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ATP 合成酶異位表現至肺癌細胞表面之運輸途徑

The Trafficking Pathways of Ectopic ATP Synthase to Lung Cancer Cell Surface

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本論文係鄭辰彥君（R03B21015）在國立臺灣大學生命科學系、所完成之碩士學位論文，於民國一零五年六月二十九日承下列考試委員審查通過及口試及格，特此證明

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誌謝

從進入實驗室，經過碩士班的兩年，到這本論文的完成，是個充滿挑戰卻也多采多姿的旅途，許多人給了我希望和力量，我才得以走到這暫時的終點。

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中文摘要

ATP 合成酶是一種以多個次單元組合而成的蛋白複合體，可以進行 ATP 的生合成，並提供細胞生存所需的能量。粒線體是細胞中重要的 ATP 合成胞器，因此，長久以來 ATP 合成酶被認為僅在粒線體中表現；然而，一些近期的研究發現 ATP 合成酶在癌組織的表皮細胞、淋巴細胞、肝細胞，以及乳癌和肺癌細胞株的細胞膜表現，這一類 ATP 合成酶我們稱它們為「異位表達 ATP 合成酶」。這些 ATP 合成酶具有在胞外合成 ATP 的功能，我們先前的研究發現透過藥物 citreoviridin 可以抑制其 ATP 產生的活性，進而引發癌細胞的死亡，然而它們是如何被運輸至細胞表面的機制至今仍然不清楚。為了找出參與在這種運輸機制的蛋白分子，我們使用 RNA 干擾為基礎的篩選實驗，首先在抑制 25 個參與運送有關的基因表現後，以細胞 ELISA 測量細胞表面的 ATP 合成酶表現量；我們同時也以流式細胞儀和細胞免疫螢光染色方法，驗證在細胞 ELISA 的實驗中被認為涉及 ATP 合成酶運輸的蛋白，是否真的可以改變細胞膜上 ATP 合成酶的表現量。結果發現有 PARK2、MFN1 以及 COPA 等基因可能在異位表現之 ATP 合成酶組裝、運輸機制中，扮演重要的角色。

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Abstract

ATP synthase is a multimeric protein complex that catalyzes the synthesis of ATP. For a long time, animal ATP synthase was believed to be found only in mitochondria, where most cellular ATP synthesis takes place. However, in recent studies ATP synthase was also found on the extracellular surface of endothelial cells in some cancer tissues, lymphocytes, hepatocytes, proliferating cell lines, breast cancer and lung cancer cells. With the property of facing out-side the cell, this kind of ATP synthase is called ectopic ATP synthase. Our previous studies found that treating lung and breast cells with drug such as citreoviridin inhibited ectopic ATP synthase and caused the cancer cell death. However, how ATP synthase expressed on plasma membrane is still unclear. Therefore, to reveal what kind of the molecules are involved in the ectopic expression of ATP synthase, the RNA interference screening in lung cancer A549 cells was performed. Twenty-five genes were silenced, and then ectopic ATP synthase expressions were measured by cell ELISA assay. Several genes are potentially involved in ectopic ATP synthase expression. The alternations of ectopic ATP synthase expression were further confirmed by flow cytometry and immunocytochemistry. These results suggest that PARK2, MFN1 and COPA may play crucial roles in the trafficking and assembling mechanism of ectopic ATP synthase.

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Chapter 1 Introduction

1.1. ATP synthase

Human ATP synthase, which is part of mitochondrial electron transport chain (ETC), is one of the most important ATP-producing protein complex. This protein complex consists of F_1 and F_o parts; those parts can be further divided into several subunits[1-3]. Almost all subunits of ATP synthase are encoded from nucleus except two of them, subunit a and subunit A6L. These two subunits are encoded by mitochondrial DNA[4] and later assembled with other subunits which are imported into mitochondria.

ATP synthase contains two domains, F_1 and F_o; F_1 motor attaches to F_o domain which anchors in the lipid bilayer. When the proton gradient across the lipid bilayer forms, the proton can flow through the transmembrane channel of F_o domain and force subunit c oligomer rotor to rotate[5], causing the rotation of γ subunit[6], a subunit locates at the axis of ATP synthase. This rotation leads to the conformation changes of α and β subunits, catalyzing the phosphorylation of ADP[7].

The fifth member of mitochondrial electron transport chain (ETC), ATP synthase complexes, typically locate on the inner membrane of mitochondria with their F_1 part facing inside matrix. The other ETC complexes pumps proton into the inner membrane of mitochondria, causing a proton gradient across inner membrane[2]. As protons flow

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into the matrix via ATP synthase complex, the ADP is converted into ATP, providing the cell with energy for survival and biological processes.

1.2. Sorting mechanisms of proteins

1.2.1. Overview

Proteins and enzymes are usually distributed in specific subcellular locations or organelles in order to function properly. Generally, the proteins are transferred to their proper location according to their signal peptides or targeting sequences. These sorting signals guide those protein to endoplasmic system, cytosol, mitochondrion or nucleus. Based on the signal peptide or targeting sequence, there are two major types of protein sorting mechanism, cotranslational translocation and post-translational translocation.

1.2.2. Cotranslational translocation

Cotranslational translocation is the mechanism that transports proteins into endoplasmic reticulum (ER). After an mRNA is shipped out of the nucleus, a ribosome in cytosol binds to the mRNA and starts translation. If this protein is destined to ER, plasma membrane or other endoplasmic system organelles, it usually contains an ER signal peptide sequence[8]. Along with the protein translation, signal recognition particles (SRP) recognize this synthesizing peptide and guide the

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translation complex to dock on ER membrane[9]. The ribosome will continue translating the protein into the ER lumen. Before the translation is finished, the signal peptide is cleaved by peptidases[10]. These proteins are folded, modified and transported to Golgi apparatus via COPII vesicles[11].

Golgi apparatus is where the proteins are further modified before they're transported to the other endoplasmic system organelles, plasma membrane or extracellular environment through the secretory pathway[12,13]. Rab GTPase proteins and SNARE proteins work together to sort proteins to their destinations[14,15]. So far many Rab and SNARE proteins have been identified, forming a complex interaction and trafficking network in eukaryote cells[16-18].

1.2.3. Post-translational translocation

For some organelles or cytosol, proteins are transferred to proper destination after finishing their translation; this mechanism is called "post-translational translocation." Similarly, these proteins contain different types of targeting sequences. For example, mitochondrial matrix-targeting sequence (MTS) and nuclear localization signal (NLS) import proteins to mitochondria and nucleus, respectively[19,20].

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1.2.4. Protein importing into mitochondria

The proteins which are destined to mitochondria usually contain mitochondria-targeting sequence[21]. Being unfolded, these protein precursors binds to Tom20/22, receptor for targeting sequence. Tom40, a pore-shaped mitochondrial outer membrane protein, helps the proteins to be imported into outer membrane at the contact site of outer and inner membrane[22]. There are different types and combinations of mitochondria-targeting sequences on different mitochondrial proteins in order to guide each protein to specific location in mitochondrion like intermembrane space or inner membrane[23].

1.3. Ectopic expression of ATP synthase

1.3.1. Biological significance

About a decade ago, many reports showed that some or even all subunits are unexpectedly expressed on the plasma membrane[21,24-27]. ATP synthase subunit β was found on the cell surface of HepG2 hepatocellular carcinoma cells, acting as HDL receptor[27]. Also, a study of lipid raft reported that ATP synthase was found in purified lipid raft[24]. In the next few years, this extraordinary discovery has been also found in many types of human cell lines and even in rat cells by using various

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methods[21,25,26]. Thus, the ATP synthase which is expressed on the cell surface is called “ectopic ATP synthase” or “ecto-ATP synthase” in short.

In our previous studies, ecto-ATP synthase, along with other ETC complexes, is found in A549 and CL1-0 lung cancer cell lines but not in IMR-90 cell line, which is a normal-like fibroblast cell line[28]. Additionally, it is also expressed on MCF-7 breast cancer cell surface[29]. Under the treatment of ATP synthase inhibitor, the extracellular ATP concentration decreases, showing that ectopic ATP synthase are functional[28,30]. Since citreoviridin, an ATP synthase inhibitor, selectively suppresses proliferation and migration of A549 and CL1-0 lung cancer cell line, ectopic ATP synthase seems to play an important role for cancer survival[28]. Another research showed that under hypoxia or acidic environment, which is probably like the microenvironment in solid tumors, the catalytic activity of ectopic ATP synthase increases[31]. Therefore, the authors proposed that the ecto-ATP synthase produced extracellular ATP and somehow help cancer cell to tolerate these stresses[31].

1.3.2. Origin of ectopic ATP synthase

The existence of ectopic ATP synthase has been discovered for many years. However, the place where the ectopic F_1F_o-ATP synthase is assembled is still unknown. Two major hypotheses are proposed to explain the ectopic expression of

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ATP synthase. One is that the subunits of ATP synthase do not enter mitochondria; instead, they are routed to plasma membrane. The other one is that ATP synthase complex is first assembled in mitochondria and later transported from mitochondria to plasma membrane.

The first hypothesis is that those ATP synthase subunits are assembled elsewhere in cell instead of mitochondria. A study has found that α subunit of ATP synthase didn't appear on the cell surface after interrupting the ER-to-Golgi transportation in neuronal cell[32], suggesting that ecto-ATP synthase are assembled in the secretory pathway and transported to the cell surface as other plasma membrane proteins usually do. However, ATP synthase subunits contains only mitochondria-targeting sequences, so they are not likely to be guide to ER. Furthermore, a recent study suggests that mitochondria-associated membranes (MAM) may be involved in exchanging lipids and proteins between ER and mitochondrion[33,34]. Interrupting ER-to-Golgi would lead to the same result even if the ecto-ATP synthase is assembled in mitochondria before moving into ER.

On the other hand, several studies support that ectopic ATP synthase is assembled in mitochondrion. As mentioned above, subunit a and A6L of ATP synthase are encoded in mitochondria; surprisingly, these two subunits are also

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found on cell surface, along with other subunits[35]. By expressing β subunit-GFP fusion protein with endogenous and truncated targeting sequences, Ma *et al.* suggested that these ecto-ATP synthases are assembled in mitochondria. Those β subunit-GFP fusion protein with truncated targeting sequence are not detectable on the cell surface, showing that they have to be translocated into mitochondria before moving to plasma membrane[31].

Nevertheless, although these studies support that mitochondria are the place for ectopic ATP synthase assembly, how it moves out of mitochondria is still unclear. Typically, mitochondria do import proteins but do not export them. Also, in the case of cytochrome b, a mitochondria-encoding protein, leakage of mRNA may let those mitochondrial protein be translated in cytosol[36], leading to a possibility that ATP synthase subunit a and A6L are translated in cytosol.

1.3.3. Transportation of ectopic ATP synthase

While the exact origin is still under debate, the transportation vehicle of ectopic ATP synthase to plasma membrane is another considerable issue. There is no doubt that the secretory pathway utilizes vesicles, especially clathrin-coated vesicles, to sort protein cargos to specific locations[37]. Although mitochondria do not release vesicles in traditional view, they are dynamic; mitochondria have the abilities of

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fusion and fission which are controlled by several proteins such as Drp1 and MFN1/2[38-40]. Recently, some studies showed that mitochondria release vesicles, which may be responsive for the quality control of mitochondria[41,42]. Those vesicles are named as “mitochondria-derived vesicles (MDV)”, which can be classified into four different types. The destinations of those MDVs are not fully discovered, yet some of them are probably sent to peroxisomes and lysosomes[43-45]. However, even if the vesicles could carry assembled ATP synthase from inner membrane to plasma membrane, there is a defect in this explanation. The F_1 domain of ATP synthase complex faces toward the mitochondrial matrix, meaning that it would face toward the cytosol instead of extracellular environment after being transported to the cell surface; it doesn’t match with the previous evidences that those ATP synthases produce ATP outward.

