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Localization and function of a mitochondrial Protein X

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This Bachelor's thesis project was initiated to learn more about the localization and function of a novel Protein X in mitochondria. The experiments took place at the Institute of Biotechnology, in Viikki, Helsinki. The laboratory work was done as a part of Team Zhao's research.

The localization of Protein X was detected by Western blot and immunofluorescence, and the oligomeric state in cells was detected using Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE). In order to elucidate the cellular function of Protein X, siRNA of protein X was used to silence the endogenous expression of Protein X and then, the changes in mitochondrial morphology were detected using immunofluorescence.

The results showed that Protein X mostly localizes to mitochondria. As a result of proteinase K digestion in isolated intact mitochondria, it was found that Protein X mainly localizes to mitochondrial inner membranes. Through BN-PAGE the oligomeric state of protein X was examined in isolated mitochondria, and the data showed that it remained as a monomer in U-2 OS cells. SiRNA silencing showed that loss of Protein X resulted in mitochondrial morphology changes from tubular to fragmented and swollen structure. In conclusion, the thesis showed that Protein X localizes to mitochondria and plays a vital role in maintaining normal mitochondrial morphology.

The results are crucial for understanding the function of mitochondria, which will provide a potential new therapeutic methods for overcoming mitochondrial diseases caused by protein dysfunction.

Keywords	protein X, mitochondria, localization, function

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Opinnäytetyön tutkimustyöt aloitettiin, jotta opittaisiin enemmän uudesta mitokondrio-proteiini X:stä, erityisesti sen sijainnista ja funktiosta mitokondriossa. Tutkimustyöt suoritettiin Biotekniikan instituutissa, Helsingin Viikissä, ja laboratoriotyöt suoritettiin osana Zhaon ryhmää.

Proteiini X:n sijainti selvitettiin käyttämällä Western blotia ja immunofluoresenssia. Oligomeerista tilaa soluissa tutkittiin natiivilla polyakryyliamidielektroforeesilla (BN-PAGE). Jotta proteiini X:n funktio soluissa selventyisi, käytettiin proteiini X:n siRNA:ta hiljentämään proteiini X:n solunsisäinen ekspressio. Immunofluoresennssia hyödyntäen havainnoitiin muutoksia mitokondrion morfologiassa.

Tutkimuksessa onnistuttiin havaitsemaan, että proteiini X sijaitsee ensisijaisesti mitokondriossa. Eristetyn mitokondrion proteaasi K digestiolla saatiin selville, että proteiini X on suurimmaksi osaksi lokalisoitunut mitokondrion sisäiseen membraaniin. BN-PAGE:lla tutkittiin proteiini X:n oligomeerista tilaa eristetyissä mitokondrioissa, ja tulokset osoittivat, että U-2 OS soluissa proteiini X on monomeeri. Proteiini X:n siRNA -hiljennyksellä saatiin selville, että mitokondrion morfologia muuttui putkimaisesta fragmentoituneeksi ja turvonneeksi rakenteeksi.

Tutkimus osoitti, että proteiini X on lokalisoitunut mitokondrion sisälle ja sillä on elintärkeä funktio mitokondrion normaalin rakenteen morfologiassa. Tulokset ovat tärkeitä mitokondrion funktion ymmärtämiseksi, sillä sen ymmärtämisellä on potentiaalia paljastaa uusia terapeuttisia menetelmiä mitokondrio-proteiinien aiheuttamien toimintahäiriöiden parantamiseksi.

Avainsanat	proteiini X, mitokondrio, sijainti, funktio

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Appendix 1 Lipofectamine® RNAiMAX reagent protocol

Abbreviations

BN-PAGE	Blue Native Polyacrylamide Gel Electrophoresis
BSA	Bovine Serum Albumin
DB	Dulbecco + 0.2% Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle's Medium pH 7.4
ECL	Enhanced Chemiluminescence
EGTA	Ethylene Glycol Tetraacetic Acid, used to adjust pH
FBS	Fetal Bovine Serum
HRP	Horseradish Peroxidase
IB_c	IB_{CELL} solution
IMM	Mitochondrial inner membrane
LSB	Loading Sample Buffer
MOPS	3-(N-morpholino)propanesulfonic acid buffer, used to adjust pH
MtDNA	Mitochondrial DNA
MQ	Milli-Q laboratory water
OMM	Mitochondrial outer membrane
PAB	Primary Antibody
PAGE	Polyacrylamide Gel Electrophoresis
PD	Parkinson's disease
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl Fluoride
Protein X	The studied protein
PVDF	Polyvinylidene Fluoride
RIPA	RIPA Lysis and Extraction Buffer
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline + 0.1% Tween® 20
TX-100	Triton® x-100 in PBS
U-2 OS	Human Bone Osteosarcoma Epithelial Cell

1 Introduction

Mitochondria are the major energy producers in the cells. Mitochondria can be 25% of the cells total volume [1]. Even though mitochondria are thought as the power sources of the cells, mitochondria have many other important roles in cells. Understanding the structure and function of mitochondria is very important for understanding the mitochondrial dysfunctional related diseases. Mitochondrial diseases are the result of a failure or dysfunction in mitochondrial function. [2]

It is predicted that a human mitochondria consists of up to 1,500 different proteins. The functions and identity of these proteins is largely unknown. Researching mitochondrial proteins is significant, and there is plenty of research to be done. Knowing all of the proteins of mitochondria is the main key for understanding the relations between their functions on the mitochondrial morphology. [3]

Team Zhao is an international group of five individuals, and the laboratory is located in Biocenter 1 in Viikki, Helsinki. The research strongly focuses on the membrane morphogenesis and how it is linked to central cellular processes. This thesis project was a part of Team Zhao's research. The research is very important to understand the complete cellular structure and function of mitochondria. The cellular function and localization of a newly found protein, Protein X, remains completely unknown. The aim of my project focuses on studying the function and localization of Protein X in cells. [4]

2 Theoretical background

2.1 History of studying mitochondria

A huge benefit to mitochondrial study, is the work of George Palade and coworkers, who developed a protocol for isolating mitochondria. The protocol was invented in the late 1940s and it is based on centrifugation. This method was revolutionary and it allowed the isolation of pure organelle with high yields. In the next 20 years, it also helped to discover mitochondrial DNA, mechanisms for protein import in mitochondria, definition of mitochondrial ultrastructure, discovery of mitochondrial membrane channels and many others. [5]

Before 1990s there was a 15 years lasting slow phase on studying mitochondria, and in the 1990s, it was discovered, that mitochondria amplify apoptosis by releasing cytochrome c and other proteins to activate effective caspases. Even though this was known, the precise mechanism of how that worked was not known. From that point onward, the study of mitochondria has risen, and mitochondria are largely studied. [5]

