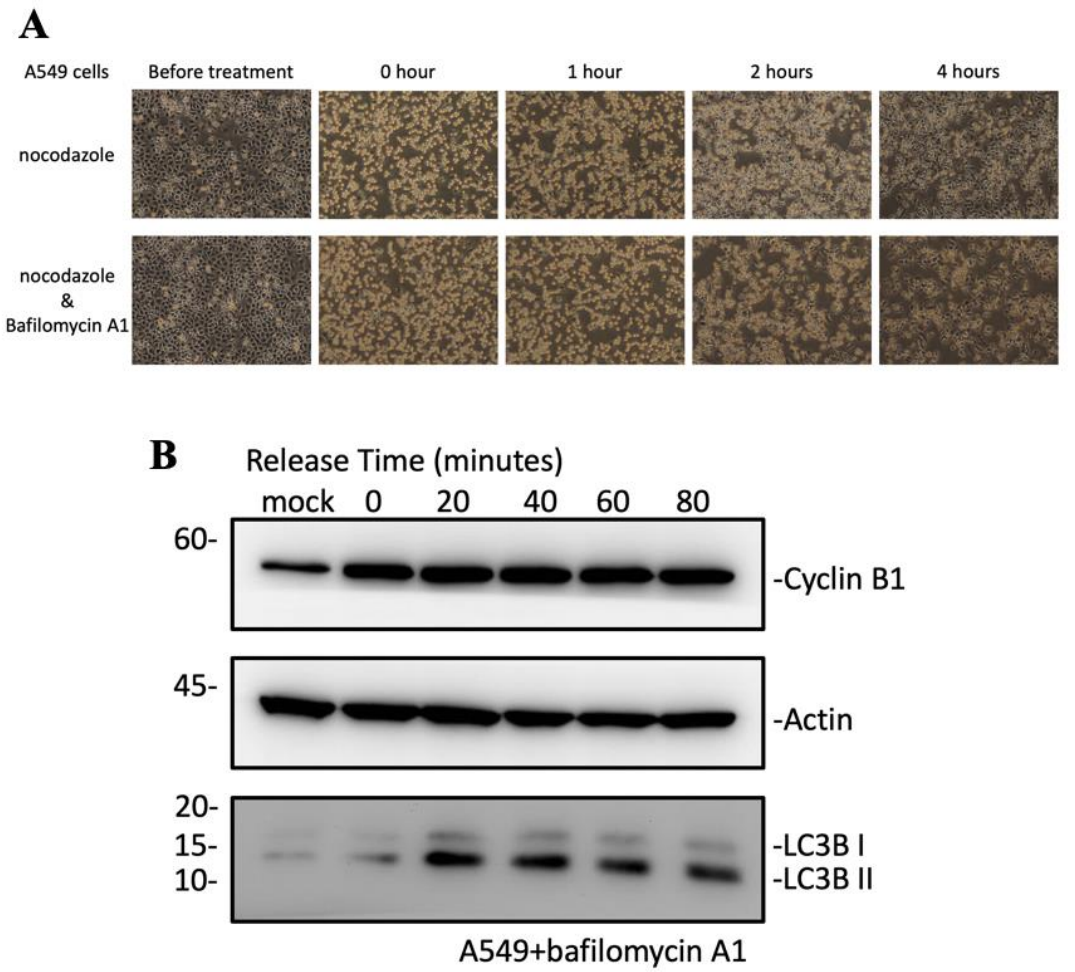

A
A549 cells
Before treatment
0 hour
1 hour
2 hours
4 hours
nocodazole
nocodazole & Bafilomycin A1
B
Release Time (minutes)
mock 0 20 40 60 80
60-
-Cyclin B1
45-
-Actin
20-
15-
10-
-LC3B I
-LC3B II
A549+bafilomycin A1
C
Release Time (minute)
A549 cells
80 90 100 110 120
nocodazole
nocodazole & Bafilomycin A1
D
NOC
NOC + Baf A
Relative G1/S phase(%)
0 1 2 3 4
*
80min 90min 100min 110min 120min