Alternatively, there is another hypothesis suggests that the inner membrane of mitochondria fuses to the cell surface and consequently redistributes all the inner membrane proteins. This hypothesis is supported by two evidences. First, the ectopic ATP synthases seemed to be patchy on the cell surface instead of random or uniform distribution[27]. Second, not only ATP synthase but also ETC complexes are detected on plasma membrane[28]. It might be caused by abnormal fission of mitochondria or

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incomplete mitochondria degradation. Some would consider about the outer membrane of mitochondria, which is a barrier for the fusion of inner membrane and plasma membrane. Indeed, inner membrane has little chance to directly contact with plasma membrane unless the outer membrane is degraded or penetrated. Since the mitochondria-associated membranes (MAM) and MDVs were found[34,41], the hypothesis that ETC was transferred to plasma membrane together might still stand a chance.

1.4. Identification of the trafficking pathway of ectopic ATP synthase

In order to discover the origin and the route of ecto-ATP synthase, here we propose a novel approach shown in Figure 1. Due to the complexity of trafficking pathways, a siRNA-based screening was conducted instead of pharmaceutical inhibition of each pathway. Several genes are selected to be silenced, including Rab proteins, vesicle coat proteins, mitochondria dynamic-related and mitophagy-related proteins. Those targets and their roles in the cellular trafficking system are listed in Table 1 and shown in Figure 2. After silencing these genes, ectopic ATP synthase expression level is measured. An ELISA-based screening can be utilized to observe the expression change of ectopic ATP synthase. Comparing with pharmaceutical inhibiting

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these pathways individually, this approach takes less time to find out the involved candidate proteins.

Furthermore, three approaches are used to validate whether these candidate proteins are responsible for controlling the trafficking of ectopic ATP synthase. Pattern of ecto-ATP synthase expression can be observed by using immunocytochemistry staining. The intensity of fluorescence immunostaining can be quantified by flow cytometry. In summary, interrupting trafficking pathways with an RNA interference technique could help us to propose a comprehensive model of the ectopic ATP synthase transportation.

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Chapter 2 Materials and Methods

2.1. Cell culture

A549 and H1975 lung cancer cell lines as well as IMR-90 human fibroblast were obtained from ATCC (USA). The frozen cells were thawed by in 37°C water-bath and seeded into a 10-cm petri dish filled with 10 mL DMEM (Thermo Fisher Scientific, USA) + 10% FBS (Thermo Fisher Scientific, USA). For IMR-90 cells, 1× glutamine and 1× non-essential amino acid solution (Thermo Fisher Scientific, USA) were added. After 24 hours of incubation in a 37°C incubator with 5% CO_2, the medium was refreshed in order to remove DMSO. When the cell culture reached 80-90% confluency, cells were detached with trypsin/EDTA solution (Thermo Fisher Scientific, USA) and transferred to new petri dishes for further incubation.

2.2. Short interfering RNA (siRNA) transfection

The siRNAs were obtained from GE Healthcare Dharmacon, UK. A siRNA was mixed with Lipofectamine 3000 reagent (Thermo Fisher Scientific, USA), then this liposome solution was mixed with serum-free DMEM. The siRNA-containing medium was added into petri dishes or cluster plates and removed 24 hours to reduce the toxicity.

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2.3. RNA extraction and cDNA preparation from cell culture

First, the cells were washed with 37°C D-PBS twice to remove the medium and any floating cells. Then harvest the cells were added with TRIzol reagent (Thermo Fisher Scientific, USA) and pipetted several times. Second, following the instruction, the RNA extraction was performed with Direct-zol RNA MiniPrep (Zymo Research, USA). In brief, the homogenized sample was mixed with 100% ethanol by vortexing. After the membrane-binding step, DNase I digestion was performed in the column. The column was later washed by PreWash and Wash Buffer (Zymo Research, USA). Elution of RNA with RNase-free water was the last step of RNA extraction.

In order to convert the extracted RNA to cDNA, RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used. 250-1000 ng RNA was mixed with oligo dT primer and other reagents and heated to 65°C for 5 minutes. After annealing, the mixture was heated to 42°C for 60 minutes, followed by 5 minutes of 70°C enzyme inactivation. The cDNA product was stored at -20°C.

2.4. Real-time polymerase chain reaction (RT-PCR)

To quantify specific mRNA expression, 2.5 ng cDNA was mixed with gene-specific primers (Table 2) and iQ SYBR Green Supermix (Bio-Rad, USA). Thermal cycles and detection were conducted by CFX Connect system (Bio-Rad, USA). First,

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the sample-SYBR green mixture was heated up to 95°C for 3 minutes to activate the DNA synthase. Second, the temperature of the mixture was set to be 95°C for 10 seconds and 60°C for 30 seconds, repeatedly. Meanwhile, the fluorescence intensity of each well was measured. After totally 40 cycles, the temperature of the mixture was slowly increased from 65°C to 95°C for melt curve analysis.

2.5. Cell enzyme-linked immunosorbent assay (CELISA)

As many reports have described[46-51], cells were first fixed by 3.7% paraformaldehyde (Sigma-Aldrich, USA) in the well without permeabilization of plasma membrane. 5% skimmed milk in PBS was used as the blocking solution. Those cells were hybridized with mouse monoclonal ATP synthase complex antibody (1:4000) (Abcam, USA; ab109867) at 4°C overnight or at room temperature for 2 hours. After washing 5 times, the cells were further hybridized with HRP-conjugated goat anti-mouse IgG secondary antibody (1:5000) (Abcam, USA; ab97023). After another 5 times of washing, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich, USA), a chromogenic substrate, was added into each well by 100 μL to be developed into blue color. To stop the reaction, 2N HCl (Aencore Chemical, USA) was added into each well, causing the color to change into yellow. At last, the plate was read by an ELISA

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reader (Bio-Rad, USA) at 450 nm. In addition, 655 nm was used as reference wavelength.

2.6. Crystal violet cell viability assay

As it was develop in late 1980s[52], the cells were washed twice and fixed by 100% methanol (Echo Chemical, ROC) and air-dried. The cells were stained with 100 μL 0.5% crystal violet (Sigma-Aldrich, USA) solution for 30 minutes at room temperature. After vigorously washing, the 96-well plate was air-dried again. The crystal violet left in the wells was dissolved with 100 μL 10% acetic acid (*J. T. Baker, USA)*. Finally, the plate was read by an ELISA reader at 595 nm.

2.7. Fluorescence immunocytochemistry (ICC)

Before the cells were seeded, a round-shaped coverslip was sterilized by heat and UV exposure to be put into the well of 12-well or 24-well cluster plate. Then the cells were seeded into the wells with coverslips as well as attached and proliferated on the coverslips.

Before the cell were fixed, MitoTracker (Thermo Fisher Scientific, USA), a mitochondria-staining reagent was added into the cell culture for 40 minutes. Cells were fixed with 3.7% paraformaldehyde after washing twice by D-PBS. If permeabilization of cell membrane was desired, the cells were treated with 0.25%

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Triton X-100 (Sigma-Aldrich, USA) for 10-15 minutes at room temperature. Next, the cells were blocked with 10% bovine serum albumin (BSA) in D-PBS for 2 hours. Similarly, those cells were hybridized with antibodies against ATP synthase complex (1:500-2000) (Abcam, USA) at 4°C for 16 hours (overnight). After the cells were washed three times, Alexa488-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, USA; A-11001) was used as the secondary antibody, which can glow under exposure of light with specific wavelength, for an hour. When the hybridization was finished, the coverslips were washed three times. Last of all, the coverslip were mounted onto glass slides with DAPI-containing mounting medium (Thermo Fisher Scientific, USA).

2.8. Flow cytometry

First, cells were detached with 1 mM EDTA (*J. T. Baker, USA*) and washed three times. Between each wash step, cells were centrifuged 300×g for 5 min under 4°C. Second, those cells were fixed with 1 ml 3.7% paraformaldehyde/PBS at 37°C water bath for 10 min. Third, the primary antibody, mouse anti-ATP synthase complex (1:500 in 10% BSA/PBS), was used to hybridize with ectopic ATP synthase. Meanwhile, the isotype IgG was added into another tube at the same conditions. After 16-18 hours under 4°C, the cells were further hybridized with Alexa488-conjugated goat anti-

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mouse IgG (1:1000 in 10% BSA/PBS) at 4°C for an hour. Last, those cell were washed and suspended with 500-1000 μL PBS. For the flow cytometry operation, BD FACSCanto II (BD Biosciences, USA) was used. Data from channel FSC, SSC, FITC were recorded.

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Chapter 3 Results

3.1. Ectopic ATP synthase expression of A549 lung cancer cell line.

First of all, the ectopic ATP synthase expression in A549 cell line was visualized by ICC. Figure 3 shows that ATP synthase complexes (green) were expressed mostly in mitochondria, which were stained by MitoTracker (red), in permeabilized cell. Colocalization of ATP synthases and mitochondria (yellow) was not observed in cells that were not permeabilized, showing these spots and circles of green signal were located on the cell surface.

3.2. To monitor the ectopic ATP synthase expression and cell viability by CELISA and crystal violate assay, respectively.

In order to find the optimized condition for CELISA, three dilution rates were used to hybridize with anti-ATP synthase primary antibody at fixed concentration. Meanwhile, IMR-90 cells with the same number, which express no ectopic ATP synthase, were also cultured and stained. Figure 4A shows that when the secondary antibody was diluted 1:5000, higher absorbance values were observed in both ectopic ATP synthase-presenting cell lines, A549 and H1975 cells; in contrast, lower absorbance was observed in IMR-90 cells.

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On the other hand, 1% BSA/PBS blocking solution were later changed to 5% milk/PBS due to the better blocking efficiency of 5% skimmed milk/PBS (see Figure 4B). The absorbance values of wells lacking of primary antibody were largely decreased. However, there was only little decrease in the signal of complete antibody hybridization, showing that 5% milk/PBS was a suitable blocking reagent for CELISA

Because the mitochondria activity may be affected due to the suppression of mitochondria proteins, MTT, MTS and other electron transport chain-dependent cell proliferation assays are not suitable. For the cell viability, measuring amount of DNA is a good approach. Similar to colony formation assay, the cells can be stained with crystal violet and dissolved in 10% acetic acid. By measuring the absorbance of acetic acid-crystal violet solution, cell viability can be evaluated. This method was verified by seeding known numbers of cells. Standard curve shown in Figure 5 suggested that the crystal violet assay was able to measure A549 cell number from 2500 to 40000 cells in a well.

3.3. Genes of interest were silenced by RNA interference.

The siRNAs were used to inhibit the protein expression by transfecting them into A549 cells. Besides, a siRNA, non-targeting control siRNA (siNTC) was used as the control of the following experiments. SiPPIB was used as positive control of RNA

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interference. After the siRNA transfection for 24 hours, the total RNA of these cells were extracted and retro-transcribed into cDNA. With the primers listed in Table 2, the expression of the targeted genes were measured by real-time PCR. The results is demonstrated in Figure 6. Clearly, all of these genes were successfully suppressed by transfected siRNAs at mRNA level.

3.4. Screening of genes involved in ectopic ATP synthase expression by RNA interference.

CELISA and crystal violet assay were able to measure the ectopic ATP synthase expression and evaluate cell viability, respectively; furthermore, these siRNAs have the suppression efficiency. Therefore, we proceed the screening step. A549 cells were seeded in 96-well cluster plates and transfected with siRNAs. Each group of wells were transfected with one siRNA pool targeting to a single gene. After transfection for 48 and 72 hours, the absorbance of colorized TMB and acetic acid-crystal violet solution were measured by the ELISA reader (see Figure 7 and Figure 8).