2.2 Structure of mitochondria

Mitochondria have two membranes which divide mitochondria into different compartments. As shown in Figure 1, the yellow smooth layer membrane structure is mitochondrial outer membrane (OMM), which forms the barrier around the rest of the organelles. Inside the mitochondria, a highly curved membrane structure is mitochondrial inner membrane (IMM), which contains many different kinds of enzymes that are responsible for oxidative phosphorylation. As shown in Figure 1, the inner membrane forms many protrusion membrane structures which are called cristae, which significantly increase inner membrane area. The inside of the inner membrane is called matrix, which contains mitochondrial DNA (mtDNA), ribosomes, granules and many enzymes that are responsible for the citric acid cycle. Between outer and inner membrane, there is a very narrow space, called the intermembrane space. Some transmembrane proteins release their domain into this space. [1] [6]

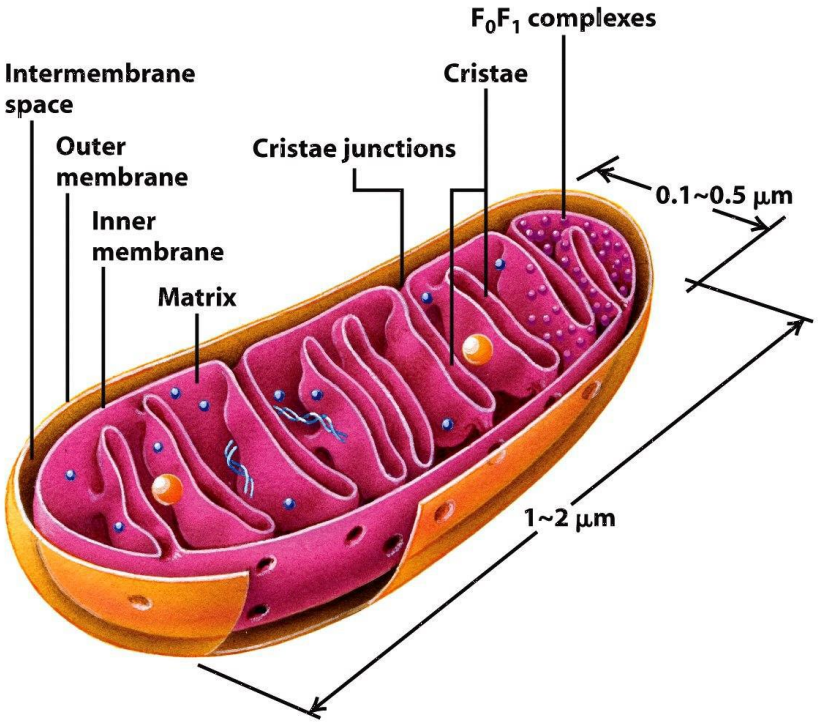

Figure 1. A simplified composition of the mitochondrion. [1]

2.3 Mitochondrial diseases

Mitochondria are highly dynamic organelles which rapidly change their morphology through fission and fusion in order to fulfill a plethora of functions. Except for their prominent role in energy metabolism, the mitochondria play key roles in various key cellular processes, such as stress response, the regulation of calcium homeostasis and cell death signaling. Therefore, it is not surprising that an impairment of mitochondrial function results in cellular damage, which further lead to aging and neurodegeneration. [7] [8]

The mitochondrial diseases may be inherited from mitochondrial DNA (mtDNA) or nuclear DNA (nDNA). The changes in mtDNA can only be transferred from the mother's egg cell, thus, they are only inherited from the mother. If the child is conceived from an egg cell that mostly contains mutated mitochondria, it is more likely to develop a disease. Because the cell only contains one nucleus and hundreds of mitochondria, only a part of the mitochondria might be mutated. The amount of mutated mitochondria in the cell will determine the state of the cell's health. [9] [10]

Recently, many researches suggest that mitochondrial dysfunction is strongly associated with the pathogenesis of Parkinson's disease (PD). For example, previous research showed that complex I inhibits one of complexes of the electron transport chain and could induce PD. Interestingly, recent research found that several PD-related genes interfere with pathways regulating mitochondrial morphology, function and dynamics. Through understanding how mitochondria maintain its normal morphology and function, it will open a novel avenue to discover new and potential mechanical theory for overcoming mitochondrial related disease. [11]

At the moment, there is no direct way to treat mitochondrial diseases. Only way to help the patients with disorders is to help the mitochondria to work with different vitamin and mineral mixes and lessen the mitochondrial stress. These therapies are debatable because of the agents and doses are not prescribed, although the therapies are fairly harmless. [9] [12]

2.4 Theoretical background on methods used to study mitochondrial proteins

The mitochondrion can be studied in many ways. The localization and expression level of proteins can be detected with Western blot and immunofluorescence. The native oligomeric state of the proteins can be detected with blue native PAGE (BN-PAGE), and siRNA silencing can be used to study the effects of proteins in cells, combined with Western blot and immunofluorescence.

Human Bone Osteosarcoma Epithelial Cell (U-2 OS) line was used for the experiments in this thesis. This cell line was chosen because it is widely used in the laboratory. The fact that it is a cancer cell line means that it doesn’t reach senescence as fast as normal cells, which is good because plenty of cells are required for the experiments. U-2 OS cells are also fairly easy to grow. Even though all the mentioned factors made the selection easy, the biggest determining function was that the mitochondria have a clear morphological structure in these cells.

The mitochondria are isolated from cells with a homogenizer, and the homogenization is done until the cell membrane is broken. The homogenization is difficult because the homogenization can also break the mitochondria, if too much force is used. In order to isolate mitochondria effectively, it is required to find the suitable homogenization method. In this thesis the mitochondria were isolated with a Teflon homogenizer (Potter-Elvehjem PTFE pestle and 3 mL glass tube, Sigma). The pestle is operated by hand, thus, the operation of the machine is different for every user. The buffers used can also affect the results and trying different buffers might work for other individuals. The pH of the buffers should be around 7.4, since it is thought to be the best one for mitochondria. The isolation should take place in a cold environment, such as a cold room or an ice box. The mitochondria are separated from the other parts of the cell by centrifuging at different speeds. [5]

Western blot is a very basic method used to identify proteins extracted from cells. In this method, the proteins were separated by their molecular weight with gel electrophoresis. These proteins were then transferred to a selected type of a membrane, and the membrane was then blocked. [13] The blocking of the membrane prevents nonspecific binding of the proteins, thus, improving the detection of the target proteins, and reducing the background interference. The membrane is then treated with primary antibody, which binds specifically to the targeted protein. Then, the membrane is washed, and incubated with a secondary antibody, which binds to the primary antibody. The secondary antibody is a horseradish peroxidase (HRP) conjugate, which can be detected with digital imaging. [14]

Immunofluorescence microscopy is a widely used method, which is used by researchers to study the localization and expression levels of proteins. Immunofluorescence which uses two antibodies is called secondary immunofluorescence or indirect immunofluorescence. In this method the primary antibody recognizes and binds to the targeted biomolecule, and the secondary antibody binds to the primary antibody indirectly. The secondary antibody contains a fluorescence label, which can be detected with fluorescence microscopy. Two or more primary antibodies can be stained at the same time to detect the localization of a protein. [15]

The localization of a studied protein can be detected by comparing it to the location of known proteins. In this thesis many protein markers were used, such as Tom20 (Santa Cruz Biotechnology), Cox IV (Thermo Scientific), Hsp60 (Stressmarq Biosciences), GAPDH (Thermo Scientific), α-Tubulin (Sigma Aldrich). Tom20 is a mitochondrial outer membrane protein, Cox IV is a mitochondrial inner membrane protein, Hsp60 is used as a mitochondrial matrix protein, GAPDH is a loading control protein (used to detect the amount of protein loaded) and α-Tubulin is a cytoskeleton marker. More information about the antibodies can be obtained from the supplier's websites.

