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Master's university study

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SVEUČILIŠTE U RIJEKA

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Diplomski sveučilišni studij

„Biotehnologija u medicini"

Klara Erceg

Ekspresija i pročišćavanje receptora mitofagije proteina BNIP3L/NIX

Diplomski rad

Rijeka, 2023.

Mentor: Prof. dr. sc. Ivana Novak Nakir

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This thesis has 41 pages, 5 pictures and 27 references.

Abstract

Homeostasis is one of the most important mechanisms that a living organism can have. It maintains and regulates the internal environment of organisms through numerous pathways. One of them is autophagy, a mechanism that balances the production and destruction of cellular organelles and components to preserve cellular integrity. A specific type of autophagy that degrades damaged and dysfunctional mitochondria is called mitophagy. There are a few types of mitophagy, but one of the most interesting ones is the BNIP3L/NIX-mediated mitophagy. The major mediator in this process is BNIP3L/NIX, a protein that comes in a monomeric and dimeric form. The BNIP3L/NIX is positioned on the outer mitochondrial membrane and is crucial for interactions with the autophagosomes and thus promoting mitophagy. To define and study the mechanisms behind this pathway, it would be of great benefit to produce the protein in vitro. As it is a transmembrane protein, this is very challenging. By using pGEX-4T1 plasmids, we successfully achieved the expression of GST-tagged BNIP3L/NIX within *E. coli* BL21 bacterial strain. Furthermore, we effectively purified the expressed protein utilizing Glutathione Sepharose beads. We have concluded that BNIP3L/NIX proteins can be induced in BL21 bacteria in a 3 hour incubation at 37°C and that they have to be extracted from the bacterial pellet rather than the supernatant. We have also concluded that the GST-NIX wt variant is the hardest to produce and purify as opposed to the mutated GST-NIX G203A and ΔTM variants. These results could potentially lead to new discoveries and research done regarding BNIP3L/NIX and its role in BNIP3L/NIX-mediated mitophagy.

Keywords: autophagy, mitophagy, BNIP3L/NIX, dimerization

Sažetak

Homeostaza je jedan od najvažnijih mehanizama koje živo biće može imati. Ona održava i regulira unutarnji okoliš organizama kroz bezbroj bioloških procesa. Jedan od njih je autofagija, mehanizam koji balansira stvaranje i razgradnju staničnih organela i komponenata kako bi se održao stanični integritet. Posebna vrsta autofagije koja razgrađuje oštećene i disfunkcionalne mitohondrije zove se mitofagija. Postoji nekoliko tipova mitofagije, no jedan od najzanimljivijih je mitofagija posredovana BNIP3L/NIX receptorom. Glavni posrednik ovog procesa je BNIP3L/NIX, protein koji se javlja u obliku monomera i dimera. BNIP3L/NIX se nalazi na vanjskoj membrani mitohondrija te je vrlo važan za interakcije s autofagosomima i pokretanje mitofagije. Kako bi proučili i definirali mehanizme iza ovog procesa, potrebno je proizvesti protein u in vitro uvjetima. BNIP3L/NIX je usidren u vanjsku mitohondrijsku membranu, stoga je njegovo pročišćavanje zahtjevan proces. Koristeći plazmide pGEX-4T1, uspjeli smo ne samo proizvesti GST-BNIP3L/NIX proteine(divlji tip i mutante transmembranske domene) u BL21 bakterijama već i pročistiti ga s glutation sefaroznim kuglicama. Došli smo do zaključka da se BNIP3L/NIX proteini mogu inducirati u BL21 bakterijama uz inkubaciju od 3 sata na 37°C te da se moraju izolirati iz bakterijskog peleta, a ne supernatanta. Također smo zaključili da je GST-BNIP3L/NIX divlji tip varijantu puno teže proizvesti i pročistiti u odnosu na mutirane varijante GST-BNIP3L/NIX G203A i ΔTM (mutante koje ne stvaraju dimere). Ovi rezultati mogu potencijalno dovesti do novih otkrića i istraživanja na BNIP3L/NIX-u i mitofagiji posredovanom BNIP3L/NIX-om.

Ključne riječi: autofagija, mitofagija, BNIP3L/NIX, dimerizacija

Contents

1. Introduction

Every living organism on Earth has a system that controls its physiological functions, something that maintains the balance within its body. An essential characteristic that, in case of a disruption, would lead to toxicity and diseases. This system that is required for survival and normal-functioning, is called homeostasis[1].

It is a complex and self-regulating ability that maintains and regulates the internal environment of an organism in a state of constancy[2]. Various homeostatic variables such as body temperature or blood sugar levels have to be regulated within a certain range and that is just what homeostasis does. Homeostatic mechanisms that are in charge are negative and positive feedback. Depending on the signals that the body gives out, they can either signal for a stimulus reduction or an increase respectively. Some of the other variables that homeostasis maintains are nutrient concentrations and fluid balance. All biological processes that are essential for surviving are under homeostatic control[3].

There are various types of homeostasis, such as systemic homeostasis which works on a systemic level and regulates variables like glucose concentration and body temperature. Another type of homeostasis is cellular homeostasis which functions on a cellular level. It maintains variables such as cell membrane potential, cytoplasmic pH, and concentrations of ions[2].

The most important goal of cellular homeostasis mechanisms is to maintain cell integrity. One of the mechanisms used is called autophagy - a pathway that balances the production and destruction of cellular components and organelles and thereby maintains cellular function and life[4].

1.1 Autophagy

The phenomenon of autophagy was first described in the 1960s by biochemist Christian de Duve who was observing organelle degradation within lysosomes. The term autophagy, which was derived from Greek, means „self-eating". His studies ultimately led him to the Nobel Prize in Medicine in 1974, but not for autophagy discovery but for the explanation of the function of the lysosomes[5]. Later on, various other scientists such as Yoshinori Oshumi started studying autophagy more thoroughly and discovered around 15 Atg (autophagy-related) genes. This research was done on yeast, during which homology in Atg genes between yeast and other organisms, including mammals, was found. Because of this discovery, it was possible to investigate autophagy in mammals, too. Some of these genes code for ubiquitin-like ATG proteins which are very important for the formation of double-membrane vesicles called autophagosomes, which contain cellular content that is up for degradation.

In the beginning, autophagy was thought to be stimulated only by starvation or any kind of nutrient stress. This process would lead to the formation of autophagosomes, which, upon merging with lysosomes, facilitated the degradation of the contents enclosed within these vesicles. In those conditions, autophagy acted as a survival mechanism as autophagosome degradation would generate nutrients for the cells[6]. After numerous studies, it is now known that it can also be stimulated by other stressors such as hypoxia, production of ROS molecules, organelle damage, misfolded proteins and intracellular pathogen infection[5]. Autophagy can not only protect cells from starvation but can eliminate toxic cellular waste, prevent necrosis and protect from genome instability. It is an important pathway during cellular development and differentiation and has a role in innate and adaptive immunity[7].