Figure 2:

(A) The A549 cells were treated with nocodazole (50ng/ml), and were released after 18 hours. Two hours before released, the cells were treated with/without bafilomycin A1 (10ng/ml) respectively. After released, the morphology of two groups of cells were examined by light microscopy respectively. (B) The A549 cells were treated with nocodazole (50ng/ml), and were released after 18 hours. Two hours before released, the cells were treated with bafilomycin A1 (10ng/ml). After released, the cells were harvested at different time intervals (0, 20, 40, 60, 80 minutes). Western blot analysis was performed to detect the level of cyclin B1, actin, and LC3B. (C) The A549 cells were treated with nocodazole (50ng/ml), and were released after 18 hours. Two hours before released, the cells were treated with/without bafilomycin A1 (10ng/ml) respectively. After released, the cells were collected at different time intervals (80, 90, 100, 110, 120 minutes). The cells were then studied by flow cytometer with propidium iodine. The result was analyzed by Attune NxT Software. (D) Comparing the relative G1/S ratio between cells treated with/without bafilomycin A1, after three independent experiments were done, and the data were analyzed by t-test. (*=$p<0.05$)

3. Figure 3

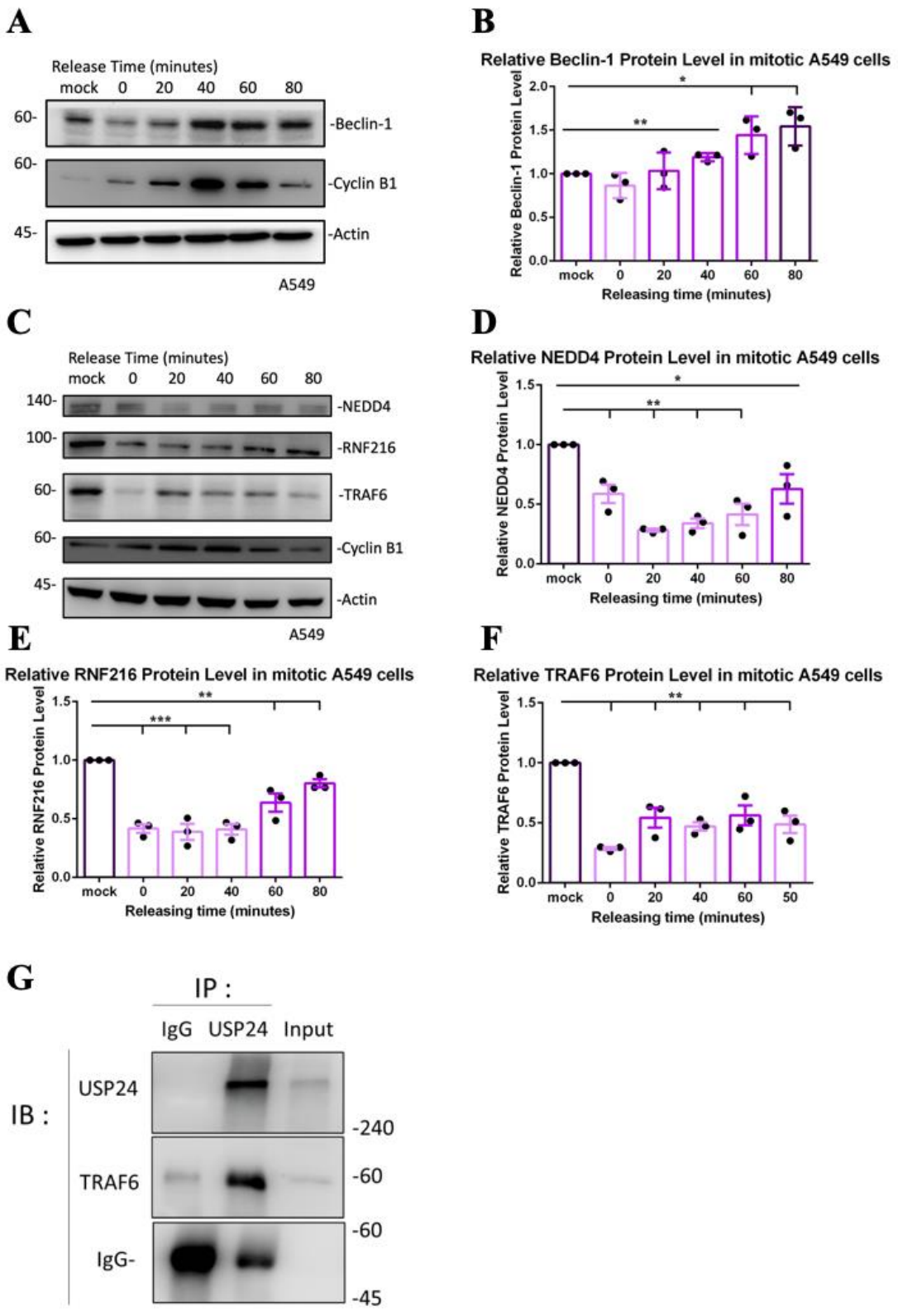

A
Release Time (minutes)
mock 0 20 40 60 80
60-
-Beclin-1
60-
-Cyclin B1
45-
-Actin
A549
B
Relative Beclin-1 Protein Level in mitotic A549 cells
Relative Beclin-1 Protein Level
Releasing time (minutes)
C
Release Time (minutes)
mock 0 20 40 60 80
140-
-NEDD4
100-
-RNF216
60-
-TRAF6
60-
-Cyclin B1
45-
-Actin
A549
D
Relative NEDD4 Protein Level in mitotic A549 cells
Relative NEDD4 Protein Level
Releasing time (minutes)