Proteinase K is an enzyme used to digest proteins and can easily be inactivated by Phenylmethylsulfonyl Fluoride (PMSF). Proteinase K is an excellent enzyme because it can maintain its activity even in 1% Triton™ X-100 (TX-100), which is important because TX-100 can be used to disrupt and break the mitochondrial membrane, so that the proteinase K can digest the proteins inside of the mitochondrial membranes. [16]

Blue native PAGE is used to analyze the native oligomeric state of proteins in isolated mitochondria. The mass range of the proteins detected can vary from 10 kDa up to 10 MDa. This method is based on the separation of proteins by their size in acrylamide gradient gels. The biological membranes are solubilized with a nonionic detergent, such as digitonin. The proteins are stained with Coomassie dye, which can be detected as blue lines in the gel. The BN-PAGE also has many other uses, such as determining the stoichiometry of multiprotein complexes and researching apoptosis. [17]

SiRNA silencing method is based on designing a siRNA against your target gene. After designing the siRNA, it needs to be delivered into the cell effectively. The siRNA silences the targeted gene, thus, stopping the expression of a specific protein. The silencing efficiency is detected with control scramble cells to make sure the silencing is specific and does not silence any other genes. This can be used to detect the function of a protein in cells. Thermo Scientific offers good protocols for efficient silencing in different cell types. [18]

3 Methods and materials

The following chapter describes the methods used for this thesis project. The research was done with basic and advanced methods, such as Western blot and immunofluorescence. The cell line used for all experiments was Human Bone Osteosarcoma Epithelial Cell (U-2 OS) line.

3.1 Cell Culture

The cells were divided maximum of 20 times in order to avoid senescence. The starting of the cell line was done with the ATCC's culture method [19]. The cells were grown on 14.5 cm plates which were cultured in Dulbecco's Modified Eagle's Medium (DMEM) including 10% Fetal Bovine Serum (FBS) and Penicillin Streptomycin Glutamine. Two plates were divided twice a week into 12 plates, after reaching more than 90% confluence, and 10 plates were used to isolate 2 mitochondrial samples.

3.2 Isolation of mitochondria

The isolation of mitochondria method is based on Nature Protocol [5]. The method was modified to fit the laboratory equipment, and it was optimized to work in optimized way in this specific lab environment. Isolating mitochondria can be troublesome and much concentration is required. Mitochondria must be isolated from freshly cultured cells.

After cell culture, the DMEM was collected from 10 plates into two 50 mL polypropylene falcon tubes, the rest was suctioned out. The cells were then washed one time with 10 mL of Phosphate Buffered Saline (PBS). After washing the cells, PBS was removed completely with suction and 1 mL of Trypsin was added on all of the plates. Trypsin was let to affect until the cells were detached (around 5 minutes), and then 9 mL of the collected DMEM was added on the plate, and it was used to collect the cells. Nine mL of DMEM was used to wash the plates for any residue of cells. After washing, the DMEM was collected and it was used to collect cells from the second plate. This was repeated until all the plates full of cells were collected into two falcon tubes.

Collected cells were then centrifuged for 5 minutes at 600g at 4 °C. While the centrifuge was running, the IB_{CELL} (IB_c) solution was prepared with the recipe on Table 1. After centrifuging, the DMEM was poured out, and the pellet was suspended in 50 mL of PBS. Washing was repeated twice, and after the second wash the falcon tube was placed downwards on a paper towel to get rid of the PBS. Once the pellet was dried, it was put on ice.

Table 1. IB_{CELL} solution for isolating mitochondria. MOPS and Tris were used to set the pH of Tris and EGTA solutions to 7.4. Solution was made in distilled water.

Name of solution	Concentration
Sucrose	200 mM
Tris/MOPS, pH 7.4	10 mM
EGTA/Tris, pH 7.4	1 mM

From this point forward, all the steps were done in an ice box (Figure 2). The cell pellet was suspended in 700 µL of IB_c solution and collected into the homogenizer (Potter-Elvehjem PTFE pestle and 3 mL glass tube, Sigma). The falcon tube was washed with 500 µL of IB_c solution, and the solution was collected into the homogenizer. After transferring all the cells into the homogenizer, 250 strokes were done with the pestle. The strokes were done with fluidity and the pestle was not twisted (twisting the pestle could break the mitochondria). When the homogenization was done, the cells were collected into 15 mL polypropylene falcon tube. The tube was filled to 14 mL mark with IB_c solution. The homogenization was repeated with the other cell pellet. The tubes and the ice box were now put into a 4 °C cold room for an hour.

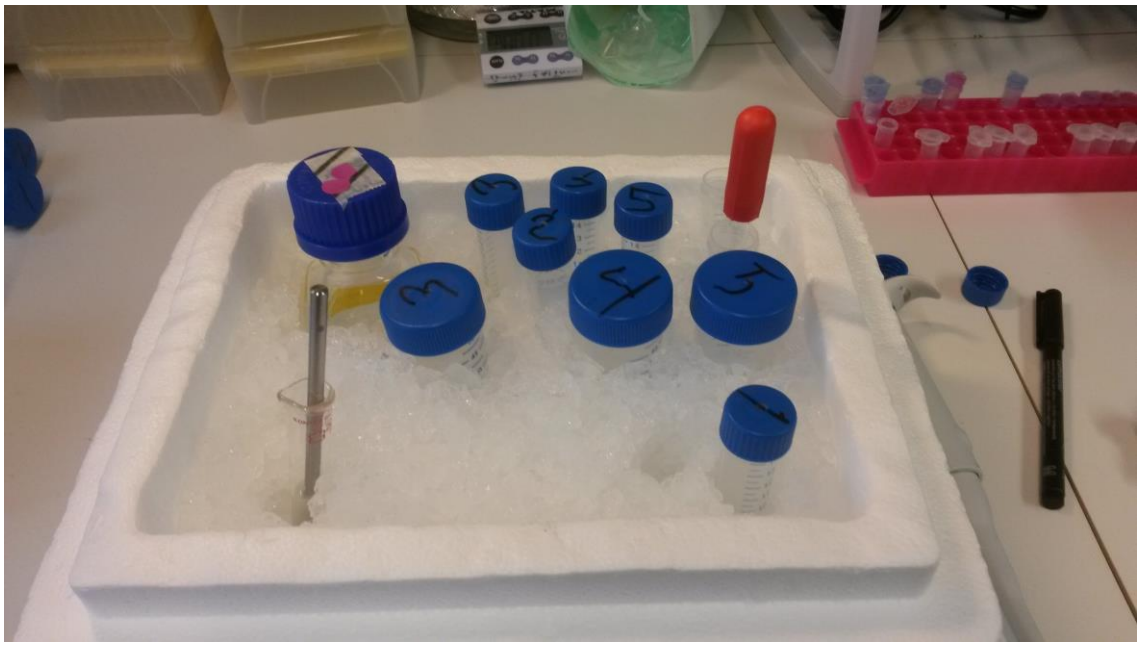

Figure 2. Picture of the setup used for isolating mitochondria.