Knockdown of these genes may affect the viability/proliferation of cells; this will leads to false judgements on expression changes. For example, inhibiting the expression of COPA and RAB33A gene caused decline in cell viabilities. Meanwhile, the siRAB33A group showed decreased ectopic ATP synthase expression. But this

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decreased expression may be caused by the decline in cell viability. In contrast, the ecto-ATP synthase expression in siCOPA group didn't change much, but due to the decline in cell number, the expression per cell is actually increased. In order to avoid false evaluation of expression changes, the raw absorbance values from CELISA were normalized with those from crystal violet assay. Furthermore, all the normalized expression levels were shown relatively to the siNTC group.

In the 48 hours-post-transfection experiment, silencing genes such as PARK2, SNAP25 decreased the expression of ecto-ATP synthase yet the suppression of COPA and DNM1L increased it. On the other hand, the 72 hours-post-transfection experiment shows that silencing RAB32, MFN1 and RAB6A decreased the expression of ecto-ATP synthase. Similarly, silencing COPA still up-regulated ectopic ATP synthase expression.

3.5. Eleven genes were further checked if they affected ecto-ATP synthase expression.

To narrow down the candidate proteins to be validated, eleven genes, including RAB6A, RAB32, RAB33A, CLTB, CLTC, SNAP23, SNAP25, MFN1, PARK2, VAMP7 and COPA, were selected to perform silencing experiments again. According to Figure 9, mRNA of most of these genes kept being inhibited after transfection for 72 hours, except RAB33A and PARK2.

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In this experiment, only PARK2 showed to be involved in transporting ectopic ATP synthase (see Figure 10 and Figure 11). Silencing COPA still resulted in up-regulation of ectopic ATP synthase after 72 hours.

3.6. Immunocytochemistry (ICC) shows that ectopic ATP synthase expression altered after silencing PARK2, MFN1 and COPA.

A549 cells were transfected with siPARK2, siMFN1 and siCOPA due to their possibilities of taking part in ectopic ATP synthase trafficking pathway. After 48 and 72 hours, these transfected cells were fixed and stained with antibodies. Knockdown of COPA gene increased ectopic ATP synthase expression as shown in Figure 13. Silencing PARK2 for 48 hours decreased ectopic ATP synthase expression (Figure 12), suggesting PARK2 may be involved in ectopic ATP synthase trafficking pathway. Surprisingly, MFN1, which didn't altered ectopic ATP synthase expression later in the second round of screening, changed the distribution of ectopic ATP synthase. As shown in Figure 3, the ectopic ATP synthase expressed on the surface in patches; however, the siMFN1 group cells expressed ectopic ATP synthase evenly on the cell surface as shown in Figure 12.

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3.7. Flow cytometry confirmed that the RNAi changed ectopic ATP synthase expression.

Due to the decent ability of quantification of cell surface protein, flow cytometry technique was used to measure ectopic ATP synthase expression. Based on the result of ICC, A549 cells were collected and hybridized with ATP synthase complex antibody for 48 hours after they were transfected with siPARK2 and MFN1; on the other hand, a group of cells were transfected with siCOPA and collected after 72 hours. According to Figure 14, suppressing PARK2 and MFN1 caused the main peak of shifted toward the lower expression, meaning that the overall expression of ectopic ATP synthase in those cell populations was decreased. Also, similar with the previous results, knockdown of COPA gene increased the ectopic ATP synthase expression in A549 cells. These results have confirmed that the PARK2, MFN1 or COPA suppression leads to the expression change of the ectopic ATP synthase.

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Chapter 4 Discussion

At the beginning, inhibition of trafficking pathways were expected to decrease ectopic ATP synthase expression. However, after silencing those genes, up-regulation was found surprisingly, especially in the siCOPA group. Coatomer subunit alpha, which is encoded from COPA gene, is one of the component of COPI vesicle, sending cargos from Golgi apparatus to endoplasmic reticulum[53,54]. This retrograde transport transfers ER-resident proteins and protein participating anterograde transport, which sends proteins from ER to Golgi apparatus. Due to its reverse direction from sending cargos toward plasma membrane, COPI vesicles was likely to be involved in a "recycling" pathway of ectopic ATP synthase. However, the detail of this pathway stays unclear.

Mitofusin-1 and mitofusin-2 are important molecules to mitochondria fusion[40,55]. The siMFN1 might disturb the process of fusion, therefore change the pattern of ATP synthase on cell surface. Under the assumption of that the inner membrane fuses with plasma membrane, stopping mitochondria fusion would probably make ecto-ATP synthase "diffused." This result also supports the idea that the ecto-ATP synthase assembly occurs in mitochondria instead of secretory pathway.

Finally, a mitophagy-related gene, PARK2, was suggested to be involved in the ectopic ATP synthase trafficking pathway. Encoded from PARK2 gene, parkin acts as an

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E3 ubiquitin ligase in the process of mitophagy. Not only initiating mitophagy with PINK1 protein (as it is shown in the appendix) but it was also found involving in mitochondria-derived vesicles formation[42]. This finding suggested that ectopic ATP synthase was assembled in mitochondria. Furthermore, it also supported that the inner membrane moved to fuse with plasma membrane, either by MDV formation or "escaping" from the mitochondria-degrading mechanism.

Nevertheless, silencing PINK1 was not shown to affect ectopic ATP synthase expression as siPARK2 did (see Figure 7 and Figure 8). In addition, the results from real-time PCR and CELISA suggested that the siPARK2 was only effective within 48 hours post-transfection and decreased ectopic ATP synthase expression in 48 hours-RNAi group. These data gave us the hint that silencing PARK2 may not sufficient to completely stop transporting ATP synthase from mitochondria to plasma membrane. Ectopic ATP synthase might be also transported by other pathways that is not covered by the initial screening.

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Chapter 5 Conclusions

Base on the results, we proposed a hypothetical explanation about the expression of ectopic ATP synthase (Figure 15). First, the MFN1 suppression changes the expression pattern of ectopic ATP synthase. This remarks that the ATP synthase subunits should be assembled inside the mitochondria instead of secretory pathway. Second, PARK2, which is responsible for the mitochondria degradation, may be involved in the transportation of ATP synthase complex toward cell plasma membrane. However, whether the assembled ATP synthase complex go through the autophagosome or mitochondria-derived vesicles is still unclear. Finally, silencing COPA gene surprisingly caused the incline of ectopic ATP synthase expression. Although it hardly helps us to understand the expression mechanism of ectopic ATP synthase, COPI-vesicles might play roles of recycling these ectopic ATP synthase.

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Chapter 6 Figures

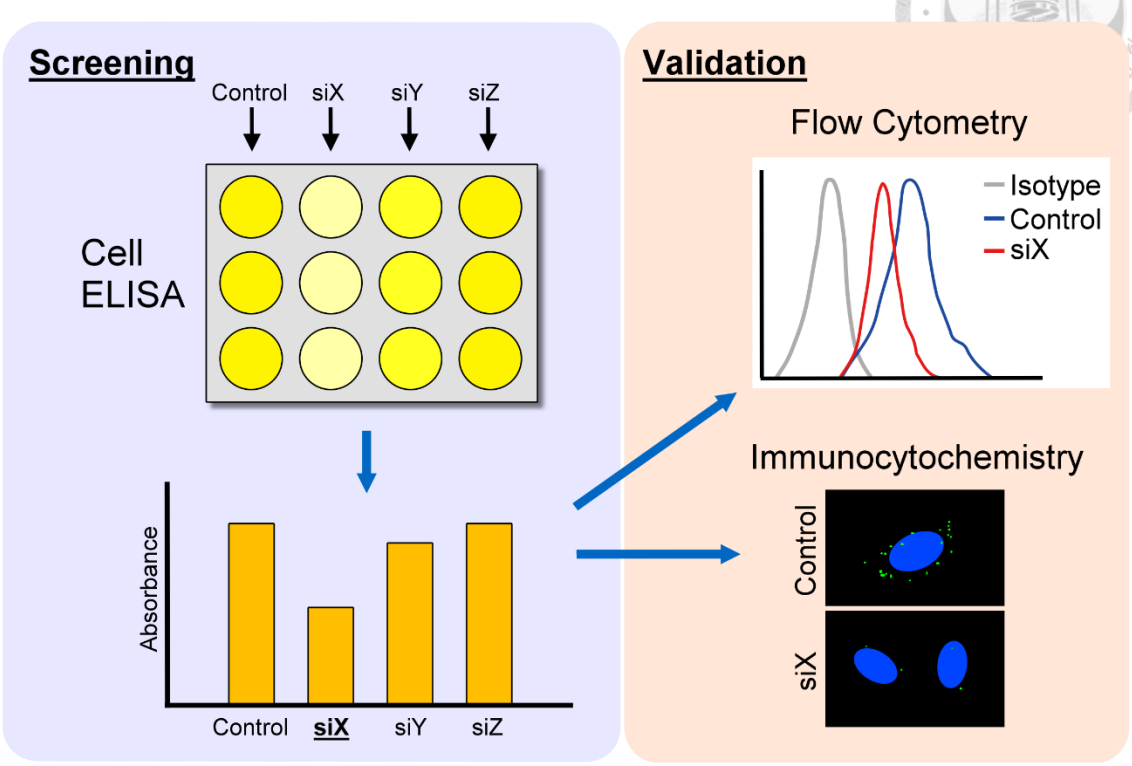

Figure 1. Experimental design.

The experimental process consists of screening and validation parts. Two approaches were used to confirm the result of screening.

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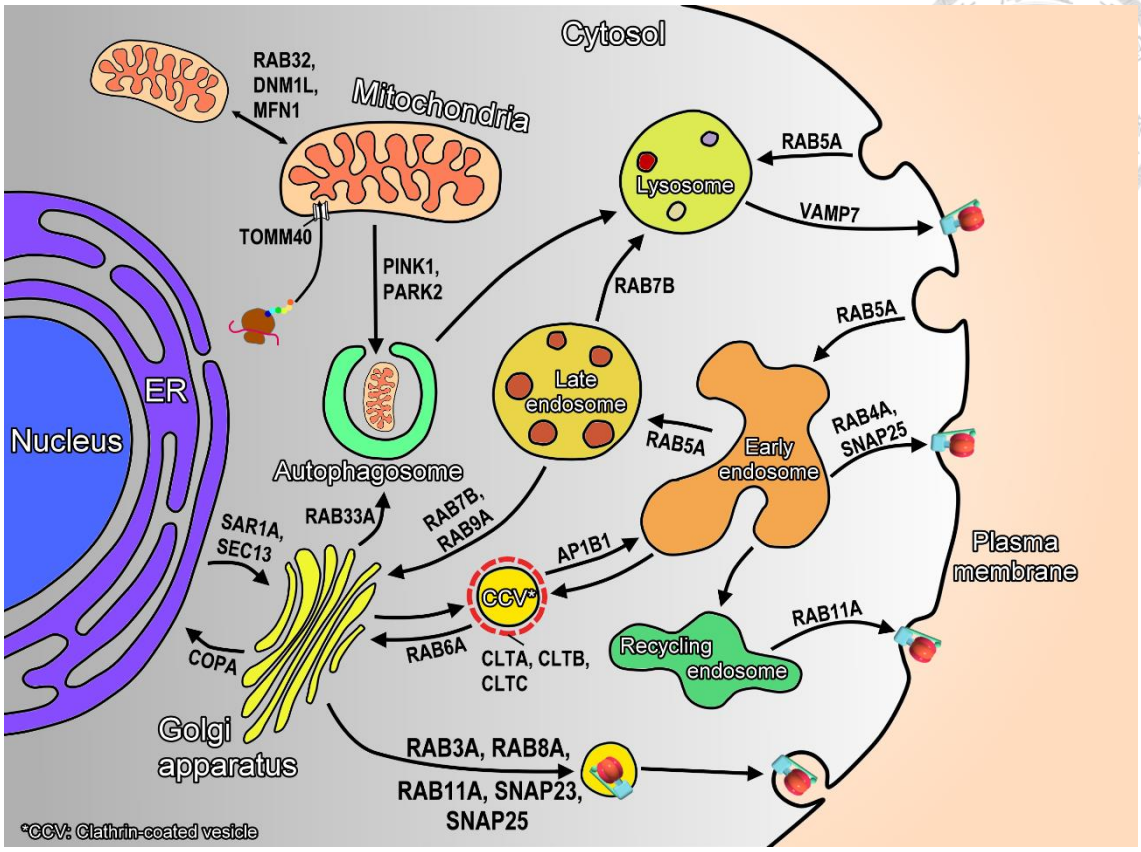

Figure 2. Focused pathways and the genes involved.