E
Relative RNF216 Protein Level in mitotic A549 cells
Relative RNF216 Protein Level
Releasing time (minutes)
F
Relative TRAF6 Protein Level in mitotic A549 cells
Relative TRAF6 Protein Level
Releasing time (minutes)
G
IP :
IgG USP24 Input
IB :
USP24
TRAF6
IgG-
-240
-60
-60
-45

Figure 3:

(A) Western blot analysis was performed to detect the level of Beclin-1, cyclin B1, and actin in sample harvested in the same condition of experiment in figure 1-A. (B) The relative level of Beclin-1 was quantified after three independent experiments were done, and the data were analyzed by t-test. (C) Western blot analysis was performed to detect the level of NEDD4, RNF216, TRAF6, cyclin B1, and actin in sample harvested in the same condition of experiment in figure 1-A. (D) The relative level of NEDD4 was quantified after three independent experiments were done, and the data were analyzed by t-test. (E) The relative level of RNF216 was quantified after three independent experiments were done, and the data were analyzed by t-test. (F) The relative level of TRAF6 was quantified after three independent experiments were done, and the data were analyzed by t-test. (G) The A549 cells were treated with nocodazole (50ng/ml), and were harvested 18 hours after treatment. The cell lysate was undergone the process of co-immunoprecipitation, in which anti-rabbit IgG (IP: IgG) and anti-USP24 antibody (IP: USP24) were used to conjugate with normal rabbit IgG protein or USP24 protein respectively. After co-immunoprecipitation, the samples were subjected to western blot analysis. During western blot analysis, the level of USP24, TRAF6, and Rabbit IgG was detected. (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$)