The falcon tubes were centrifuged at 600g for 5 minutes at 4 °C. The supernatant was collected carefully and then centrifuged again to ensure that there is not any pellet in the supernatant. The supernatant was collected into a sterile high speed centrifuge tube. The cells were centrifuged at 7,000g for 10 minutes at 4 °C. All supernatant was removed and the pellet was suspended in 700 µL IB_c. The suspension was collected into a sterile 1.5 mL Eppendorf tube. The centrifuge tube was washed with 500 µL IB_c. The suspension was collected into the same Eppendorf tube and centrifuged at 7,000g for 10 minutes at 4 °C.

After centrifuging, the supernatant was removed completely with suction, in order to get rid of the cytosolic fraction (the cytosolic fraction was collected on the first times to detect if the Protein X was localized to it). The pellet was pure mitochondria and it could be frozen for later use. The pellet was suspended in different amounts of IB_c solution, depending on the following experiment.

3.3 Experiments on isolated mitochondria

3.3.1 Western blot

The mitochondria pellet was suspended in 120 µL IB_C solution and divided into 4 Eppendorf tubes. The tubes were sonicated for 5 cycles and 30 seconds each cycle. Next, 4X loading sample buffer (LSB) was added into the tubes, and the tubes were boiled at 100 °C for 10 minutes.

The SDS-PAGE machine was now set-up and the boiled samples were loaded into the SDS gel. Gel with 15 wells was used and 18 µL of sample were loaded into each well. The voltage was set to 80 V, and after the blue line had reached the lower gel, the voltage was set to 120 V. The samples were run until the blue line almost reached the bottom of the gel.

The proteins were transferred from the gel into a nitrocellulose membrane at 100 V at 4 °C for an hour. After transferring, the membrane was blocked with 5% milk solution. Next, the membrane was incubated with the primary antibody overnight. The primary antibodies used, can be found on Table 2.

Table 2. Dilution ratios and suppliers of used antibodies.

Primary antibody	Dilution ration	Supplier
Tom20	1:500	Santa Cruz Biotechnology
Cox IV	1:500	Thermo Scientific
Hsp 60	1:500	Stressmarq Biosciences
Protein X	1:500	-
GAPDH	1:500	Thermo Scientific
α-Tubulin	1:500	Sigma Aldrich
Secondary antibody	1:10,000	Life Technologies

On the next morning, the primary antibody solution was collected into a tube for later use. The membrane was then washed 3 times with around 10 – 15 mL of Tris-buffered Saline + 0.1% Tween® 20 (TBST). While washing, the horseradish peroxidase (HRP) secondary antibody was prepared, the HRP was diluted 1:10,000 in 1% milk/TBS. The membrane was incubated with the HRP secondary antibody solution for 1 hour, and then it was washed 3 times with TBST. After washing, the membrane was rinsed 3 times with Tris-buffered Saline (TBS), and 2 mL of Enhanced Chemiluminescence (ECL) (MERCK) solution was added on the membrane (TBST can react with ECL). Pictures were taken with FujiFilm LAS-3000 Imaging System, and chemiluminescence was used to detect the bands.

3.3.2 Proteinase K digestion with and without 1% Triton® X-100 pre-treatment

The isolated mitochondria were suspended with 30 µL of IB_C solution, and the suspension was divided into two tubes. One tube was treated with 1.5 mL of 10% Triton® x-100 (TX-100), and then the digestion was started by adding 0.6 mL of 20 mg/mL Proteinase K into both tubes. The digestion happened for 30 minutes on ice. After 30 minutes, the digestion was ended by adding 0.5 µL of 100 mM PMSF. The samples were solubilized with 2 µL of 10% SDS.

The cells were lysis with sonication, and the tubes were sonicated for 30 seconds with 5 cycles. After sonication, 5 µL of 4X LSB was added into both samples and they were boiled for 10 minutes at 100 °C. After boiling, the samples were ran with Western blot.

3.3.3 Alkaline extraction

All samples were kept in an ice box during the experiment. After mitochondria were isolated, 30 µL of 0.2 M Na_2CO_3 (pH 11, 11.5 and 12) was mixed with 30 µL of mitochondrial pellet. When the different pH value of Na_2CO_3 was added, the solutions were incubated for 30 minutes on ice with occasional vortex.

The samples were transferred to ultracentrifuge tubes and centrifuged at 51,000 rpm at 4 °C for 30 minutes. After centrifuging the pellet and the supernatant were collected into separate tubes.

Fifteen µL of RIPA and 5 µL of 4X LSB were added into the pellet tubes. For the supernatant, 120 µL of 100% acetone was added in each tube and then the tubes were incubated for 30 minutes in a freezer. Next, the supernatant tubes were centrifuged at 15 000g at 4 °C for 15 minutes. The pellet was collected, and 15 µL of RIPA and 5 µL of 4X LSB was added into the tubes. The samples were now boiled for 10 minutes at 100 °C. After boiling, the samples were loaded on a SDS-PAGE gel, and Western blot was done.

3.3.4 Determining the native oligomeric state of Protein X

The BN-PAGE protocol is based on Nature Protocol [17]. The protocol presented here was made to fit the laboratory equipment. All the solutions were prepared according to Table 3 before starting to do the experiment.

Table 3. Solutions for BN-PAGE.

Name of solution	Amount
Solubilization buffer A, pH 7.0 (4 °C)	
Sodium chloride	50 mM
Imidazole/HCl, pH 7.4	50 mM
6-Aminohexanoic acid	2 mM
EDTA	1 mM
Coomassie dye	
Brilliant Blue G	5% (wt/vol)
6-Aminohexanoic acid	500 mM
Cathode buffer B, pH 7.0	
Tricine	50 mM
Imidazole	7.5 mM
Brilliant Blue G	0.02% (wt/vol)
Cathode buffer B/10, pH 7.0	
Tricine	50 mM
Imidazole	7.5 mM
Brilliant Blue G	0.002% (wt/vol)
Electroblotting buffer, pH 7.0	
Tricine	50 mM
Imidazole	7.5 mM

After isolating mitochondria, 40 µL of solubilization buffer A was added to the pellet. The pellet was homogenized by twirling a tiny spatula. Twelve µL of 20% digitonin was added, and the samples were left to solubilize for 10 minutes. The samples were centrifuged for 15 minutes at 4 °C at 100,000g. Five µL of 50% glycerol and 6 µL of Coomassie dye was added into the tube. Samples were loaded on the BN-PAGE gel (Bio-Rad, MINI-PROTEAN® TGX Stain Free™ Gel).