Autophagy can also serve as a modulator of disease progression especially in the case of tumors. While it has a role in suppressing tumors through

cell cycle arrest, necrosis and inflammation inhibition, it can also have an oncogenic role by promoting cell survival during nutrient stress[5].

Defective autophagy can bring numerous problems to the organism. When there is no functioning mechanism that keeps balance within the cells, dysfunctional cellular components accumulate and lead to thedevelopment of diseases. It impacts the immune system which makes the organism more prone to some diseases. It can lead to the development of neurodegenerative diseases as protein aggregates can't be degraded. It is also linked with diabetes and obesity. Defective autophagy is usually associated with aging and age-related diseases[8].

There are three main types of autophagy, chaperone-mediated autophagy, microautophagy, and macroautophagy.

Chaperone-mediated autophagy works by making a complex of chaperone proteins with targeted proteins that are going to be degraded. The targeted proteins are led by chaperones across the lysosomal membrane until they are recognized by a special lysosomal membrane receptor which activates the degradation process[7]. This type of autophagy also only happens in mammal cells[9].

In microautophagy, cellular waste is degraded by direct invagination of the lysosomal membrane[5].

A classic form of autophagy, also known as macroautophagy, starts with the formation of autophagosomes. They can contain damaged cellular organelles, misfolded proteins, nuclear fragments, lipid droplets and so on. The autophagosome fuses with a lysosome so the lysosomal enzymes can degrade the cellular content. The degraded content is then released into the cytosol which can be reused as nutrition for the cell for example[7].

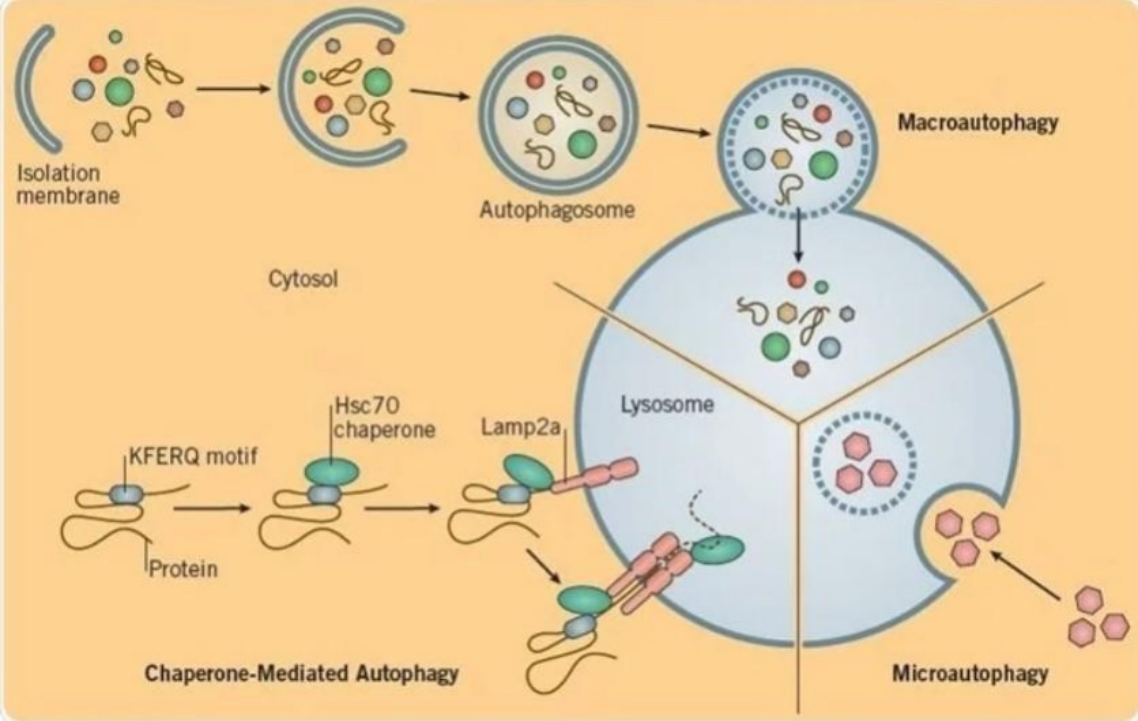


Image 1. *Mechanisms behind macroautophagy, chaperone-mediated autophagy and microautophagy. Taken from News-Medical.net. 2019. Function and Mechanisms of Autophagy. Available from: https://www.news-medical.net/whitepaper/20190817/Function-and-Mechanisms-of-Autophagy.aspx*

Autophagy can also be non-selective and selective. Non-selective autophagy occurs during starvation conditions where a bulk of cytoplasm is engulfed into the autophagosome. Selective autophagy targets certain damaged and superfluous organelles[5]. There are various types such as pexophagy which targets peroxisomes, ribophagy for ribosomes, lysophagy for lysosomes and one of the most important autophagy process which is mitophagy, selective removal of mitochondria[9].

1.2 Mitophagy

Mitophagy is a type of selective autophagy that targets damaged and superfluous mitochondria for degradation. For example, this process is crucial during terminal mammalian erythropoiesis in which reticulocytes differentiate into mature erythrocytes[4]. The term „mitophagy" was first described in 2005. and since then, significant breakthroughs have been

made such as the discovery of essential proteins that mediate the whole process of mitophagy[10].

Mitochondria are double-membrane organelles that have several important roles in the organism. Among these are iron metabolism, cellular calcium level regulation for signaling, or the regulation of proliferation and apoptosis. However, the most significant function is the production of ATP[10]. Mitochondria supply all living cells with energy and ensure normal cellular function. As much as these organelles are the powerhouse of the cell and participate in a fundamental process of supplying cells with energy through oxidative phosphorylation, they also produce harmful ROS molecules[11]. Accumulation of these molecules can cause disbalance in homeostasis and oxidative stress which damages mitochondrial proteins, lipids and mitochondrial DNA. Further damage to mitochondria increases ROS production even more which finally can lead to inflammation. Furthermore, cytochrome c and other pro-death factors are released into the cell cytosol which ultimately leads to apoptosis and death of once perfectly healthy mitochondria[12]. Mitochondrial malfunction is linked to aging and is associated with diseases such as diabetes, various neurodegenerative diseases like Alzheimer's and Parkinson's disease and cardiovascular disorders.

Since mitochondria are constantly challanged with oxidative stress, it has a system that maintains its health. In stressful conditions, the organelles undergo mitochondrial fusion and fission. Mitochondrial fusion allows mixing of the matrix content between healthy and partially dysfunctional mitochondria while fission separates damaged mitochondrial components from the healthy ones. Mitophagy also contributes to maintaining the mitochondrial quality control system alongside the fusion and fission processes.