The arrows indicates directions of transportation. All these annotated genes are listed in the Table 1 with their protein names and abbreviates. Briefly, the Tom40 serves as a tunnel for peptide importation into mitochondria. Rab-32, Mfn1, Drp1 are responsible for mitochondria dynamics. Pink1 and parkin cooperates in mitochondria degradation. Components of COPI and COPII vesicles are Sar1a, Sec13 and α-COP. The other molecules are involved in vesicle trafficking system through the endomembrane system. The protein products of gene CLTA, CLTB and CLTC form the clathrin-coated vesicles.

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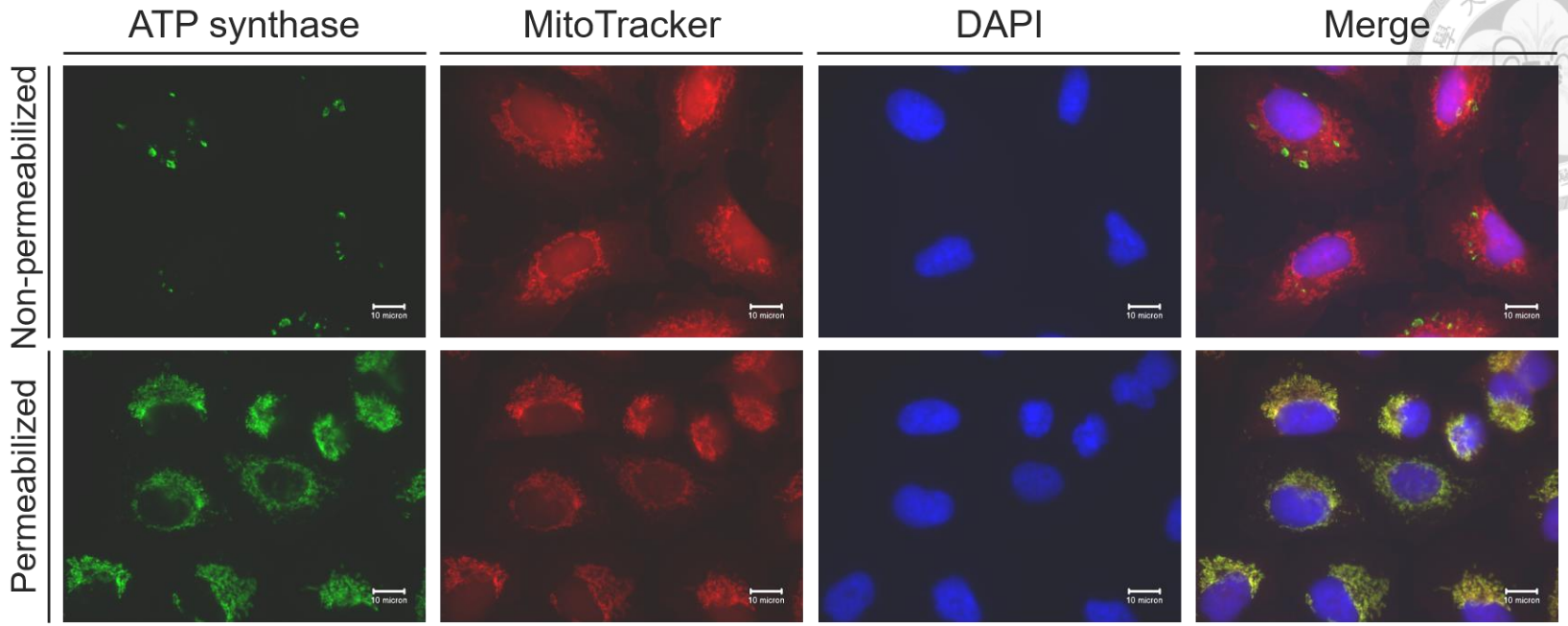

Figure 3. Immunostaining of ectopic ATP synthase in A549 cell line.

Both non-permeabilized and permeabilized A549 cells and stained with ICC technique. Yellow color indicates the colocalization of ATP synthase and mitochondria. An oil lens (100×) was used. Scale bar = 10 μm.

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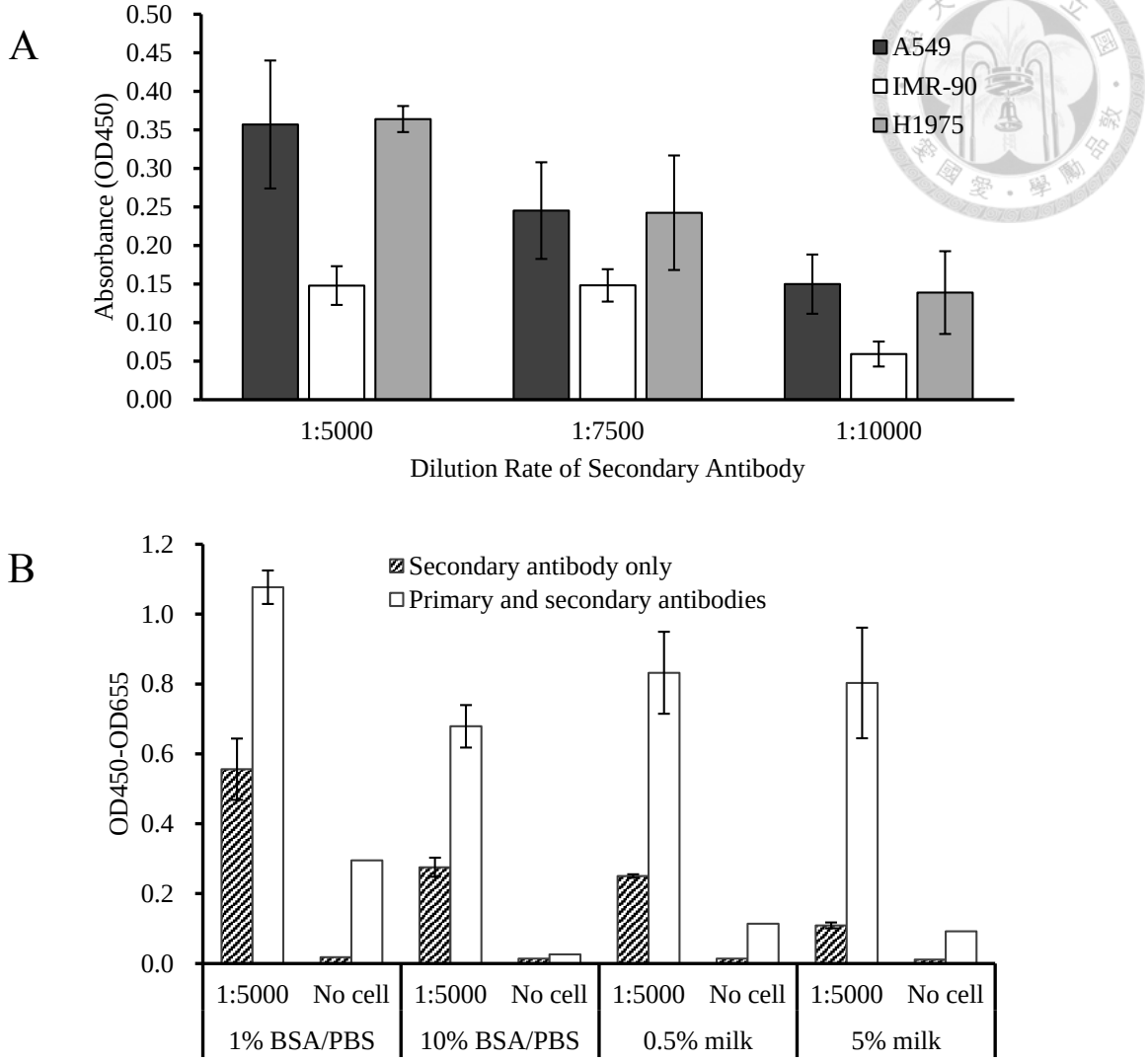

Figure 4. Preliminary test of CELISA.

(A) Ectopic ATP synthase expression of A549 and IMR-90 under the detection of CELISA with three dilution rates. (Mean ± SD)

(B) CELISA experiment results by using different blocking buffer on A549 cell line. "1:5000" means the wells that were seeded with A549 cells and used HRP-conjugated goat anti-mouse IgG secondary antibody at 1:5000 dilution for hybridization. (Mean ± SD)

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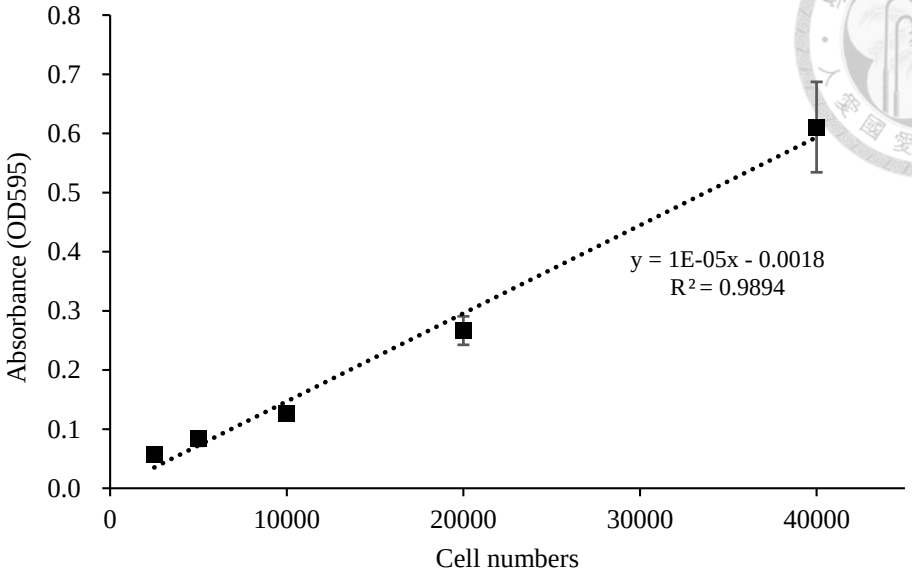

Figure 5. Standard curve of crystal violet assay.

Certain numbers of cells were used to determine a standard curve of crystal violet assay.