4. Figure 4

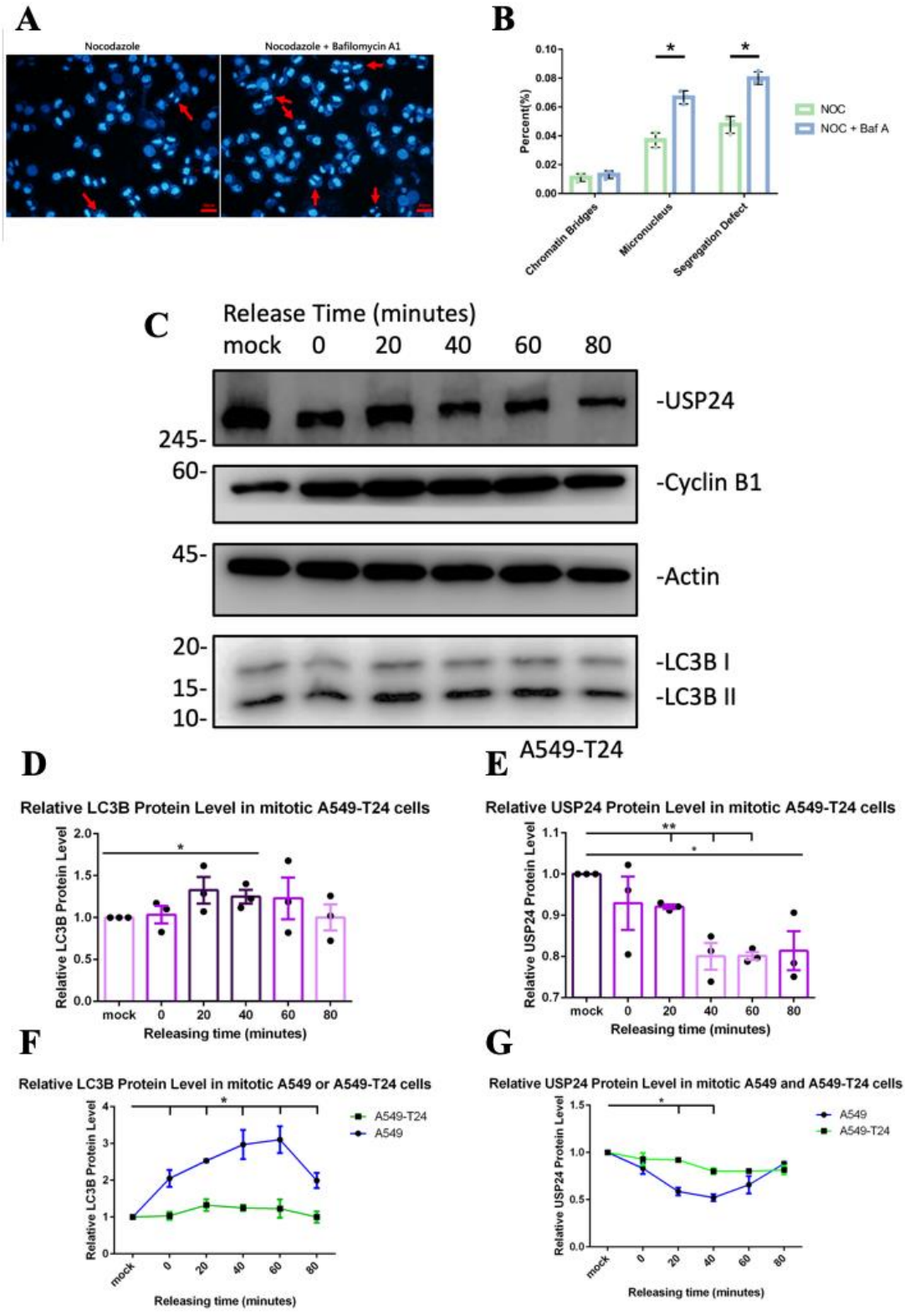

A
Nocodazole
Nocodazole + Bafilomycin A1
B
Percent(%)
NOC
NOC + Baf A
Chromatin Bridges
Micronucleus
Segregation Defect
C
Release Time (minutes)
mock 0 20 40 60 80
245-
-USP24
60-
-Cyclin B1
45-
-Actin
20-
15-
10-
-LC3B I
-LC3B II
A549-T24
D
Relative LC3B Protein Level in mitotic A549-T24 cells
Relative LC3B Protein Level
Releasing time (minutes)
E
Relative USP24 Protein Level in mitotic A549-T24 cells
Relative USP24 Protein Level
Releasing time (minutes)
F
Relative LC3B Protein Level in mitotic A549 or A549-T24 cells
A549-T24
A549
Relative LC3B Protein Level
Releasing time (minutes)
G
Relative USP24 Protein Level in mitotic A549 and A549-T24 cells
A549
A549-T24
Relative USP24 Protein Level
Releasing time (minutes)

Figure 4:

(A) The amount of segregation defects in A549 cells treated with nocodazole (50ng/ml) and nocodazole (50ng/ml) plus bafilomycin A1 (10ng/ml) were quantified by immunofluorescent analysis with DAPI. The segregation defects were indicated with red arrows and the proportional scale were shown below. (B) The amount of chromatin bridges, micronucleus, and segregation defects were quantified and analyzed by t-test after three independent experiments were done. (C) A549-T24 cells were released with fresh medium after they were synchronized by nocodazole (50ng/ml) for 18 hours. After released, the cells were harvested at different time intervals (0, 20, 40, 60, 80 minutes). Western blot analysis was performed to detect the level of USP24, cyclin B1, actin, and LC3B. (D) The relative level of LC3B was quantified after three independent experiments were done, and the data were analyzed by t-test. (E) The relative level of USP24 was quantified after three independent experiments were done, and the data were analyzed by t-test. (F) Comparing the relative level of LC3B between sample 1-A and 4-C after three independent experiments were done, the data were analyzed by t-test. (G) Comparing the relative level of USP24 between sample 1-A and 4-C after three independent experiments were done, the data were analyzed by t-test. (*=$p<0.05$, **=$p<0.01$)