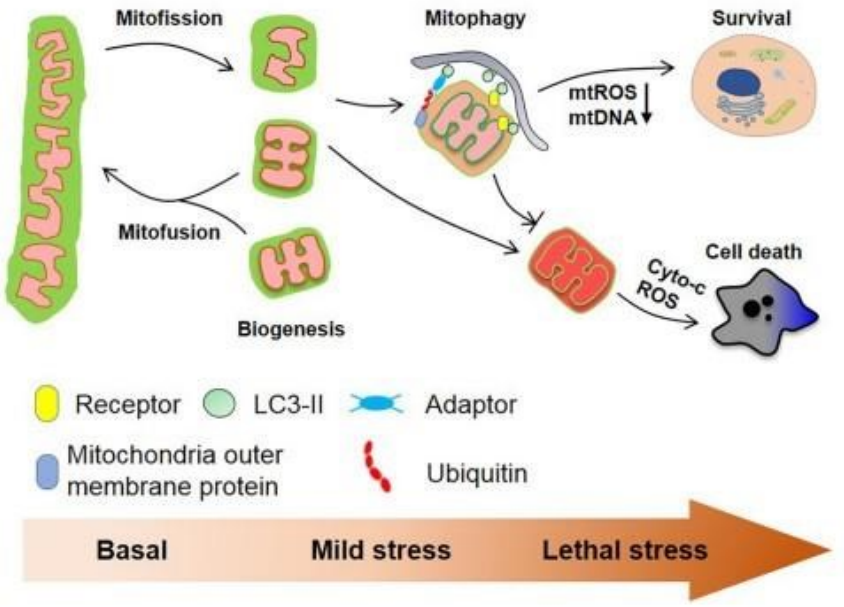


Image 2. *Mitochondrial quality control system and its outcomes. Taken from Ma K, Chen G, Li W, Kepp O, Zhu Y, Chen Q. Mitophagy, Mitochondrial Homeostasis, and Cell Fate. Frontiers in Cell and Developmental Biology. 2020;8.Available from: https://www.frontiersin.org/articles/10.3389/fcell.2020.00467*

There are two types of mitophagy pathways, ubiquitin-mediated and receptor-mediated.

Ubiquitin-mediated pathway, as the name implies, uses the process of ubiquitination to target damaged and dysfunctional mitochondria. The best described pathway of this kind is called PINK1/Parkin-mediated-pathway which is activated by stress[10].

PINK1 (PTEN-induced putative kinase 1), a serine/threonine kinase, is used for maintaining membrane potential for healthy mitochondria. It enters the organelle, is recognized and cleaved by an inner membrane protease PARL (Presenilin-associated Rhomboid-like) and is then released into the cytosol where a proteosome degrades it. When mitochondria are damaged, PINK1 can no longer enter it and therefore it accumulates on the OMM (outer mitochondrial membrane)[13]. After its stabilization on the membrane, PINK1 phosphorylates ubiquitin. Phosphorylated ubiquitin recruits Parkin, an E3

ubiquitin ligase, which is also phosphorylated and activated by PINK1. Activated Parkin now has the ability to ubiquitinate more of the OMM proteins as to label the damaged mitochondria for degradation. Ubiquitin signaling initiates the formation of autophagosomes. Autophagosomal membranes are decorated with LC3/GABARAP (light chain 3/ GABA type A receptor-associated protein) proteins that are covalently attached to the lipids and are main adaptors between cargo that needs to be degradad and autophagosomes. The interaction between LC3/GABARAPs is achieved through the LIR (LC3-interacting region) domain on the phosphorylated OMM proteins. This initiates mitophagy and only then can the autophagosome engulf the organelle and degrade it when fused with a lysosome[4].

Receptor-mediated mitophagy is observed more during other stressors alongside mitochondrial damage, such as cellular differentiation or hypoxia[14]. The receptors located on the OMM receive signals that trigger the beginning of mitophagy. There are many of these receptors, some of them being BNIP3, FUNDC1, BCL2L13 and BNIP3L/NIX. The similarities between them lie in the fact that they are all integral membrane proteins that interact with autophagosomal membrane proteins through the LIR domain[10].

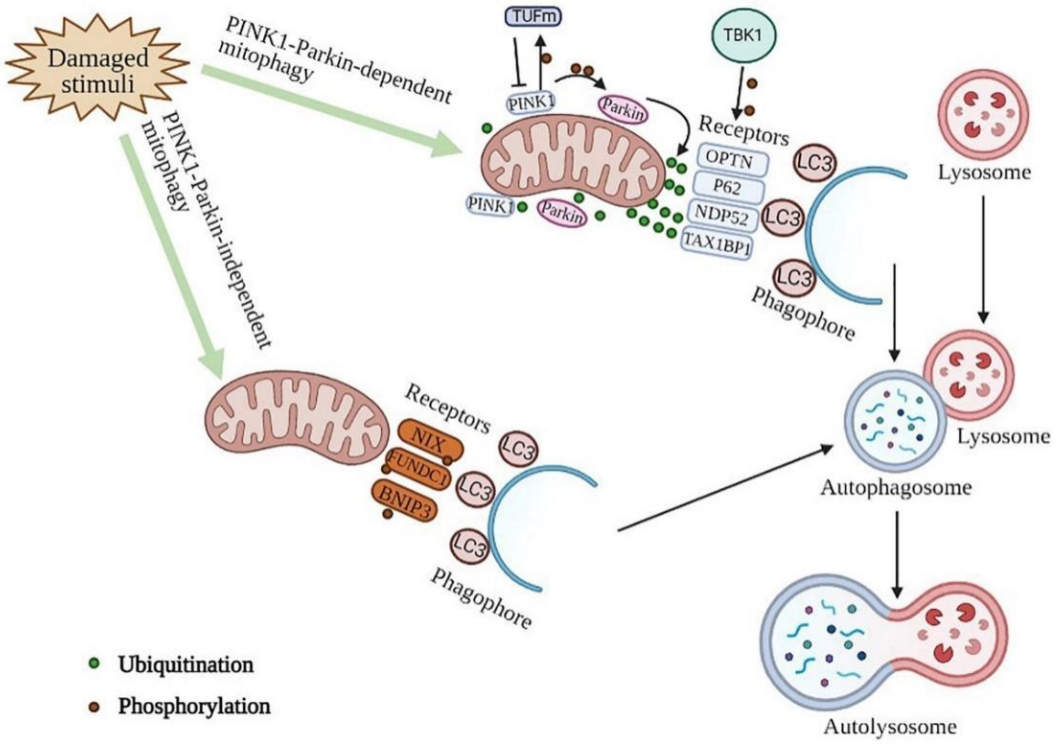


Image 3. *The difference between stress-activated mitophagy and receptor-mediated mitophagy. Taken from Zhu C long, Yao R qi, Li L xi, Li P, Xie J, Wang J feng, et al. Mechanism of Mitophagy and Its Role in Sepsis Induced Organ Dysfunction: A Review. Frontiers in Cell and Developmental Biology. 2021;9. Available from: dd*[*https://www.frontiersin.org/articles/10.3389/fcell.2021.664896*](https://www.frontiersin.org/articles/10.3389/fcell.2021.664896)

Even though the PINK1/Parkin pathway is the most investigated mitophagy pathway, this thesis focused on the receptor-mediated mitophagy pathway and most importantly, one of its most studied receptor, BNIP3L/NIX.