(Mean ± SD)

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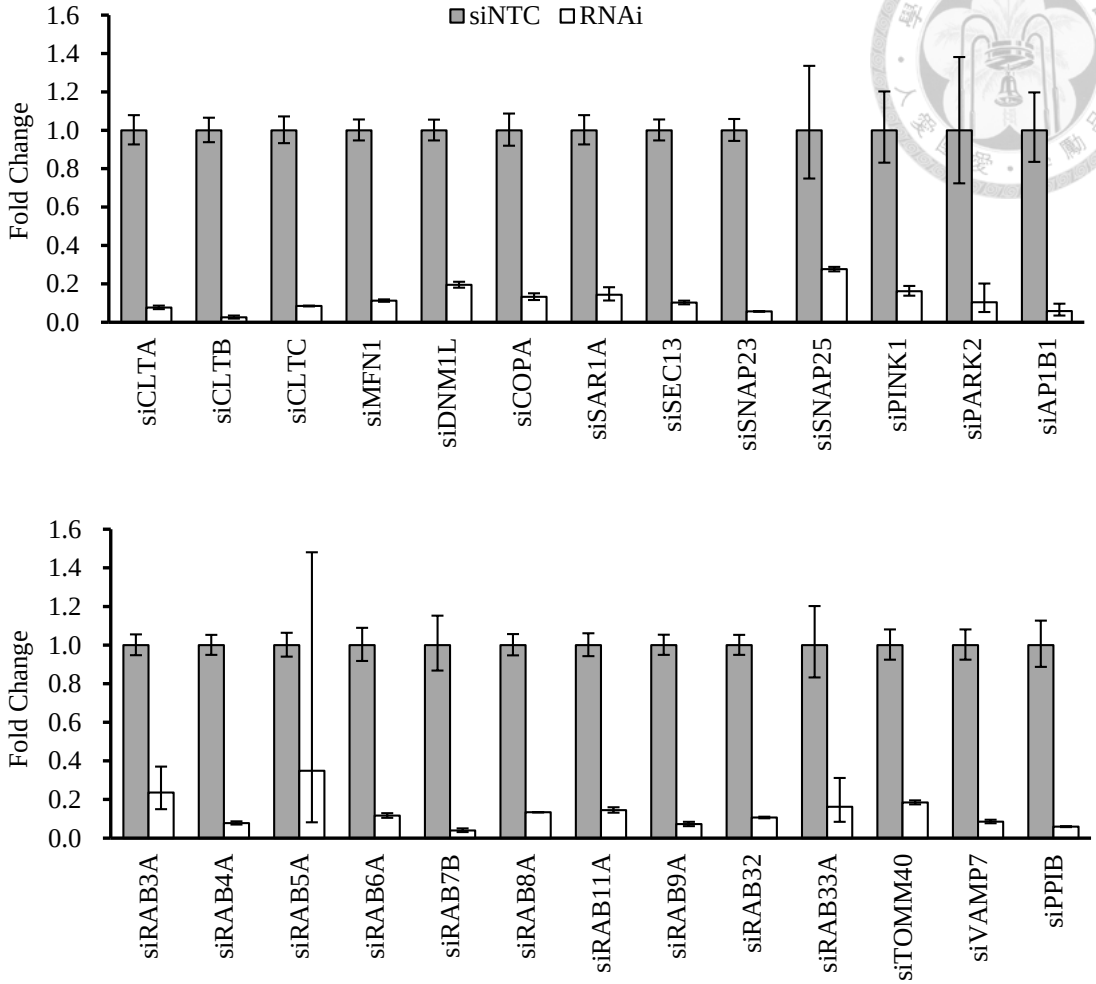

Figure 6. Target genes were silenced by RNA interference of 25 genes which have potentially involved in ATP synthase trafficking.

The RNAi efficiency after transfection for 24 hours were validated by real-time PCR. All the mRNA expression were normalized with non-targeting control siRNA (siNTC). (Mean ± SD)

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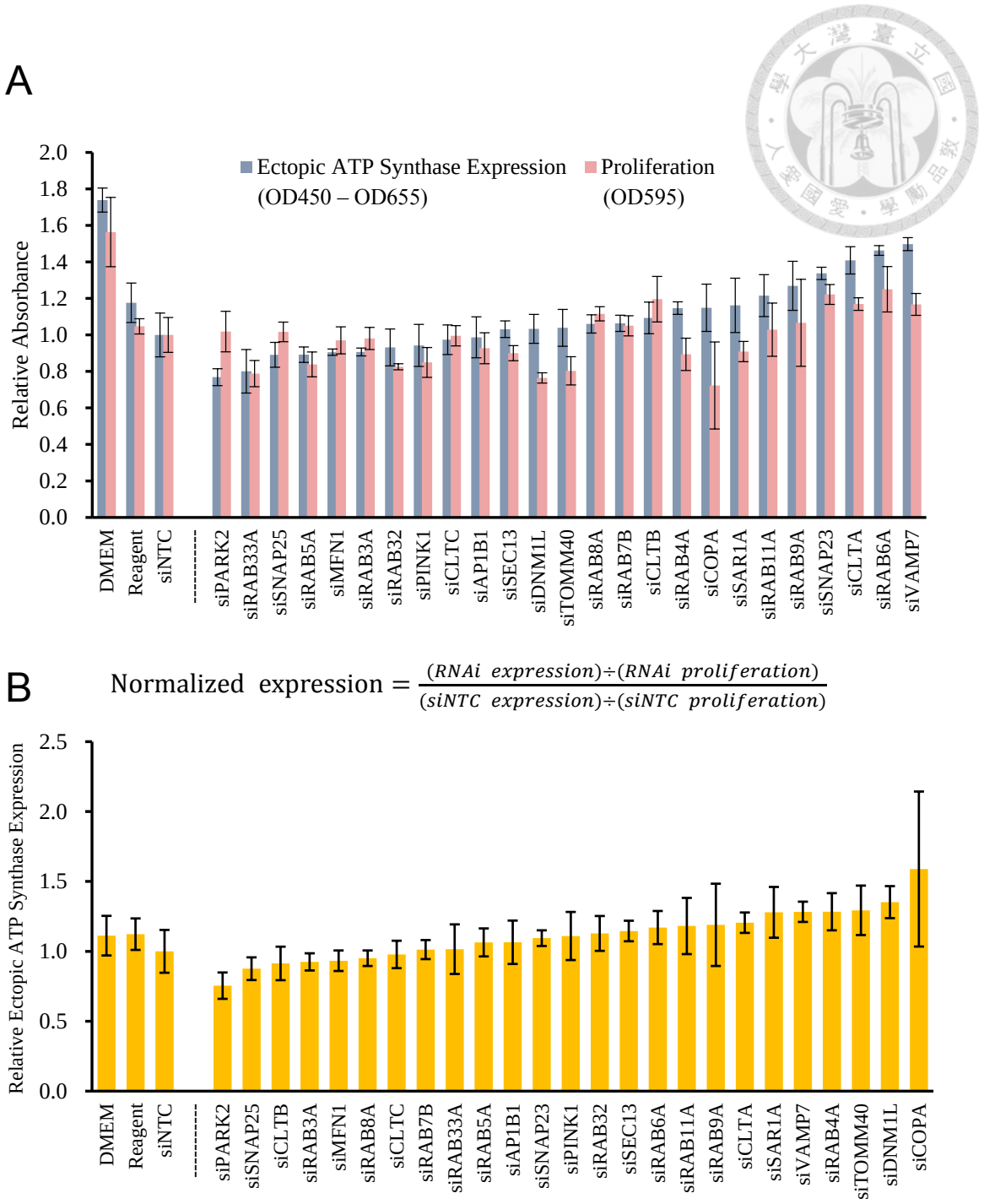

Figure 7. Ectopic ATP synthase expression after transfection with various RNA interferences for 48 hours.

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(A) A549 cells were transfected with siRNAs after 48 hours, the expression level of ectopic ATP synthase (blue) and the cell viability (red) were measured with CELISA and crystal violet assay, respectively.

(B) To calculate the ATP synthase expression level for each cell, we normalized CELISA values with crystal violet absorbance values; the result is shown as yellow bars. DMEM, reagent, siNTC indicates medium only, transfection reagent (without any siRNA) and non-targeting control siRNA, respectively. (Mean±SD)

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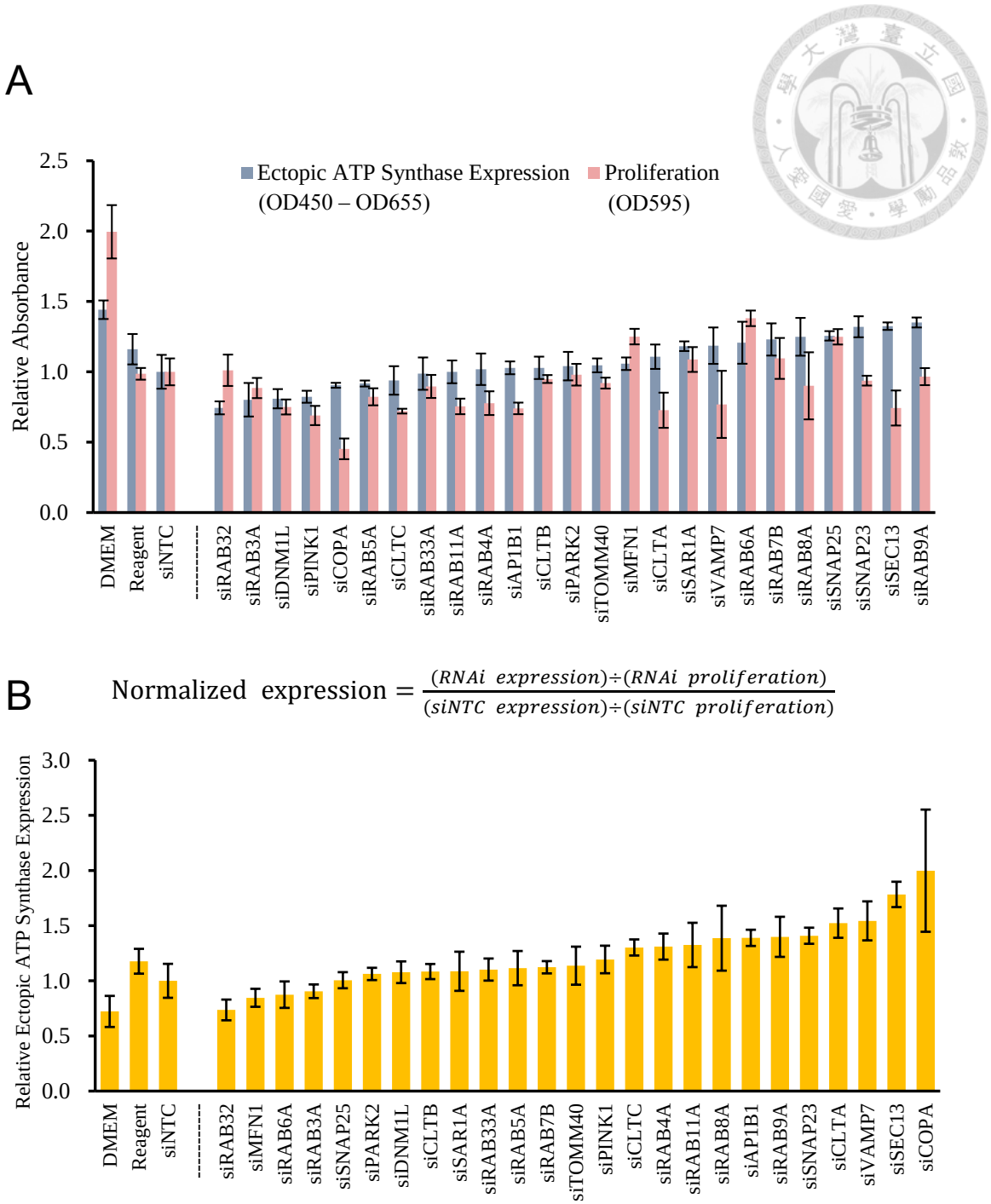

Figure 8. Ectopic ATP synthase expression after transfection with various RNA interferences for 72 hours.

doi:10.6342/NTU201601624

(A) A549 cells were transfected with siRNAs after 72 hours, the expression level of ectopic ATP synthase (blue) and the cell viability (red) were measured with CELISA and crystal violet assay, respectively.