三、研究討論

1. The down-regulation of USP24 and activation of autophagy is important for mitotic progression

As earlier mentioned, previous study has revealed that the decrease in USP24 during mitosis is crucial for the metaphase – anaphase transition *via* the declining securin level (Wang *et al.*, 2017). Also, there have been some studies implying that autophagy and autophagy-related factors might play a critical role during mitosis (Loukil *et al.*, 2014; Mathiassen *et al.*, 2017). In addition, our recent preliminary results also indicated that targeting USP24 by its specific inhibitor, USP24-i, could significantly induce autophagy in lung cancer cells (data not shown). These studies have enlightened our curiosity about whether autophagy plays an important role during mitosis. During this study, we not only confirmed that the level of LC3B, an autophagy marker, does increase during mitosis, but also revealed that the inhibition of autophagy could lead to delay of mitotic progression. Hence, our result has provided a reasonable demonstration to the importance of mitotic autophagy. Upon previous studies about autophagy, the majority of them had focused on autophagy occurring during interphase, whereas a few of them had focused on mitotic autophagy. However, during our study, we had concentrated on mitotic autophagy and its contribution to mitotic progression. Therefore, our results may provide some details about the correlation between autophagy and genomic integrity, which contributes to our subsequent experiments.

2. Discussion on potential E3 ligase which may bridge mechanism by which USP24 negatively regulates autophagy during mitosis