1.3 BNIP3L/NIX protein

BNIP3L (BCL2/adenovirus E1B 19-kDa-interacting protein 3) or NIX protein is an OMM receptor. It has been first identified and sequenced more than twenty years ago when it was recognised to belong to the Bcl-2 family of proteins. These proteins regulate apoptosis and have either pro-apoptotic or anti-apoptotic function. BNIP3L/NIX differs from the rest of the proteins by having only a BH3 (BCL2-homology 3) domain which initiates apoptosis and autophagy, specifically mitophagy[15]. Alongside the BH3 domain,

BNIP3L/NIX sequence also contains LIR domain, MER domain and TM domain.

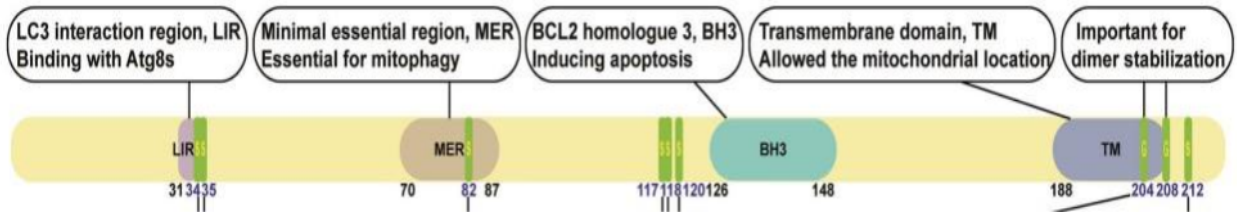


Image 4. *Main domains in the BNIP3L/NIX sequence. Taken from Li Y, Zheng W, Lu Y, Zheng Y, Pan L, Wu X, et al. BNIP3L/NIX-mediated mitophagy: molecular mechanisms and implications for human disease. Cell Death Dis. 2021 Dec 20;13(1):14.*

Like all other mitophagy receptors, BNIP3L/NIX sequence has the LIR domain near the N-terminal ending in its sequence. It's positioned on the cytoplasmic side of the cell for the direct recruitment of autophagosomes. It's composed of four amino acid motif which is needed for interacting with LC3 on the autophagosomal membrane[4].

The MER (Minimal essential region) domain, which is located on BNIP3L/NIX's cytoplasmic domain, is also recognized for its involvement in BNIP3L/NIX activity. It was shown through a study that the deletion of this domain impedes the initiation of BNIP3L/NIX mitophagy. Nevertheless, its exact role in this process is still unclear[16].

The final important region of the BNIP3L/NIX sequence is the TM (transmembrane) domain on the C-terminal ending which allows it to be positioned on the OMM. This domain is very important for localizing BNIP3L/NIX on the OMM and is crucial for BNIP3L/NIX dimer stabilization[16].

As previously mentioned, mitophagy is vital in the process of erythropoiesis and BNIP3L/NIX protein was one of the proteins which were studied. BNIP3L/NIX knockout mice have shown inadequate mitochondrial removal during erythrocyte maturation which caused them to develop anemia[17,18].

Various other studies have shown that BNIP3L/NIX is important for many other essential physiological processes. BNIP3L/NIX is vital for mitochondrial removal during neurogenesis of retinal ganglion cells and proinflammatory macrophages[19]. It participates in cardiac progenitor cell differentiation where it helps a formation of a mitochondrial network in cardiomyocytes[4]. Knockdown of BNIP3L/NIX would lead to mitochondrial fission and an increase in dysfunctional mitochondria[4].

As mentioned before, during the first studies of autophagy in yeast, ATG proteins were identified as having a key role in this process[6]. Atg32 protein was found to mediate mitophagy in yeast similarly to receptor-mediated mitophagy in mammals. This gene has a homolog in mammals as some of the OMM proteins have been found to display identical functions as Atg32, one of them being BNIP3L/NIX. This correlation allowed further studies of BNIP3L/NIX and its interactions with autophagosomal membrane proteins.

BNIP3L/NIX comes in at least two forms, as a monomer and as a dimer. Both forms are located on the OMM. The molecular weight of the monomer form, when performing SDS-PAGE electrophoresis, is ~42 kDa while the molecular weight of the dimer form doubles at ~80 kDa[4].

The difference between the monomer and the dimer form is that BNIP3L/NIX dimers are more potent recruiters of autophagosomes and bind to their LC3 proteins more strongly. As said earlier, the TM domain is crucial for BNIP3L/NIX dimerization and location on the OMM[13]. Studies showed that, when the deletion of the TM domain occurred, BNIP3L/NIX lost its position on the membrane and formed in the cytosol as a monomer[16].

However, if point mutations are made on this domain, such as in 6xHis-NIX G204A variant, BNIP3L/NIX is a transmembrane protein but in a monomeric form[15]. In this protein, point mutations have been made on the 204th Glycin which mutated into Alanin.

Since BNIP3L/NIX was identified, it was recognized as a part of a family of pro-apoptotic mitochondrial membrane proteins with the tendency to make

oligomers. Because of this, we can also hypothesize about BNIP3L/NIX's potential of forming oligomers as higher order structures[4].

As much as BNIP3L/NIX is important for mitochondrial clearance and maintaining cell integrity, it can cause diseases if it becomes dysfunctional. It can lead to a disabled mitochondrial quality control system which can no longer keep balance in the cell. In stressful conditions such as starvation, BNIP3L/NIX can overexpress and lead to mitochondrial damage and cell death[16].

Impaired BNIP3L/NIX is linked to many diseases such as metabolic, cardiovascular and neurological disorders, and cancer. The best described BNIP3L/NIX-related diseases are the ones regarding the heart. Studies have been done on mice where overexpressed BNIP3L/NIX caused lethal perinatal cardiomyopathy[13]. On the other hand, mice with conditional cardiac deletion of BNIP3L/NIX grew normally but later on developed massive cardiac enlargement. The number of mitochondria in their hearts increased and varied in size and internal structure[13].

In other diseases such as cancer, BNIP3L/NIX has a dual role. For example, in melanoma and glioma, it induces autophagic cancer cell death[20,21]. However, the deletion of the protein can delay pancreatic cancer[22]. On the other hand, the upregulation of the protein can delay mitophagic apoptosis in breast cancer[23]. In neurodegenerative diseases, like Parkinson's disease, it can accelerate its development[24]. BNIP3L/NIX is even involved in acute brain injuries where it can, for example, prevent cerebral ischemia wih mitophagy[25].

Although many studies have been made regarding the link between BNIP3L/NIX and various diseases, there is no proof that BNIP3L/NIX has a fundamental role in these diseases. They only show its involvement in the disease phenotype.

1.3.1 BNIP3L/NIX-mediated mitophagy

This type of receptor-mediated mitophagy is also thought of as programmed mitophagy. It involves planned mitochondrial degradation during cell development and differentiation processes, aswell as hypoxia, starvation or any other stressful condition. It has been found in various cells such as retinal ganglion cells, natural killer cells, neurons and some types of tumor cells[16].