The BN-PAGE was run in a 4 °C cold room. Cathode buffer B was used as the first buffer. Voltage was set to 100. After the blue line reached 1/3 of the running distance, the buffer was removed and changed to the cathode buffer B/10. The BN-PAGE was stopped after 4.5 hours.

Polyvinylidene Fluoride (PVDF) membrane was put into 100% methanol for one minute, and then it was washed one time with distilled water and transferred into electroblotting buffer. The casket was prepared like in Western blot, but the PVDF membrane and electroblotting buffer was used. The membrane was transferred overnight at 4 °C. The transfer was completed at the current limited to 15 mA. The PVDF membrane was destained with methanol for 3 minutes, and then transferred to Western blot buffer. The membrane was then pictured.

3.4 Cellular localization of Protein X by immunofluorescence

This immunofluorescence method was used to detect the localization of the target protein in the cell. Six well, flat bottom cell culture plates were used to grow the cells.

After the cells had grown for 3 to 4 days, the coverslips were placed in 24 well, flat bottom cell culture plate, the cell side of the coverslip facing up. The cells were fixed for 20 minutes in 4% Paraformaldehyde (PFA). After fixing, the cells were permeabilized by 0.1% Triton x-100 in PBS. The cells were then washed 1 time with PBS and 2 times with Dulbecco + 0.2% Bovine Serum Albumin (DB), each wash lasting for 10 minutes. Between washes the primary antibody solution was prepared, and the container for coverslips. All of the primary antibodies were diluted 1:50, except for Tom20 which was diluted 1:100. The container was a 12.5 cm petri dish which was covered with a tin foil. The bottom of the dish was covered with a wet paper and on top of it was a piece of parafilm. After washing, the coverslips were placed on the parafilm, which contained 15

µL of primary antibody solution for each coverslip. The coverslips were placed cell side down and they were incubated for 1 hour at 37 °C.

The coverslips were then put back into the 24 well plate, cell side up. The coverslips were washed 3 x 10 minutes with PBS. After washing, the cells were incubated with the secondary antibody, same way as the primary antibody. The coverslips were washed 3 times 10 minutes with PBS. The coverslips were attached on microscope slides with 6 µL of mounting medium. The mounting medium was heated on a heat block at 60 °C to keep it running. The coverslips were placed on the slides cell side down with the help of a scalpel and tweezers.

The cells were detected with a fluorescence microscope. Before detecting or taking the pictures, the surfaces of the coverslips were washed with a cotton swab soaked in 70% ethanol. Immersion oil was used to enhance the resolution.

3.5 SiRNA silencing method

SiRNA silencing was used to detect the function of Protein X in mitochondrial morphology. The protocol is based on the protocol on Appendix 1. After the confluence of U-2 OS cells reached to 90%, the cells were split and seeded on a 6 well plate with 2 x 10^5 cell/well. Three coverslips were put into one well. The siRNA mix solution was prepared by adding 30 µL of 1 µM Protein X or control scramble siRNA, 470 µL of Opti-MEM® medium and 5 µL of Lipofectamine® RNAiMAX reagent. The solution was gently vortex, and then incubated at room temperature for 20 minutes. The solution was added into corresponding well. Three wells were prepared, and one was incubated for 24 hours, the second for 48 hours and the third was incubated for 72 hours at 5% CO_2 incubator at 37 °C. At the same time, a control scramble siRNA was used as a control. Coverslips were detected with immunofluorescence method for analyzing mitochondrial morphology, and the rest of the cells were collected and used by Western blot to detect siRNA silencing efficiency.

4 Results and Discussion

4.1 Subcellular localization of Protein X

Localization of Protein X was detected with immunofluorescence. As shown in Figure 3, mitochondria were labeled with red color by staining the cell with Tom20 primary antibody (PAB) (Santa Cruz Biotechnology), which is a mitochondrial outer transmembrane membrane protein. The green color is Protein X PAB staining showing the endogenous protein. Merging of the red and green images showed clear co-localization of protein X and Tom20, suggesting that Protein X localizes predominantly to mitochondria.

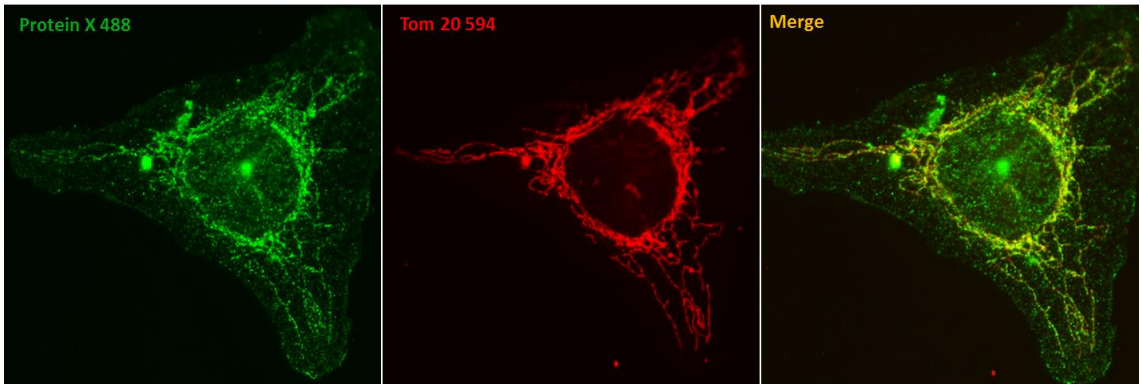

Figure 3. A cell stained with antibodies. This picture illustrates a cell which has been stained with Protein X and Tom20 primary antibodies, and the separate pictures have been merged into one on the third picture.

The localization of Protein X was further confirmed by Western blot to analyze the expression level of Protein X in cytosolic fraction and mitochondria (Figure 4). In this method, mitochondria were isolated from U-2 OS cells. The cytosolic fraction and the mitochondria were separately collected and used to detect the subcellular localization of Protein X. The membrane was separately incubated with Protein X PAB, mitochondria marker Tom20 PAB, protein loading control GAPDH (Thermo Scientific) PAB and cytoskeleton marker α-Tubulin (Sigma) PAB.