(B) To calculate the ATP synthase expression level for each cell, we normalized CELISA values with crystal violet absorbance values; the result is shown as yellow bars. DMEM, reagent, siNTC indicates medium only, transfection reagent (without any siRNA) and non-targeting control siRNA, respectively. (Mean ± SD)

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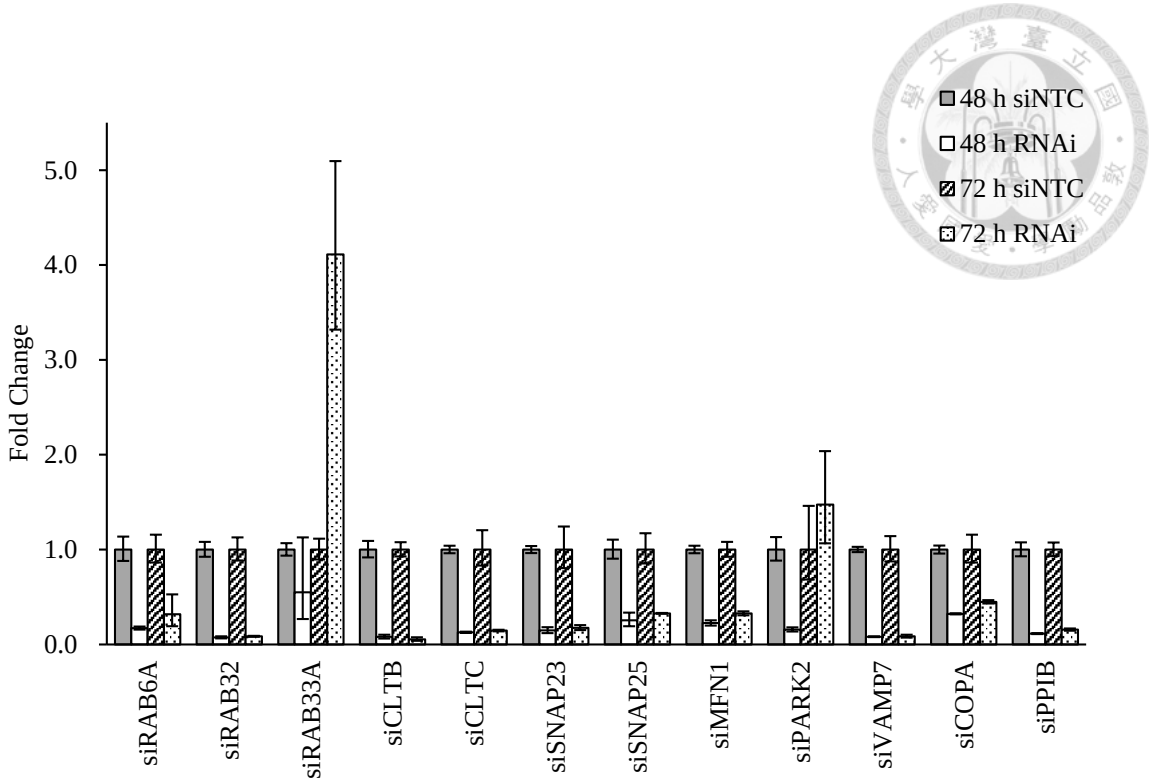

Figure 9. Eleven candidate genes were silenced again to verify the RNAi screening results. The 48 and 72 hours-RNAi efficiency were validated by real-time PCR. All the mRNA expression for each gene was normalized with non-targeting control siRNA (siNTC). (Mean ± SD)

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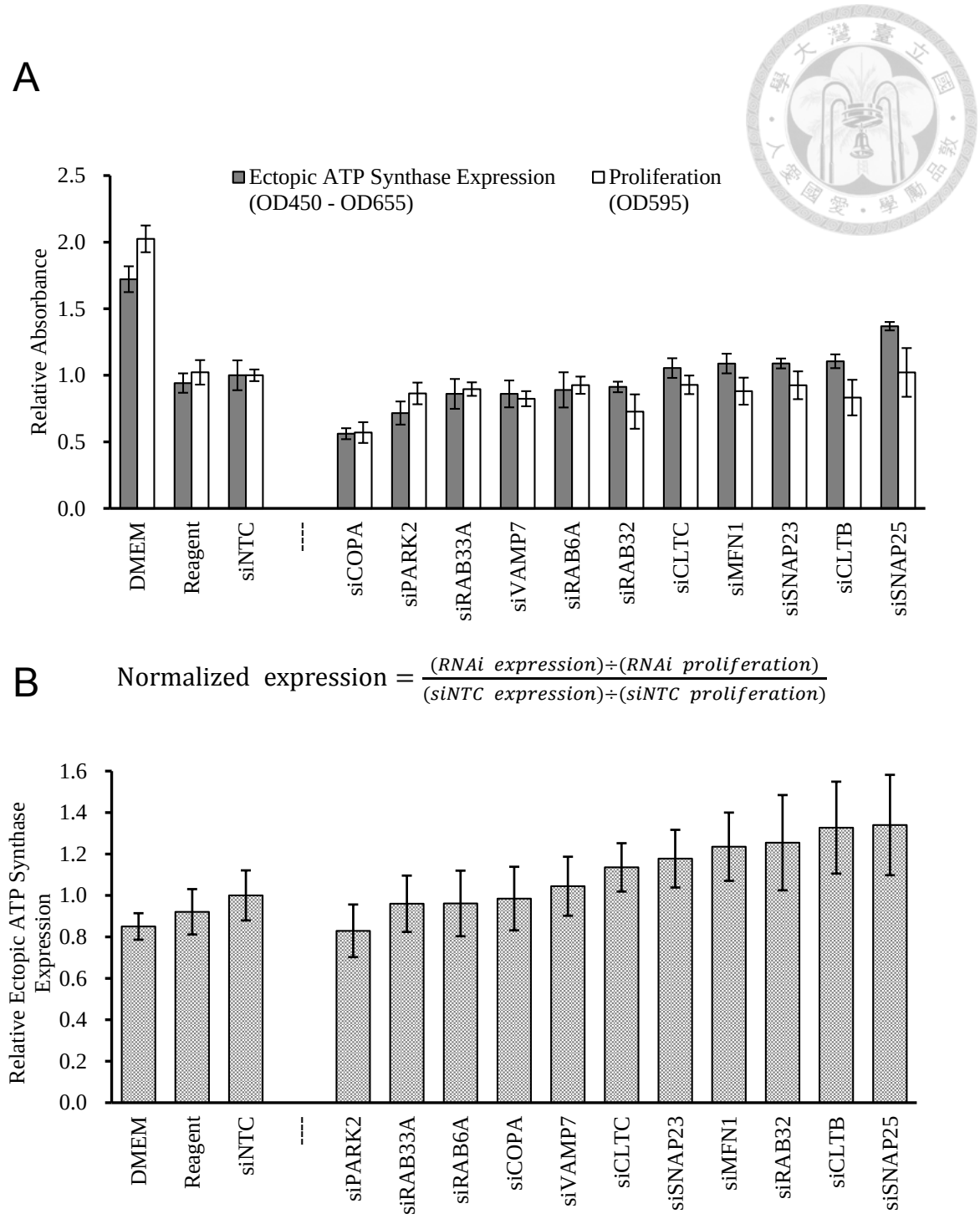

Figure 10. Ectopic ATP synthase expression after eleven candidate genes were silenced for 48 hours.

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(A) 48 hours post-transfection of siRNAs, the raw expression level of ectopic ATP synthase (gray) and the cell viability (white) was measured with CELISA and crystal violet assay, respectively.

(B) The raw expression was normalized with cell viability; the result of normalization is shown as bars. DMEM, reagent, siNTC indicates medium only, transfection reagent (without any siRNA) and non-targeting control siRNA, respectively. (Mean ± SD)

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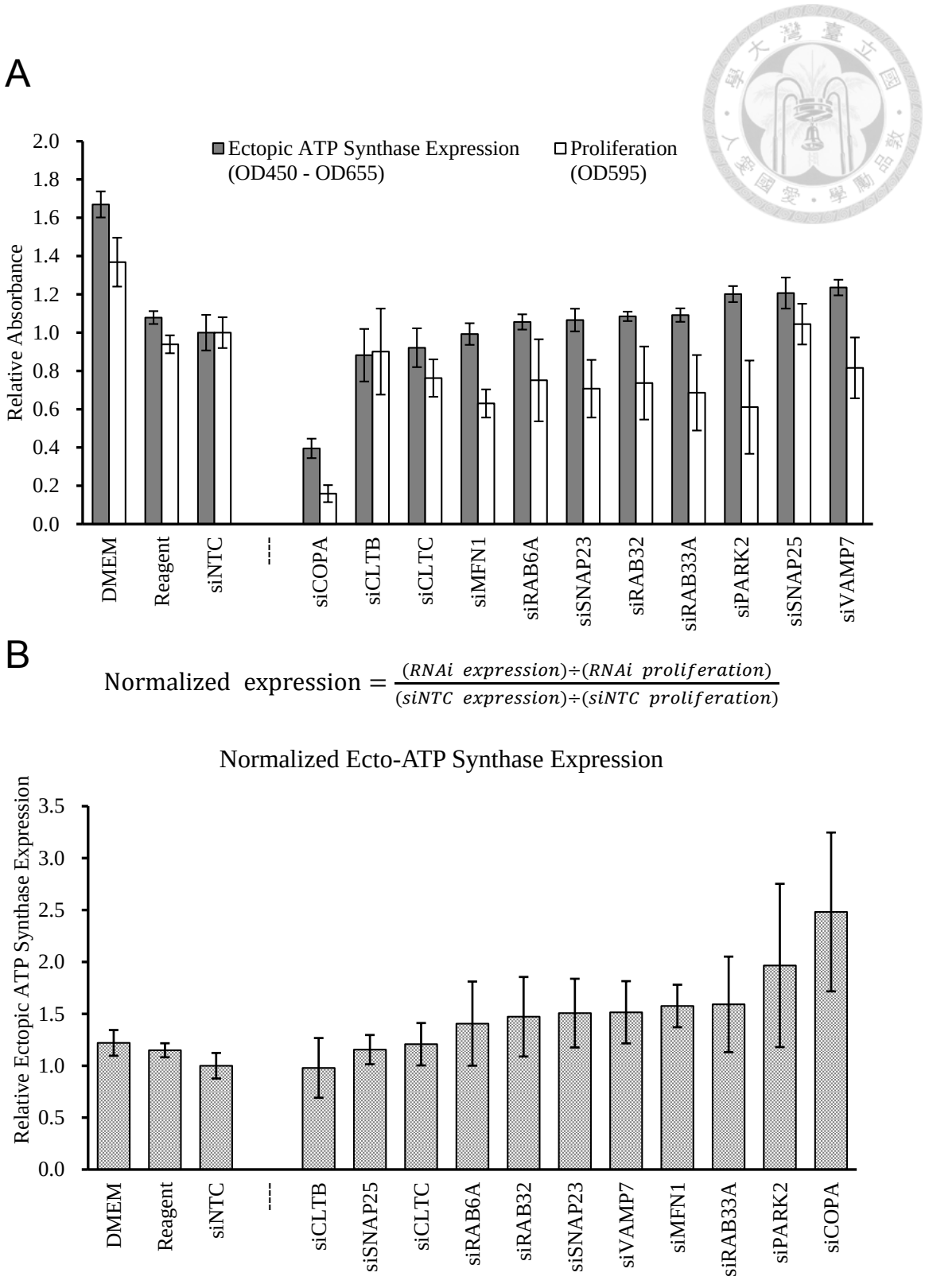

Figure 11. Ectopic ATP synthase expression after eleven selected genes were silenced for 72 hours.

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(A) 72 hours post-transfection of siRNAs, the raw expression level of ectopic ATP synthase (gray) and the cell viability (white) was measured with CELISA and crystal violet assay, respectively.