The most known mechanism of this mitophagy is through interaction with proteins from the Atg8 family. Their homologs in mammals are the LC3/GABARAP proteins on the autophagosomal membrane[4].

Firstly, the BNIP3L/NIX dimer, which is positioned on the OMM, senses certain intracellular or extracellular signals which trigger recruitment of autophagosomes. BNIP3L/NIX can also be phosphorylated to enhance this recruitment[4]. After that, BNIP3L/NIX interacts with LC3/GABARAP proteins on the autophagosome through its LIR domain[16]. After the engulfment, lysosome fuses with with the autophagosome and releases its enzymes so the mitochondria can be degraded.

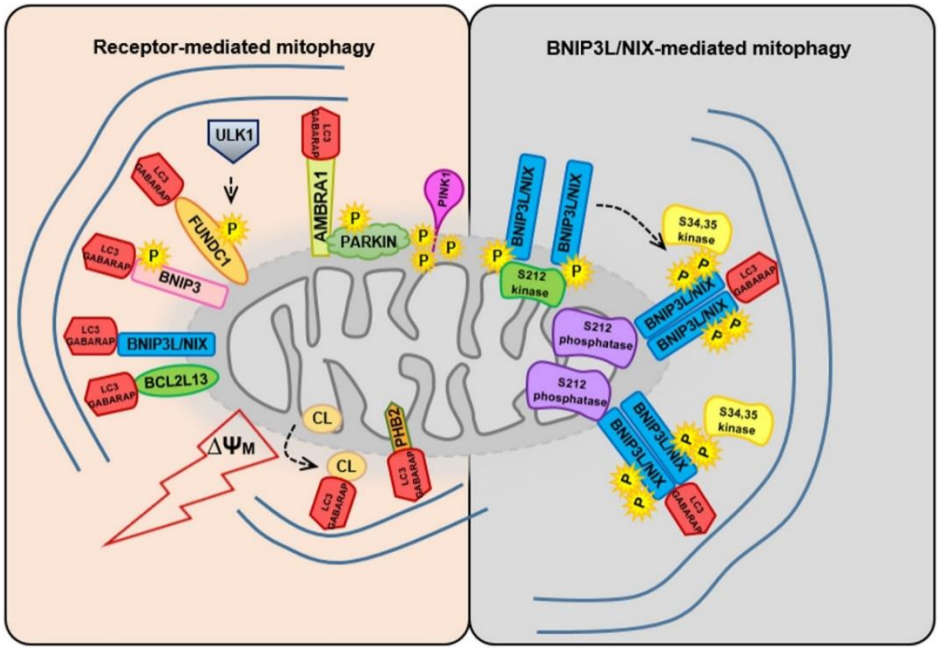


Image 5. *The molecular mechanism behind BNIP3L/NIX-mediated mitophagy. Taken from Marinković M, Novak I. A brief overview of*

BNIP3L/NIX receptor-mediated mitophagy. FEBS Open Bio. 2021 Dec;11(12):3230–6.

There are various ways in which BNIP3L/NIX-mediated mitophagy is regulated and so far the most studied posttranslational modification is, phosphorylation.

Juxtaposed to the LIR domain, there are amino acid residues S34 and S35. When phosphorylated, they promote mitophagy and enhance the autophagosome recruitment to mitochondria by acquiring a higher affinity to LC3 protein[16].

In the MER domain, there is S82 which also requires phosphorylation to induce mitophagy[16].

Near the TM domain, which is important for BNIP3L/NIX dimerization, there is S212. Dephosphorylation of this amino acid residue enhances the homodimerization of BNIP3L/NIX and autophagosome recruitment[4].

Aside from posttranslational modifications, the accumulation of ROS molecules triggers BNIP3L/NIX-mediated mitophagy. Due to highly active oxidative phosphorylation, a small GTPase called Rheb is recruited to the OMM where it forms a complex with BNIP3L/NIX and LC3 to strengthen their bond and form a mitophagosome[10].

There are a lot of things that are known about BNIP3L/NIX mitophagy but there are also some that are still unknown. It is still not clear by which molecular mechanisms BNIP3L/NIX dimerization happens and how its dimer form initiates mitophagy[16]. To understand and define these key mechanisms behind BNIP3L/NIX mediated mitophagy, a BNIP3L/NIX dimer has to be expressed and purified. This is a challenging mission as this protein form is anchored in a membrane and is difficult to isolate from cellular extracts. In this thesis, the focus will be on producing and purifying a BNIP3L/NIX dimer to complete one step in the journey of understanding BNIP3L/NIX-mediated mitophagy.

2. Aims

The main aim of this thesis is to induce expression, produce and purify BNIP3L/NIX protein dimer, produced in BL21 *E. coli* strain. BNIP3L/NIX proteins are labeled with either 6xHis- or GST-tags, depending on the type of plasmid used for production. This thesis focuses more on expressing and purifying a GST-tagged BNIP3L/NIX protein from plasmid pGEX-4T1.

Additionally, the aim is to express and purify BNIP3L/NIX dimer in GST-NIX wild type and GST-NIX G203A, as well as in GST-NIX ΔTM. The G203A and the ΔTM variants both come in a monomeric form but have different location as the G203A variant is located on the OMM while the ΔTM variant is in the cytosol.

3. Materials and methods

3.1 Premade chemicals and antibodies

His-Lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH=8.0)

GST buffer 1 (20 mM Tris/HCl, pH=7.5; 10 mM EDTA, pH=8.0; 5 mM EGTA, pH=8.5; 150 mM NaCl)

GST buffer 2 (20 mM Tris/HCl, pH=7.5; 10 mM EDTA, pH=8.0; 150 mM NaCl, 0,5% Triton X-100)

GST buffer 3 (20 mM Tris/HCl, pH=7.5)

Monoclonal primary Anti-GST-Mouse antibody (1:1000, Santa Cruz Biotechnology)

Monoclonal primary Anti-6xHis-Mouse antibody (1:1000, Novagen)

Monoclonal primary Anti-NIX-Rabbit antibody (1:1000, Santa Cruz Biotechnology)

Polyclonal secondary Anti-Mouse antibody (1:10000, Bio-Rad)

Polyclonal secondary Anti-Rabbit antibody (1:6000, Dako)

3.2 Samples

pET21 and pET23 plasmids were used to express 6xHis-NIX wt and 6xHis-NIX G204A, and plasmid pGEX-4T1 to express GST-NIX wt, GST-NIX G203A and GST-NIX ΔTM.

3.3 PCR and DpnI treatment

Before performing site-directed *In vitro* mutagenesis, the plasmids for wild type BNIP3L/NIX were sent for sequencing to Microsynth. With the NCBI „BLAST" program, their accuracy was confirmed.