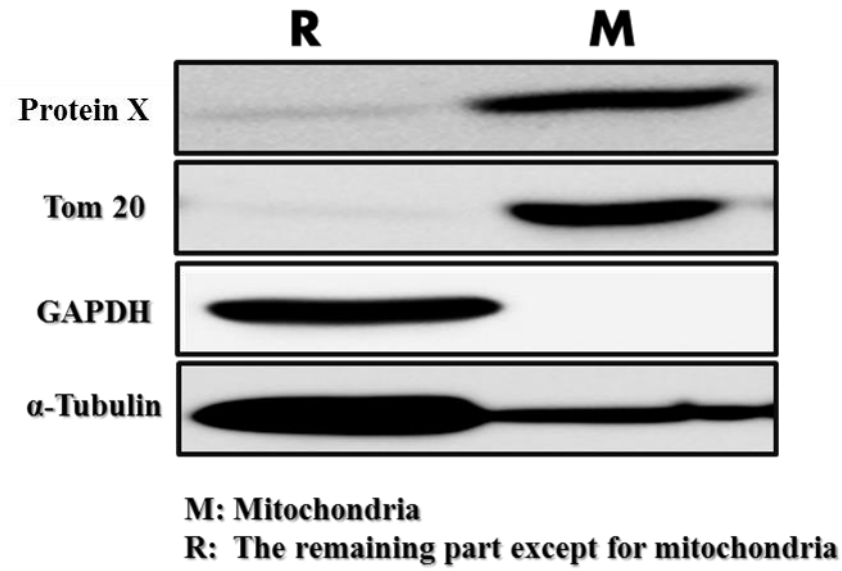

Figure 4. Western blot result for isolated mitochondria. This picture illustrates the localization of Protein X and other marker proteins in mitochondria and cytosolic fraction. Picture was obtained with Western blot using the indicated antibodies.

The Western blot data in Figure 4 showed that Protein X is mainly located in mitochondrial fraction in comparison with the fraction containing the remaining parts. Combined with immunofluorescence results, it was concluded that Protein X mainly localizes to mitochondria.

4.2 Sub-mitochondrial localization of Protein X

Sub-mitochondrial localization of Protein X was detected in isolated mitochondria by detecting the resistance of Protein X with externally added proteinase K with or without Triton® X-100 to soluble the mitochondrial membrane. Subsequently Western blot was used to examine the sub-mitochondrial localization of protein X.

As shown in Figure 5, Protein X remained protected against proteinase K in intact mitochondria. Tom20 is a mitochondrial outer membrane protein which is completely degraded when treated with proteinase K, however, inner membrane protein Cox IV (Thermo Scientific) and matrix protein Hsp 60 (Stressmarq Biosciences) remained protected and only degraded in disrupted mitochondrial membrane. Protein X showed the same trend. This result shows that Protein X localizes inside of mitochondria.

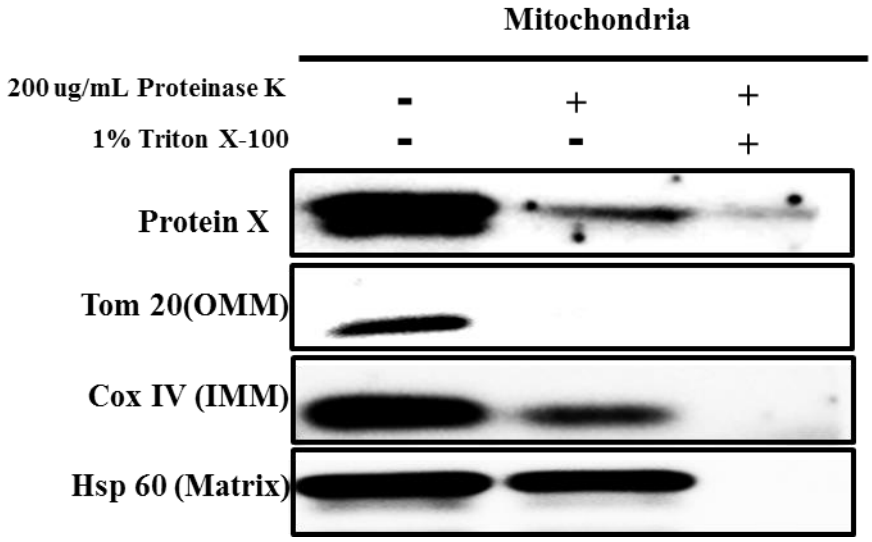

Figure 5. Western blot result for Proteinase K and TX-100 treatment. This picture illustrates the localization of Protein X when the mitochondria had been treated with different ways. Picture was obtained with Western blot using the indicated antibodies.

Alkaline extraction was used to examine, if Protein X is a membrane binding protein. As shown in Figure 6, at low pH value (pH 11), Protein X mainly resided in the pellet fraction, indicating that it can bind to mitochondrial membrane through electronic interference. However, along with the increase of pH value, Protein X was gradually released into the supernatant fraction. In combination with positive control protein analysis, it was seen that mitochondrial inner transmembrane protein, Cox IV, always remained in the pellet fraction (membrane fraction). However, mitochondrial matrix soluble protein, Hsp 60, was only detected in the supernatant fraction. This results suggests that Protein X is a membrane binding protein. Combined with proteinase K digestion experiments, conclusion was that Protein X localizes in the mitochondrial inner membrane.

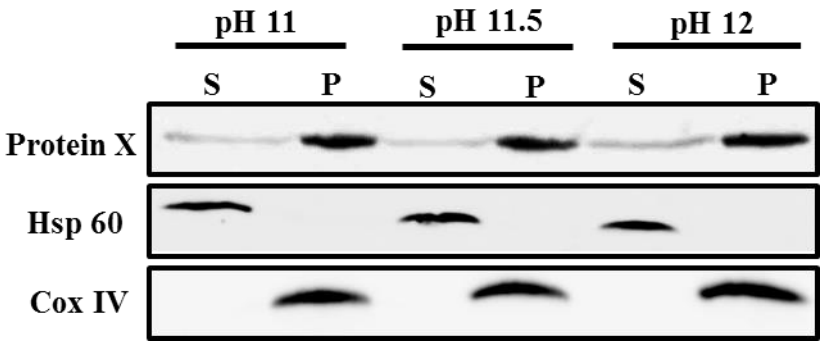

Figure 6. Western blot result of alkaline extraction. This picture illustrates the membrane binding ability of Protein X in mitochondria. Isolated mitochondria were treated with different pH value of sodium carbonate solutions and then separately detect the protein level of Protein X in supernatant fraction and pellet fraction. Picture was obtained with Western blot using the indicated antibodies.

4.3 Native oligomeric state of Protein X

The native oligomeric state of Protein X was determined through BN-PAGE analysis. Isolated mitochondria were lysed with digitonin, and directly loaded on blue native-polyacrylamide gel electrophoresis (BN-PAGE) without boiling to determine the native protein complexes. Previous researches show that the proteins in electron transport chain forms different protein complexes, for example, complex IV (Cox IV) could be dimerized or oligomerized on mitochondrial inner membrane .[20]

As shown in Figure 7, except for monomer of Cox IV, there was also detected dimer and oligomer states of Cox IV, which suggests that the method was able to successfully distinguish different molecular weights of protein complexes, without breaking their native protein structures.