(B) The raw expression was normalized with cell viability; the result of normalization is shown as bars. DMEM, reagent, siNTC indicates medium only, transfection reagent (without any siRNA) and non-targeting control siRNA, respectively. (Mean ± SD)

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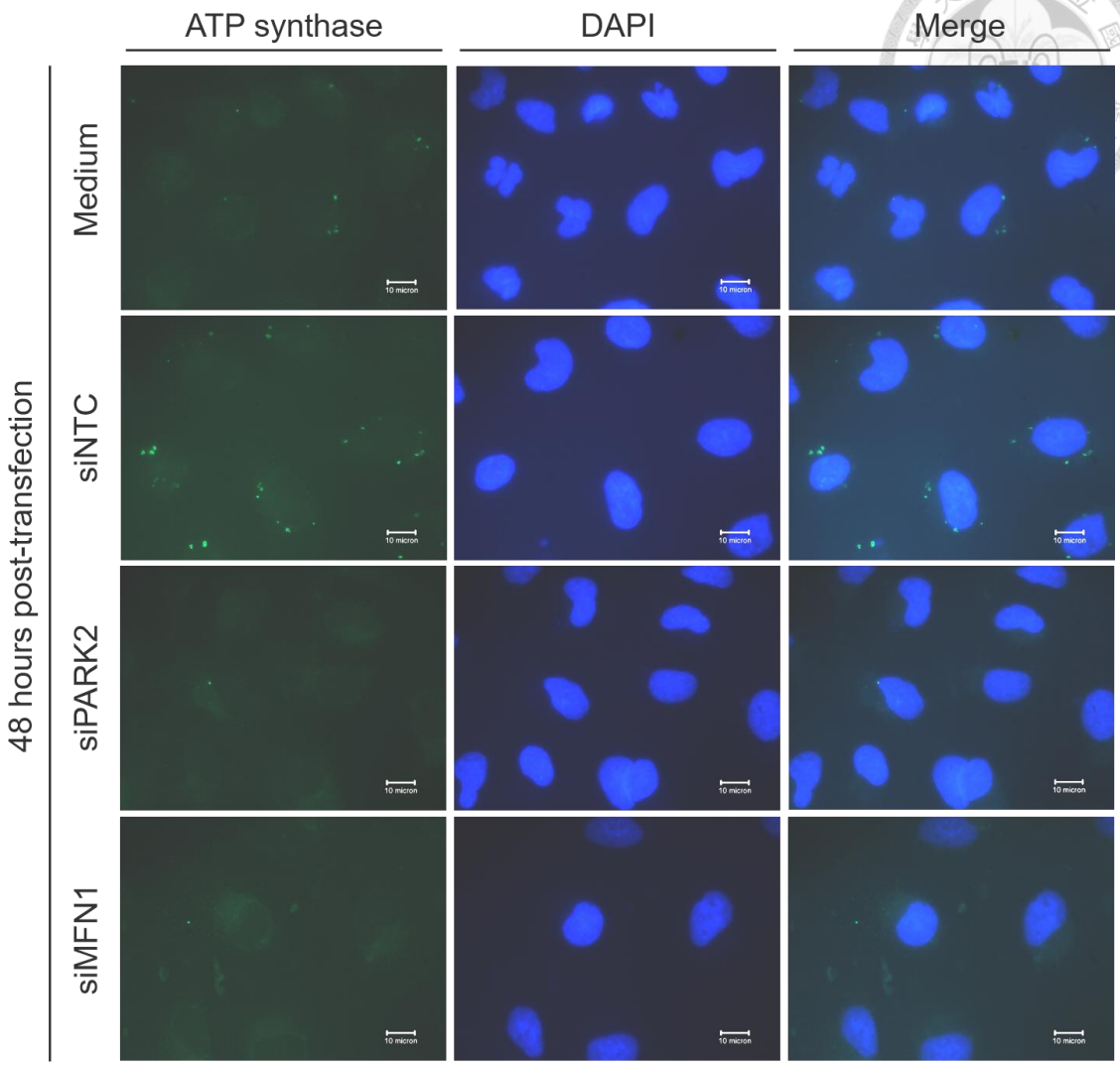

Figure 12. Immunostaining of ectopic ATP synthase after siPARK2 and siMFN1 transfection for 48 hours.

Immunocytochemistry were used to visualize the ectopic ATP synthase expression on the cell surface after those cells were transfected with siPARK2 or siMFN1 for 48 hours. Cell were stained for ATP synthase (green) and DAPI (blue). An oil lens (100×) was used. Scale bar = 10 μm.

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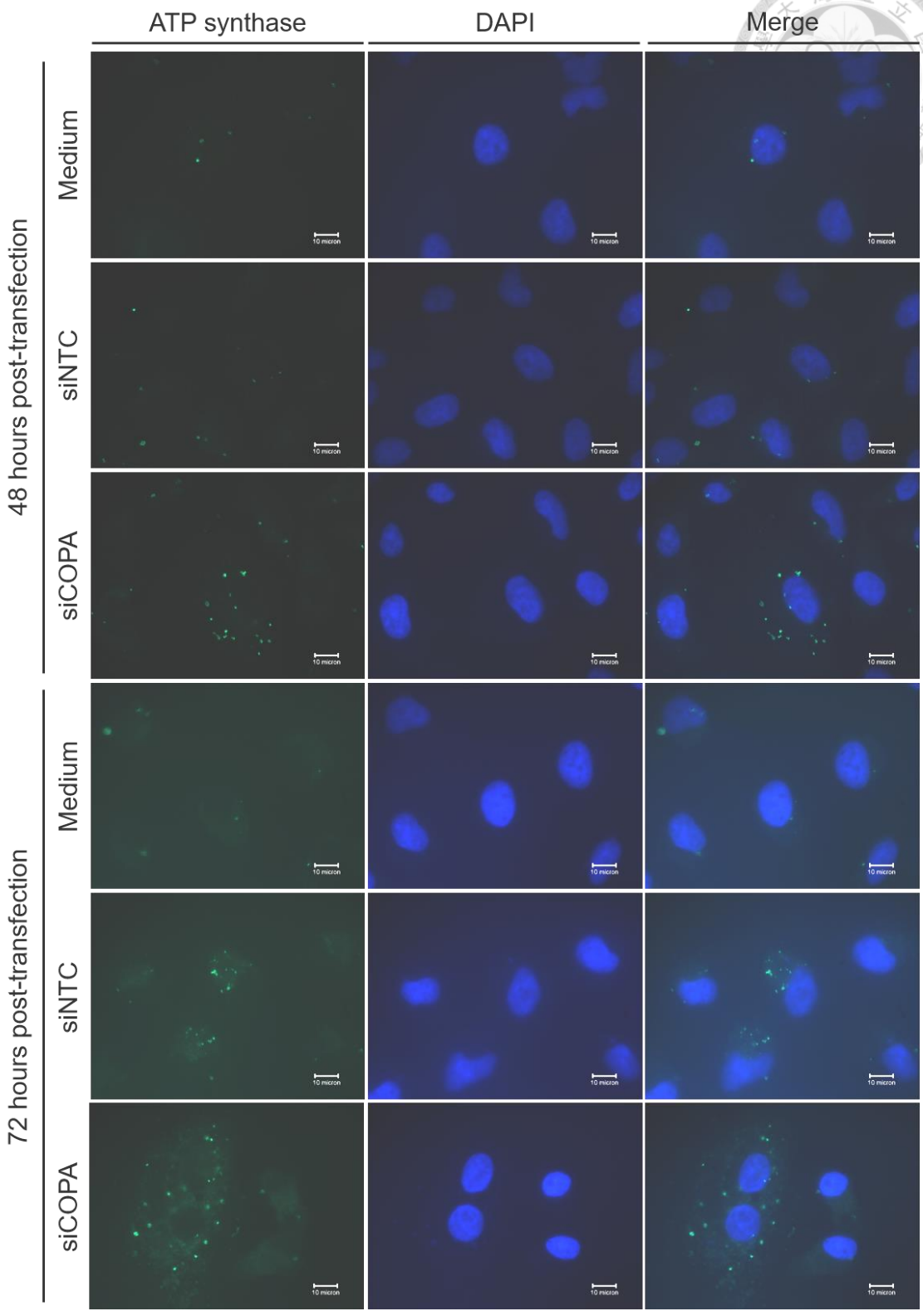

Figure 13. Immunostaining of ectopic ATP synthase after siCOPA transfection for 48 hours.

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Immunocytochemistry were used to visualize the ectopic ATP synthase expression on the cell surface after those cells were transfected with siCOPA for 48 hours. Cell were stained for ATP synthase (green) and DAPI (blue). An oil lens (100×) was used. Scale bar = 10 μm.

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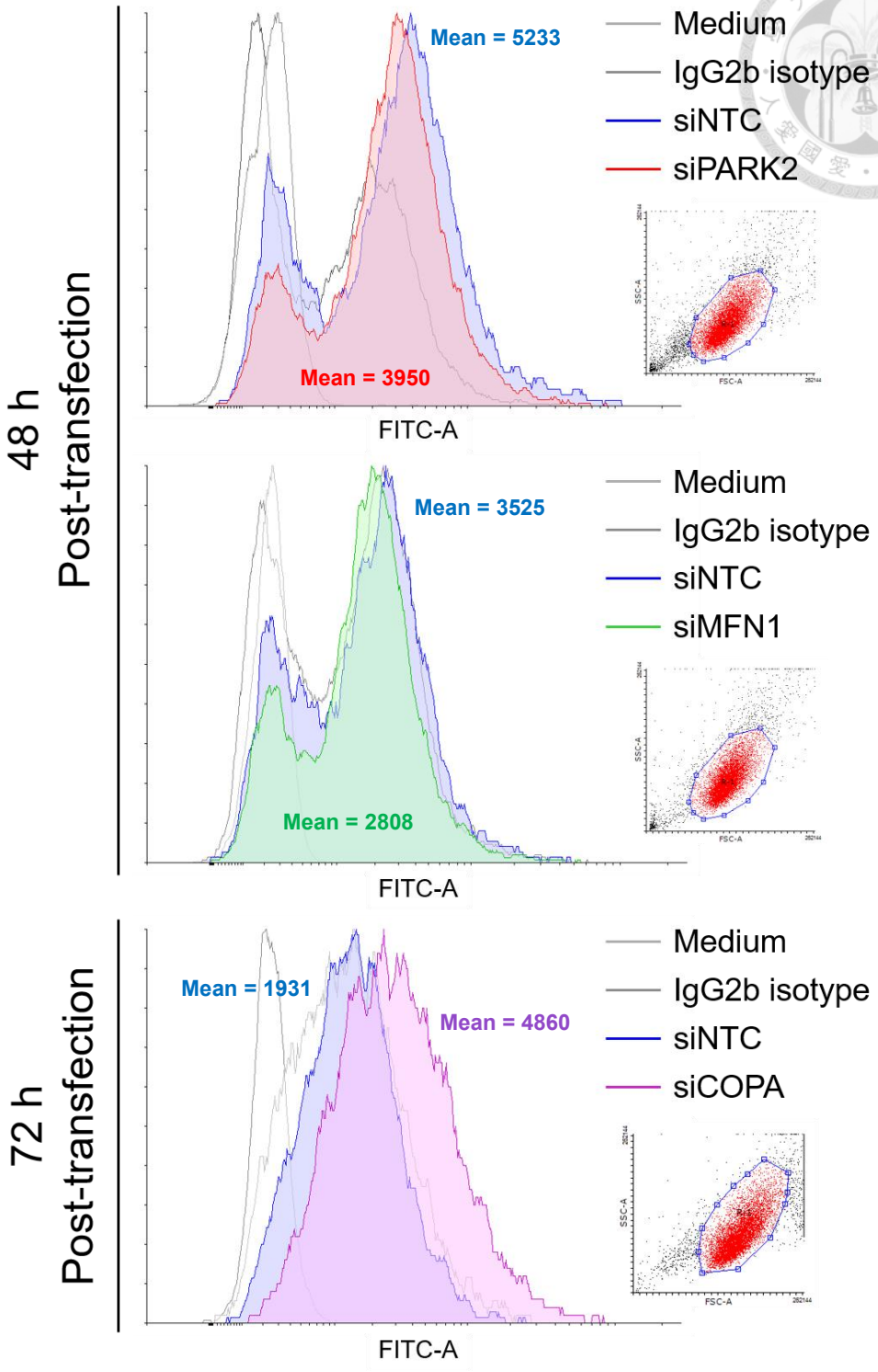

Figure 14. Flow cytometry analysis of ectopic ATP synthase after transfection with PARK2, MFN1 and COPA RNAi, respectively.