To begin site-directed In vitro mutagenesis, a PCR mixture had to be made first. It consists of 33,5 µl of ddH_2O, 5 µl of Pfu buffer (10x PfuUltra II reaction buffer, Agilent), 5 µl of 20 ng/µl of DNA template, 1 µl of dNTPs (Thermo Fisher Scientific), 1 µl of forward and reverse primer, 2,5 µl of

DMSO (Sigma) and 1 µl of Pfu polymerase (PfuUltra II fusion HS DNA polymerase, Agilent).

The tubes containing the mixture were put in the PCR machine (T100 Thermal Cycler, Bio-Rad). The denaturation and annealing steps were set to 95°C for 3 minutes and a minute respectively, the extension step was set to 60°C for 1 minute, both elongation steps were set to 72°C for 12 minutes and the last step was set to 4°C until the end of the program.

Subsequently, DNA electrophoresis was done so we could see whether PCR was successful. An agarose gel was made by mixing 1% agarose and melting it with 60 ml of 1x TAE buffer. When the mixture cooled down, 1,5 µl of etidium bromide was added to it and poured into the gel cast. After 30 minutes, the agarose gel was solid and put into the electrophoresis chamber which was filled with 1x TAE buffer. The samples were prepared by mixing 5 µl of PCR product and 1 µl of DNA Loading buffer. Samples and DNA marker were then pipetted into the gel wells and electrophoresis was set for 80 V for 30 minutes. After electrophoresis, the gel was visualized on Uvidoc HD6 (Uvitec Cambridge).

If PCR was successful, a DpnI treatment could be done to the PCR product so methylated DNA could be removed. A mixture was made by pipetting 44 µl of PCR product, 5 µl of Tango buffer (Thermo Fisher Scientific) and 1 µl of DpnI (Thermo Fisher Scientific) which was left for incubation for 1 hour, at 37°C.

3.4 Bacteria transformation

Bacteria transformation was done by heat-shock method. Firstly, BL21 bacteria were thawed in ice. A mixture was made with 100 µl of bacteria and no more than 100 ng of plasmid which was incubated on ice for 20 minutes. After 20 minutes, the cells were heat-shocked for 2 minutes at 42°C.

Next, around 1 ml of LB media was pipetted into the tube and put for incubation for an hour while shaking at 450 rpm, at 37°C. Once incubation was over, the cells were centrifuged for 5 minutes at 1500x g.

After that, most of the supernatant was removed while the bacteria pellet was resuspended in the remaining supernatant. Lastly, the whole mixture was pipetted onto ampicillin-selective agar plates and put for overnight incubation at 37°C.

Before protein induction, a bacterial culture is made by pouring 20 ml of LB media into a flask and adding 100 µg/ml of ampicillin (Roth) to it. One bacterial colony was picked from the plate with a pippette tip and put in the flask for overnight incubation at 220 rpm, at 37°C.

These samples were also later sent for sequencing to confirm the mutagenesis was performed correctly.

3.5 Protein induction

Following an overnight incubation period, a new bacteria culture with a bigger LB media volume was cultivated by passaging the overnight culture into it. OD_{600} measurements were then performed to assess the bacterial growth phase's optimization.

A spectrophotometer (Beckman Coulter DU 530) was used in which an OD_{600} measurement in the range of 0.350 to 0.600 had to be reached.

First, a blank reading was made with LB media so the machine could be callibrated. Next, each of the samples that were incubated at 37°C overnight was measured. Measurements were done until a result was obtained in the optimal range previously mentioned. Then, 0.5 mM IPTG (Roth) was added to the bacteria culture and was left for a 3 hour incubation on 220 rpm, at 37°C.

Following incubation, the bacterial cultures were centrifuged at 2000x g for 20 minutes at 4°C to obtain the bacterial pellet and remove the supernatant.

Samples for SDS-PAGE were also taken, one before IPTG induction and one after it.

3.6 Protein purification

Protein purification varied depending on the used tag.

3.6.1 6xHis-tagged protein purification

For 6xHis-NIX purification, a 6xHis lysis buffer was used. From this premade chemical, two buffers were prepared by adding a certain amount of imidazole (Sigma-Aldrich) to them, the lysis buffer and the wash buffer. The lysis buffer had to have a 10 mM concentration of imidazole while the wash buffer had to have 20 mM concentration of it. The amount of imidazole depended on the volume of the buffers which at the same time depended on the number of samples. After adding imidazole to the lysis buffer, protease inhibitors were pipetted like 1 mM Leupeptin, 1 mM Aprotinin, 1 mM Na_3VO_4, 1 mM Lyzozime and 1 mM PMSF. 50 µg/ml of DNAse and 5 mM $MgCl_2$ were also added to the mixture. Using this buffer, bacterial pellets were resuspended and incubated on ice for an hour. Next, the pellets were taken for sonication. An MS 73 sonde (Sonopuls HD 2200) was used with a 2 minute cycle sonication on each sample.

Meanwhile, Ni-NTA agarose beads (Cube Biotech) that the 6xHis-tagged proteins were going to stick to were washed. A mixture was made with 3 ml of 6xHis lysis buffer and 20 µl of the beads. We centrifuged it for 3 minutes at 500x g, at 4 °C. This step was repeated 2 more times after which the supernatant was mostly removed each time.

After that, sonicated pellets were centrifuged for 30 minutes at 8500x g, at 4 °C. The supernatant was put into a new tube and prewashed Ni-NTA beads were added to it. The mixture was put on a rotator for an hour, at 4°C

After an hour, the mixture was centrifuged for 3 minutes at 500x g, at 4 °C. Most of the supernatant was removed and we added around 3 ml of wash buffer to it. the mixture was once again centrifuged at the same

setting on the centrifuge after which most of the supernatant was removed. This step was repeated 3-5 times.

At the end of this step, all of the supernatant was removed with the insulin syringe and the same volume of His lysis buffer without imidazole as the amount of the beads was added to the mixture.

After each of the steps of purification, a sample was taken for SDS-PAGE electrophoresis.

3.6.2 GST-tagged protein purification

For GST tagged proteins, GST buffers were needed. GST buffer 1 was used and the volume was dependent on the number of samples. 10 ml per sample was pippeted and various protease inhibitors were added such as 1 mM Leupeptin, 1 mM Aprotinin, 1 mM Na_3VO_4, 1 mM Lyzozime, 1 mM PMSF as well as 50 µg/ml of DNAse and 5 mM $MgCl_2$. With this mixture, bacterial pellets were resuspended and were left for incubation on ice for one hour with occasional vortexing.

During the incubation, Glutathione Sepharose beads (GE Healthcare) were washed three times with 1x PBS and centrifuged for 3 minutes, at 4°C and 400x g as a way to cleanse them. Volume of beads was also measured by the number of samples so usually 20 µl of beads was used per sample.

Following the incubation, bacterial samples were sonicated in a 2-minute cycle with a 55% power. Samples were later once again left for an ice incubation for approximately 20 minutes.

After that, the bacterial samples were centrifuged for 20 minutes at 8500x g, at 4 °C. Following this, the supernatant was collected and prewashed Gluthatione Sepharose beads were added to it. The mixture in the tube was put on a rotator for two hours, at 4°C.