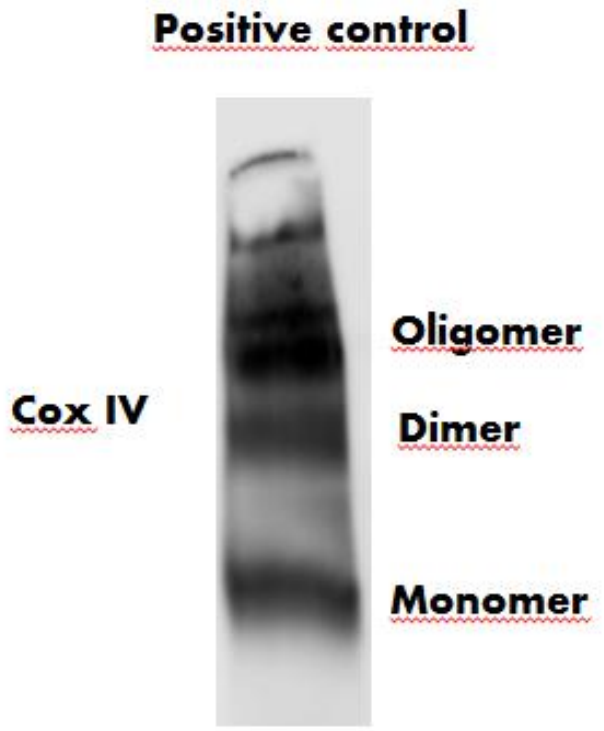

Figure 7. Oligomeric state of Cox IV. Cox IV is a positive control that showed the BN-PAGE experiment did successfully determine the oligomeric state of the native proteins.

When the oligomeric state of Protein X was detected (Figure 8), only one band could be traced, which suggests that Protein X does not form dimer or oligomer structure in cells. In conclusion, the Protein X remains as a monomer to fulfill its cellular function in mitochondria.

Figure 8. BN-PAGE result for Protein X. This picture illustrates the native oligomeric state of Protein X in mitochondria with BN-PAGE analysis. Sample was loaded 4 times in order to see variance.

4.4 Cellular function of Protein X

In order to study the cellular function of Protein X, the expression of Protein X was knockdown through siRNA silencing technology. As shown in Figure 9, after 120 hours of silencing, the same amount of cells and mitochondria were collected, which was verified from the same protein level of Cox IV and GAPDH in the cells. However, the expression level of Protein X was significantly different, In comparison with control scramble cells, the expression level of Protein X was almost completely decreased in Protein X siRNA cells. This result suggests that this siRNA target sequence was able to successfully knockdown the protein expression of the Protein X.

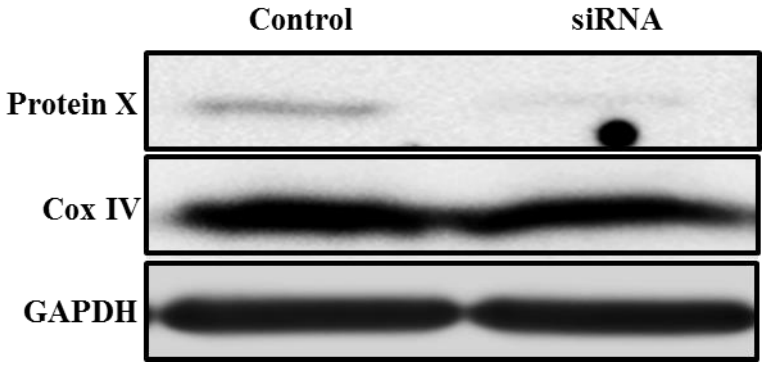

Figure 9. Western blot result for verifying silencing efficiency. This picture illustrates the Protein X targeting siRNA sequence was able to efficiently silence the protein expression of Protein X. Picture was obtained with Western blot using the indicated antibodies.

After silencing the expression of Protein X, the effect on mitochondrial morphology changes was detected with immunofluorescence. Interestingly, as shown in Figure 10, the mitochondria had the normal tubular structure in control scramble cells, while severely changed from tubular shape into rounded, swollen and fragmented pieces after losing the expression of Protein X. These results revealed that loss of Protein X induced remarkable mitochondria dysfunction which indicate that the Protein X is vital for maintaining the normal mitochondrial morphology in cells.

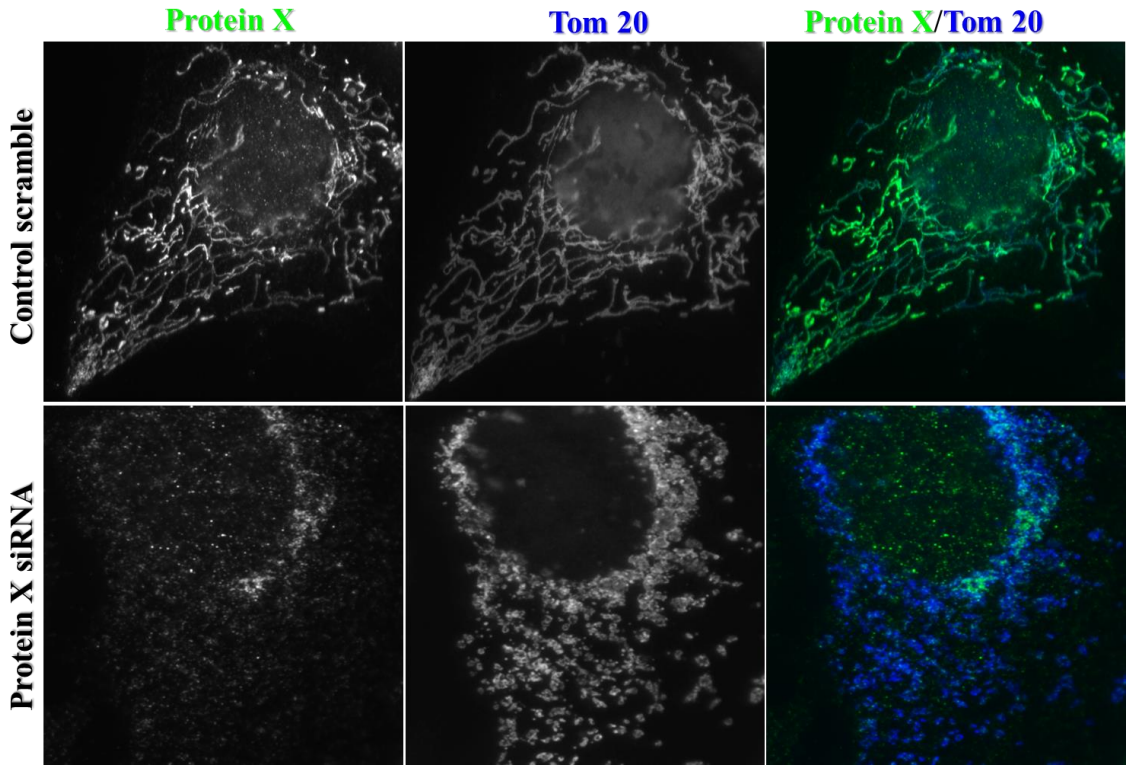

Figure 10. Immunofluorescence result for Protein X siRNA silenced cells. This picture illustrates the function of Protein X by siRNA induced gene knockdown. The upper lane is control scramble cells which show normal tubular mitochondrial structure. The lower lane is Protein X siRNA silencing induced cells. The cells were stained with Protein X antibody and Tom20 antibody, and the two separate pictures on each row have been merged into one on the third picture.