Since we had known that USP24 down-regulation during mitosis might induce autophagy. Further, we discovered that the level of beclin-1, a pro-autophagy protein, is increased during mitosis. We hypothesized USP24 might regulate the level of autophagy by manipulating the level of beclin-1. Previously, several studies had demonstrated the correlation between some distinct ubiquitin E3 ligases

and beclin-1, and their results indicates that TRAF6, RNF216, NEDD4, HERC5, AMBRA-1-containing CUL4-ligase complex, and KLHL20-contianing CUL3-ligase complex serve as the ubiquitin E3 ligases of Beclin-1 (Boutouja *et al.*, 2017). Further, our western blot results indicated that during mitosis, the level of TRAF6, RNF216, NEDD4 are decreased. Hence, we hypothesized that the down-regulation of USP24 might induce autophagy by decreasing the level of one (or some) of these ubiquitin E3 ligases, which destabilized Beclin-1. Moreover, our co-immunoprecipitation result revealed that USP24 interacts with TRAF6 during mitosis, suggesting that USP24 might serve as the deubiquitinase of TRAF6. As a whole, we suggest that down-regulation of USP24 during mitosis might induce autophagy by destabilizing TRAF6, which used to destabilize Beclin-1. In other words, USP24 stabilizes TRAF6, subsequently destabilizing Beclin-1, causing the down-regulation of autophagy ultimately.

3. Autophagy during mitosis maintains genomic integrity and prevents the development of drug resistance in lung cancer cells *in vitro*

Previous studies have demonstrated that autophagy prevents chromosome instability and aneuploidy (Mathiassen *et al.*, 2017) and maintains nuclear and mitochondrial genomic integrity (Vessoni *et al.*, 2013). Since our results had shown that autophagy is induced and plays an indispensable role during mitosis, we then assumed that autophagy might play a role in maintaining genomic integrity during mitosis. In our immunofluorescent analysis result, we discovered that the amount of segregation defects in mitotic cells is dramatically reduced when compared to that in autophagy-inhibit-mitotic cells, suggesting that autophagy facilitates the maintenance of genomic integrity during cell division. However, the mechanism by which autophagy reduces the amount of segregation defects still remains unknown. We have proposed two possible mechanisms, one is that autophagy might prevent segregation defects from happening during mitosis, resulting in reduction of segregation defects. The other is that since autophagy is known for its degradative and recycling function, it is possible that autophagy might reduce

the amount of segregation defects by simply removing the segregation defects. In any case, it is necessary that further investigation should be done to fully understand the interaction between autophagy and segregation defects.

Since we knew that autophagy facilitates the maintenance of genomic integrity during cell division. Also, we had demonstrated that USP24 negatively regulates the level of autophagy during mitosis. Further, previous study had proven that genomic instability will contribute to the phenomenon of intratumoral genetic heterogeneity (Andor *et al.*, 2017). Moreover, previous study suggested that intratumoural genetic heterogeneity serves as a driver of chemotherapy resistance in cancer patients (Saunders *et al.*, 2012). Hence, we hypothesized that autophagy might be able to prevent the development of drug resistance, and USP24 might promote drug resistance by negatively regulating the level of autophagy. In our western blot analysis comparing the level of USP24 and LC3B between drug-sensitive A549 cells and drug-resistant A549-T24 cells, we found that down-regulation of USP24 and up-regulation of LC3B in A549-T24 cells are minor than those in A549 cells, suggesting that USP24 is up-regulated and LC3B is down-regulated in drug-resistant cells. We proposed that those cells which possess higher levels of USP24 will reduce the level of mitotic autophagy in itself, resulting in a larger amount of segregation defects, jeopardizing the genomic integrity of the cell line, ultimately leading to the development of drug resistance.

To sum up, although the mechanism which autophagy reduces segregation defects still remains unclear, it is without a doubt that mitotic autophagy reduces the amount of segregation defects, meaning that mitotic autophagy facilitates the maintenance of genomic integrity. Further, our result suggested that the higher level of USP24 results in the decrease of mitotic autophagy, causing the genome much more unstable, leading to the development of drug resistance ultimately.