After two hours, the mixture was centrifuged for 3 minutes at 500x g, at 4 °C. This step was repeated 3-5 times and each time the supernatant was removed and around 3 ml of GST buffer 2 was added to it. After the final

centrifugation, the supernatant was removed completely using an insulin syringe. The added volume of GST buffer 3 to the tube was the same as the volume of Gluthatione Sepharose beads was added.

After each step of the purification, samples have also been taken for SDS-PAGE electrophoresis.

3.7 SDS-PAGE electrophoresis

After all of the samples were obtained, SDS-PAGE electrophoresis method was used where the proteins were separated and denatured by the anionic detergent SDS. Since proteins were tagged with either 6xHis or GST tags, their molecular weight was easily identified using the protein marker (Proteintech). Firstly, a running gel had to be made. The percentage of the gel was determined by which tag the protein was tagged with. Usually, the separation of 6xHis-tagged proteins works best with 12% running gel while GST-tagged proteins are best separated on a 10% gel. 12% running gel consists of 3 ml 40% Acrylamide, 2,5 ml of lower buffer, 4,5 ml of autoclaved H_2O, 50 µl of 10% APS and 10 µl of TEMED. 10% running gel is made of 2,5 ml 40% Acrylamide, 2,5 ml of lower buffer, 5 ml of autoclaved H_2O, 50 µl of 10% APS and 10 µl of TEMED.

After making the running gel, a 4% stacking gel was made by pipetting 1 ml 40% Acrylamide, 1,875 ml of upper buffer, 5 ml of autoclaved H_2O, 37,5 µl of 10% APS and 7,5 µl of TEMED.

The SDS-PAGE electrophoresis was performed with Mini Protean 3 cell (Bio-Rad) which was filled with 1x running buffer. The electrophoresis power pack got connected to the tank and was set to work for 90 minutes and 90 V. After 15 minutes, it was turned up to 150 V.

3.8 Membrane transfer

After SDS-PAGE electrophoresis, membrane transfer using nitrocellulose membranes was prepared. Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) was used and its tank was filled with 1x transfer buffer. The power pack was connected to it and set to work for 90 minutes at 200 mA.

After the transfer was done, the membrane was stained with Ponceau solution for visualisation and was washed out with TBST (1x TBS 0,05% Tween buffer) a few times. After washing, blocking buffer was poured onto the membrane which was left to incubate over night in a 4°C fridge.

3.9 Coomassie gel dying

Coomassie gel dying is a simple technique in which the gel is stained with the Coomassie Brilliant Blau dye. This is done to simply visualize the protein bands on the gel instead of performing membrane transfer for western blot later. Gel dying took approximately 15 minutes after which the gel had to be destained with a Coomassie destainer. After one hour of destaining, the protein bands were easily visualised with UV light.

3.10 Western blot

Following overnight incubation with blocking buffer, the primary antibody was poured onto the membrane. The antibody type depends on the tag of the protein so GST- or 6xHis-Mouse primary antibody could be used. After one hour of incubation, the membrane was washed out once again with TBST. Anti-mouse secondary antibody was used next on the membrane which was left for a 45-minute incubation. After the incubation, the final washing with TBST was done. Following this, the membrane was visualized on Chemi Doc XRS+ (Bio-Rad) with Image Lab software. Before visualization, the membrane was wet with a 1:1 solution of Lumigen ECL Ultra Solution A and B (Lumigen). With this, an exact position and molecular weight of a GST- and 6xHis-tagged proteins can be observed. If the results were satisfying, the membrane was blotted again with rabbit-anti-BNIP3L/NIX primary antibody and anti-rabbit secondary antibody following the same protocol previously described. This way we could see whether BNIP3L/NIX protein was produced and purified correctly.

4. Results

Firstly, to confirm the accuracies of the plasmids for wild type BNIP3L/NIX that were going to be used in the experimental setup, several of them were sent for sequencing. After performing the BLAST analysis for them, we have concluded that mice-derived pGEX-4T1/NIX wt as well as human pET23/NIX wt and pET21/NIX wt sequences were accurate.

After the bacteria transformation, we had to induce the production of BNIP3L/NIX with IPTG. We added it to the proteins and left them for a 3-hour incubation and a overnight one at 37°C to observe protein production time. The results of the induction were visible after performing SDS-PAGE electrophoresis and Coomassie gel staining.

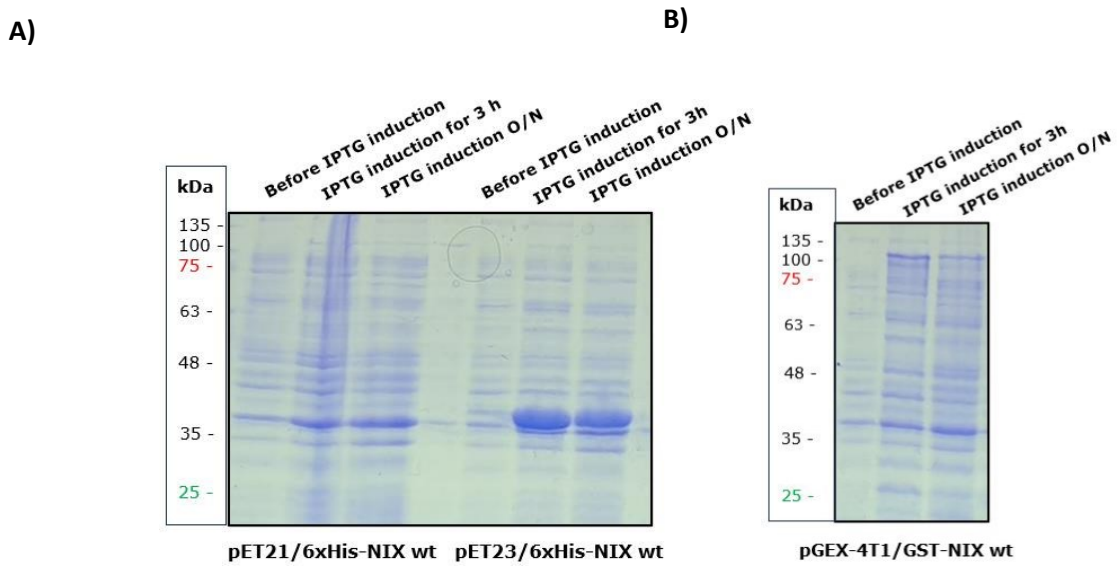


Figure 1. Coomassie Blue gel staining for samples pET21/6xHis-NIX wt, pET23/6xHis-NIX wt and pGEX-4T1/GST-NIX wt. ***(A)*** *Protein detection on Coomassie Blue stained gel before IPTG induction, 3 hours after IPTG induction and over night IPTG induction on samples pET21/6xHis-NIX wt and pET23/6xHis-NIX wt.* ***(B)*** *Visualization of produced proteins on Coomassie Blue stained gel before IPTG induction, 3 hours after IPTG induction and over night IPTG induction on sample pGEX-4T1/GST-NIX wt.*

In Figure 1., each of the samples show a strong signal of protein detection. 6xHis-tagged proteins are strongly detected at ~38 kDa for both pET21/6xHis-NIX wt and pET23/6xHis-NIX wt (Fig. 1A). GST-tagged proteins have a signal at both ~68 kDa and ~102 kDa (Fig. 1B). Both 3 hour incubation and an overnight incubation show similar signals in each sample.