5 Conclusions

This thesis focused on studying the localization and function of Protein X in mitochondria. The results in this thesis revealed that the experiments were successful. The results from Western blot and immunofluorescence show that the Protein X localizes predominantly to mitochondrial inner membrane. The BN-PAGE analysis suggests, that the Protein X is a monomer in U-2 OS cells. Detection of siRNA silencing with immunofluorescence shows that the Protein X plays a vital role in maintaining a normal mitochondrial morphology in cells.

Isolation of mitochondria was difficult at first. The team spent much time to master the art of isolating mitochondria. If the isolation of mitochondria does not work or it does not yield enough mitochondria to work with, the method should be refined to fit the personnel doing it. The biggest impact on the yield was the amount of strokes done, and the power used to do the strokes.

In the past 6 months, plenty of new data about the Protein X was discovered. The research is ongoing, and Zhao's Team will work on this project. The ongoing research will include studying the cellular function of Protein X in different cell types and detecting the structure of Protein X. Also, studying the function of Protein X with other mitochondrial proteins is remarkable.

The research of this thesis provided essential new information about the mitochondrial role of Protein X. Further research on Protein X might lead us to a greater understanding on how mitochondrial morphogenesis is affected by different proteins. Furthermore, this thesis provides a very good protocol for isolating mitochondria from cells and various ways to study mitochondrial proteins.

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Appendix 1 Lipofectamine® RNAiMAX reagent protocol

Protocol Pub. No. MAN0007825 Rev.1.0

Lipofectamine® RNAiMAX Reagent

Package Contents

Catalog Number	Size
• 13778-100	0.1 mL
• 13778-030	0.3 mL
• 13778-075	0.75 mL
• 13778-150	1.5 mL
• 13778-500	15 mL

Storage Conditions

Store at 4°C (do not freeze).

Required Materials

- siRNA or miRNA (10 µM stock)
- Opti-MEM® Reduced Serum Medium
- Eppendorf tubes

Timing

Preparation: 10 minutes
Incubation: 5 minutes
Final Incubation: 1-3 days

Selection Guide

Lipofectamine® Reagents

Go online to view related products.

Product Description

- Lipofectamine® RNAiMAX Transfection Reagent is a proprietary formulation for transfecting small RNAs (e.g., siRNA, Silencer® Select siRNA, Stealth® RNAi, mirVana™ miRNA mimics and inhibitors) into a wide range of eukaryotic cells.

Important Guidelines

- RNA-Lipofectamine® RNAiMAX complexes must be made in serum-free medium such as Opti-MEM® Reduced Serum Medium and can be added directly to cells in culture medium, in the presence or absence of serum/antibiotic.
- It is not necessary to remove complexes or change/add medium after transfection.
- Use 10 nM RNAi duplex as a starting point. BLOCK-iT™ Alexa Fluor® Red Fluorescent Oligo (Cat. no. 14750100) can be used to determine transfection efficiency.

Online Resources

Visit our product page for additional information and protocols. For support, visit www.lifetechnologies.com/support.

For Research Use Only. Not for use in diagnostic procedures.

invitrogen by life technologies

Print Options

Protocol Outline

A. Plate cells so they will be 60-80% confluent at the time of transfection.
B. Prepare RNA-lipid complexes.
C. Add RNA-lipid complexes to cells.

Lipofectamine® RNAiMAX Transfection Protocol

See page 2 to view a typical RNAiMAX transfection procedure.

Transfection Amounts

	96-well	24-well	6-well
Final siRNA used per well	1 pmol	5 pmol	25 pmol
Final Lipofectamine® RNAiMAXused per well	0.3 µL	1.5 µL	7.5 µL

Reverse Transfection of RNAi

Reverse transfection is faster to perform than forward transfection and is the method of choice for high-throughput transfection. Perform reverse transfection by preparing complexes inside the wells, and then adding cells and medium. Because the cells and siRNA-lipid complexes are prepared on the same day, we recommended using 2.5× more cells than for a regular transfection method.

Scaling Up or Down Transfections

Limited Product Warranty and Disclaimer Details

life technologies™

Typical RNAiMAX Transfection Procedure

Transfect cells according to the following table. The transfection is designed for one RNA amount combined with one amount of Lipofectamine® RNAiMAX. **The prepared mix is enough to have triplicates (96-well), duplicates (24-well), and single well (6-well) transfections, and account for pipetting variations.** For additional information on scaling your transfection reaction, see page 1.

Timeline		Steps	Procedure Details			
			Component	96-well	24-well	6-well
Day 0	1 	Seed cells to be 60-80% confluent at transfection	Adherent cells	1–4 × 10^4	0.5–2 × 10^5	0.25–1 × 10^6
	2 	Dilute Lipofectamine® RNAiMAX Reagent in Opti-MEM® Medium	Opti-MEM® Medium	25 µL	50 µL	150 µL
			Lipofectamine® RNAiMAX Reagent	1.5 µL	3 µL	9 µL
	3 	Dilute siRNA in Opti-MEM® Medium	Opti-MEM® Medium	25 µL	50 µL	150 µL
			siRNA (10 µM)	0.5 µL (5 pmol)	1 µL (10 pmol)	3 µL (30 pmol)
Day 1	4 	Add diluted siRNA to diluted Lipofectamine® RNAiMAX Reagent (1:1 ratio)	Diluted siRNA	25 µL	50 µL	150 µL
			Diluted Lipofectamine® RNAiMAX Reagent	25 µL	50 µL	150 µL
	5 	Incubate	Incubate for 5 minutes at room temperature.			
			Component	96-well	24-well	6-well
	6 	Add siRNA-lipid complex to cells	siRNA-lipid complex per well	10 µL	50 µL	250 µL
			Final siRNA used per well	1 pmol	5 pmol	25 pmol
			Final Lipofectamine® RNAiMAX used per well	0.3 µL	1.5 µL	7.5 µL
Day 2–4	7 	Visualize/analyze transfected cells	Incubate cells for 1–3 days at 37°C. Then, analyze transfected cells.			