肆、結論與應用

In this study, we have demonstrated that autophagy does play a critical role during mitosis. The inhibition of autophagy during mitosis can delay mitotic progression and result in increase of segregation defects, which is one of the primary causes of genomic instability, ultimately leading to drug resistance. Further, we discovered that USP24 might negatively regulate autophagy by stabilizing TRAF6, a ubiquitin E3 ligase of Beclin-1 during mitosis. Hence, we proposed that USP24 facilitates the development of drug resistance by inhibiting mitotic autophagy. Moreover, after comparing the difference between A549 and its drug-resistant cell line, A549-T24, we revealed that up-regulation of USP24 during mitosis and down-regulation of mitotic autophagy in A549-T24 might be the primary cause of its genomic instability, and further contributes to the development of its drug resistance. To sum up, our results suggest that targeting USP24 during lung cancer treatment may induce autophagy, which can improve genomic integrity and prevent the development of drug resistance (figure 5). During this study, however, there are still some problems that remain unclear, such as the mechanism by which autophagy reduces segregation defects. As a result, some more direct experimental design such as targeting USP24 by siRNA or USP24 inhibitor need to be addressed to study the molecular mechanism in-depth. Therefore, further investigation should be done in order to reveal the big picture of the role which autophagy plays during mitosis in a molecular aspect.

Figure 5:

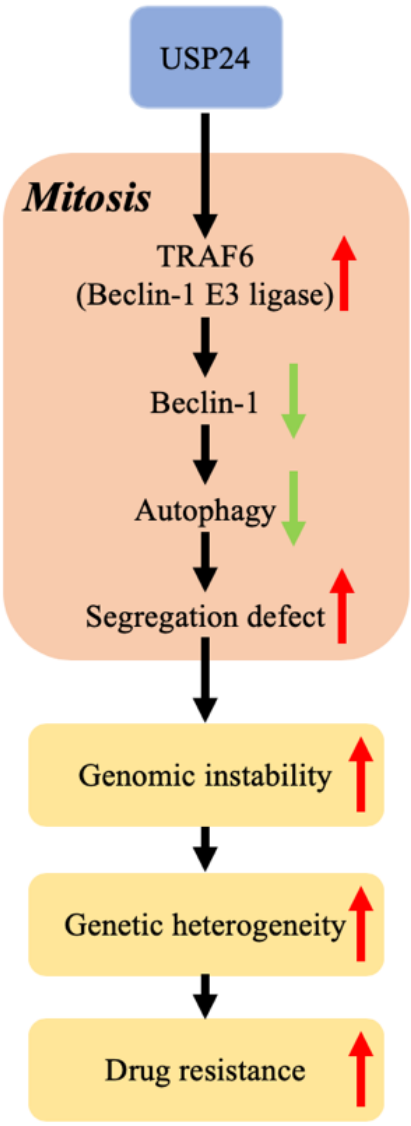


Figure 5:

The down-regulation of USP24 decreases the level of TRAF6, a ubiquitin E3 ligase of Beclin-1, hence increases the level of Beclin-1, subsequently induce autophagy, which reduces the amount of segregation defects. Therefore, USP24 induces genomic instability, resulting in genetic heterogeneity, ultimately leading to drug resistance of cancer.

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【評語】090012

報告的整體結構完整，研究動機以及背景闡述明確，研究結果描述清楚，而圖表的呈現、排版可以調整一下（有些圖過小以及不清楚。整體而言，研究內容扎實，簡報清楚，問題回答表現也相當良好，為一傑出的科展報告。此作品觀察 mitosis 進行過程中，USP24 與 TRAF6 表現減少可能參與 autophgy 的形成。然而在對化療藥物 taxol 產生抗藥性的肺癌細胞中，這些蛋白分子的變化則不明顯，顯示可能與抗藥性形成有關。USP24 經由 TRAF6 增加減少 Beclin1 表現而導致 autophagy 相關的研究不多，若能證明其與 autophagy 之間的直接關聯性，甚至為抗藥性形成的主因，USP24 或可成為新穎的治療標的，以解決臨床上癌症治療常見的化療抗性難題。