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The ectopic ATP synthase expression levels of the cells transfection with siRNAs were measured with flow cytometry. The dark grey lines of three graphs represent the basal expression. The light grey lines are the signals from IgG2b isotype. The ectopic ATP synthase expression of siNTC-, PARK2-, MFN1- and COPA-silenced cells are indicated by the blue, red, green and purple lines, respectively.

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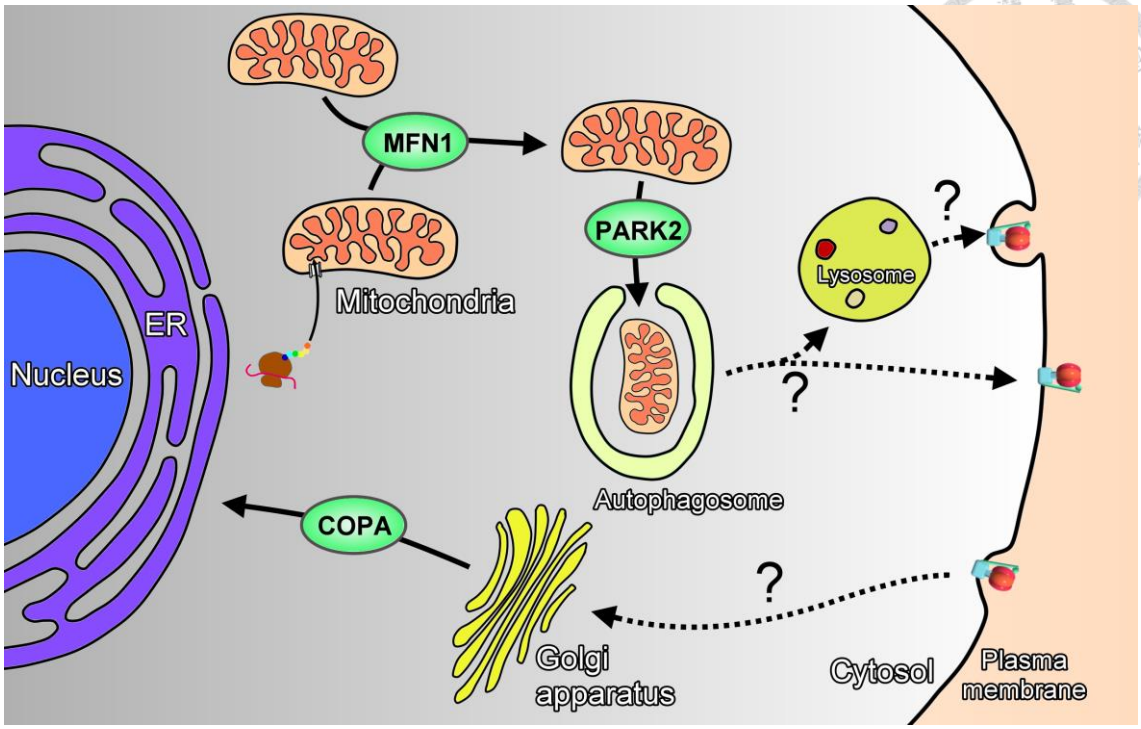

Figure 15. Possible trafficking pathways of ATP synthase from mitochondria to cell surface.

A hypothetical model that may explain the mechanism of ectopic ATP synthase trafficking. The dotted arrows show the pathways that are not known so far. After the ATP synthase subunits were imported into mitochondria, the ATP synthase complex was assembled inside mitochondria. All or fragments of the mitochondria transferred through the mitochondrial degradation process, bringing these complexes to cell surface; however, whether they enter the lysosomes before reaching the plasma membrane is not clear. Mitofusins control the dynamics of mitochondria, therefore affect the pattern of ectopic ATP synthase. The COPI vesicles may play a role in the mechanism of recycling the ectopic ATP synthase complex from the cell surface.

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Chapter 7 Tables

Table 1. Target genes and their involved pathways.

Pathways	SiRNA Targets	UniProt Protein Names	Common Abbreviates	References
Mitochondria				
Import of peptide	TOMM40	Mitochondrial import receptor subunit TOM40 homolog	Tom40	56
Fission	RAB32	Ras-related protein Rab-32	Rab-32	57
	DNM1L	Dynamin-1-like protein	Drp1	58
Fusion	MFN1	Mitofusin-1	Mfn1	55
Mitophagy	PARK2	E3 ubiquitin-protein ligase parkin	Parkin	59
	PINK1	Serine/threonine-protein kinase PINK1, mitochondrial	Pink1	59
ER to Golgi apparatus	SAR1A	GTP-binding protein SAR1a (COPII-associated small GTPase)	Sar1a	60
	SEC13	Protein SEC13 homolog	Sec13	61
Golgi apparatus				
to ER	COPA	Coatomer subunit alpha	α-COP	62
to early endosome	AP1B1	AP-1 complex subunit beta-1	β-adaptin 1	63
to plasma membrane	RAB3A	Ras-related protein Rab-3A	Rab-3A	64
	RAB8A	Ras-related protein Rab-8A	Rab-8A	65
	RAB11A	Ras-related protein Rab-11A	Rab-11	66
	SNAP23	Synaptosomal-associated protein 23	SNAP-23	67
	SNAP25	Synaptosomal-associated protein 25	SNAP-25	67

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Table 1. Target genes and their involved pathways. (continue)

Pathways	SiRNA Targets	UniProt Protein Names	Common Abbreviates	References
Early endosome				
to Golgi apparatus	RAB6A	Ras-related protein Rab-6A	Rab-6	68
to late endosome	RAB5A	Ras-related protein Rab-5A	Rab-5A	69
to plasma membrane	RAB4A	Ras-related protein Rab-4A	Rab-4A	70
	SNAP25	Synaptosomal-associated protein 25	SNAP-25	67
Late endosome				
to Golgi apparatus	RAB7B	Ras-related protein Rab-7b	Rab-7B	71
	RAB9A	Ras-related protein Rab-9A	Rab-9A	72
to lysosome	RAB7B	Ras-related protein Rab-7b	Rab-7B	73
Lysosome to plasma membrane	VAMP7	Vesicle-associated membrane protein 7	VAMP-7	74
Recycling endosome to plasma membrane	RAB11A	Ras-related protein Rab-11A	Rab-11	66
Endocytosis	RAB5A	Ras-related protein Rab-5A	Rab-5A	75
Clathrin-coated vesicle formation	CLTA	Clathrin light chain A	Lca	76
	CLTB	Clathrin light chain B	Lcb	76
	CLTC	Clathrin heavy chain 1 (Clathrin heavy chain on chromosome 17)	CLH-17	76

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Table 2. Primers for real-time PCR.

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
CLTA	GAGCAGCTACAGAAAACAAAAGCAA	GGCCACCCGTTCCCA
CLTB	AGGCATAGAGAACGACGAGG	GTTGGCCTCCTGAAACACATC
CLTC	ACGTTATTTTGTTTTGCAGTTCGGG	CTGGTTCCCTGTAGGTGGTG
MFN1	GGCATCTGTGGCCGAGTT	ATTATGCTAAGTCTCCGCTCCAA
DNM1L	CTGTCATAAACAAGCTCCAGGACG	CTCTGCGTTCCCACTACGAC
COPA	GGTGATGTGTCAGAGCGTGT	CATGATAGGTGCAGGTGGCT
SAR1A	ACTACATCCGACATCAGAAGAGC	GATTCCACGAGGCGAGAATGA
SEC13	AGATCCTTATCGCCGACCTCA	CATGCTCGTGGCTCTTCTCC
SNAP23	TCCTGGGTTTAGCCATTGAGT	AAAGGCCACAGCATTTGTTGA
SNAP25	CTGGAGGAGATGCAGCGAAG	CATACGACGGGTGCTTTCCA
PINK1	GGACACGAGACGCTTGCA	TTACCAATGGACTGCCCTATCA
PARK2	CTGATCGCAACAAATAGTCGGAAC	GTTGAGGGTCGTGAACAAACTG
AP1B1	GCTATTGGGACCTGCGGAAG	ACTGACATCTTTGCCCACGG
RAB3A	GGTGGTGTCATCAGAACGTGG	GTTAATGTTGTCCTTGGCGCTTG
RAB4A	ATGGGATACAGCAGGACAAGAAC	CATTGTAGGTTTCTCGGCTGGT
RAB5A	TCATGGAGACATCCGCTAAAACATC	GGTAAGGTCTACTCCTCTTCCTCT
RAB6A	CCGGGAATGGAAAGCACACA	GTGAGCTTCTGAAGAAGGTTGAAGA
RAB7B	GGCCAGCATCCTCTCCAAGATTATC	GATGCAGCCATCGGAGCCCTTGT
RAB8A	CCTGTGTCCTGTTCCGCTT	CCCCTGTAGTAGGCCGTTG
RAB9A	ACACTTTTCCCGTGTCGTTTGA	ATAAGTGAACTCTTCCCAACTCCAC
RAB11A	TTCGCGTCTGTCTGACATTT	TTGAAACTTCGGCCCTAGAC
RAB32	TGGCTTTGCCGGATGGTT	ACTGGGATTTGTTCTCTGCTCTC
RAB33A	CAACGTACATGCCGTGGTCT	TTTCAGGGCTAAGTTGGAGGG
VAMP7	GACTACTTACGGTTCAAGAGCACA	ACCATGATTCCTTTCAGTTCATCCA
PPIB	GCCGGGTGATCTTTGGTCT	CGCTCACCGTAGATGCTCTTT

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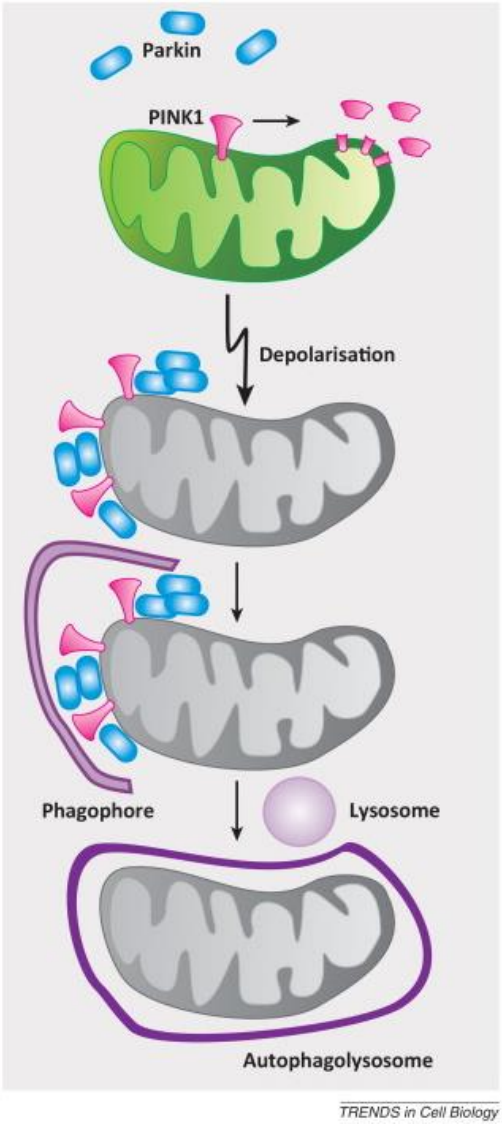
Appendix

Summarized process of mitophagy.



The PINK1 recruits PARK2 to initiate mitophagy together[77].

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