After Coomassie gel staining comes Western blot which shows whether BNIP3L/NIX was actually produced.

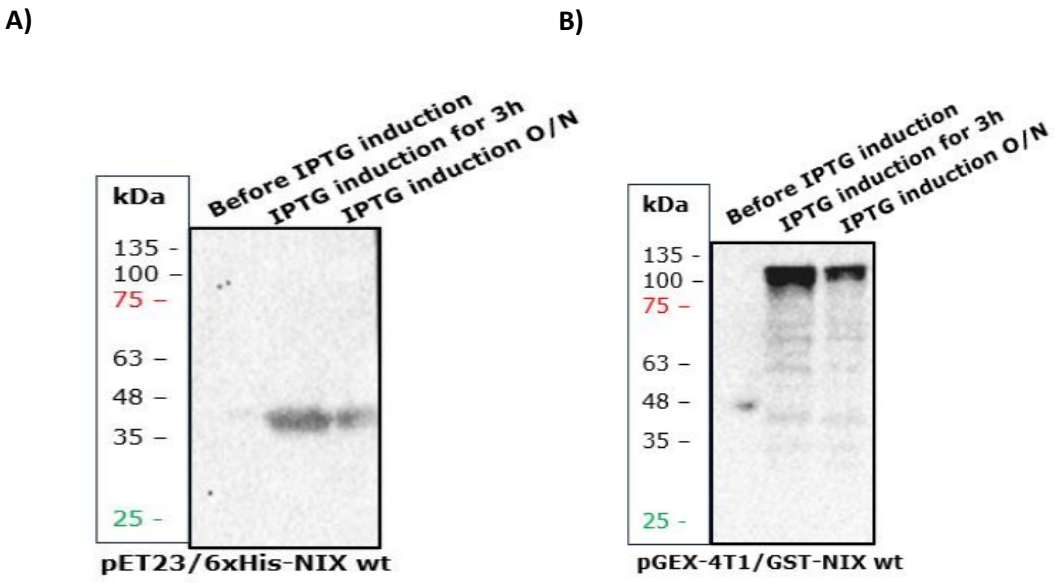


Figure 2. pGEX-4T1/GST-NIX wt signal is stronger compared to pET21/6xHis-NIX wt and pET23/6xHis-NIX wt. ***(A)*** *Visualization of His-tagged proteins after Western blot using anti-His antibody. Used on samples before IPTG induction, 3 hours after IPTG induction and overnight IPTG induction for pET23/6xHis-NIX wt. Image Lab software was used for visualisation.* ***(B)*** *GST-tagged protein visualization following Western blot using anti-GST antibody. Used on samples before IPTG protein induction, 3 hours after IPTG induction and IPTG induction over night. Visualised with Image Lab software.*

Westen blot of pET23/6xHis-NIX wt with anti-His antibody shows a weak signal at ~38 kDa for both 3 hour and overnight incubation (Fig. 2A). On

the other hand, the western blot for pGEX-4T1/GST-NIX wt shows an increadibly strong band at ~102 kDa (Fig. 2B). Western blot for pET21/6xHis-NIX wt didn't show any signal.

To confirm whether BNIP3L/NIX was produced, another western blot was done, this time with anti-NIX antibody. After this experiment, none of the samples showed signal on the membrane.

Following western blot, we have done protein purification on the samples and performed SDS-PAGE electrophoresis to visualize the result. In this experiment, a variation of the normal protein purification protocol was done to determine whether BNIP3L/NIX is contained in the bacterial pellet or supernatant after centrifugations.

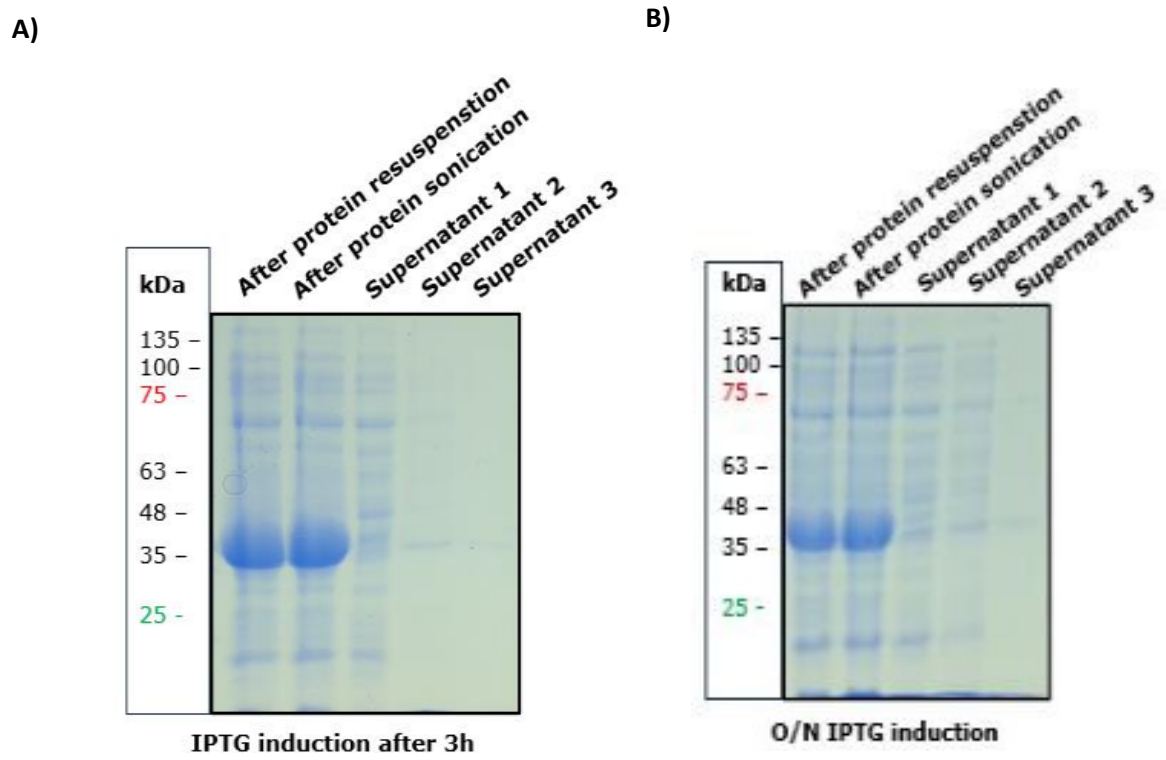


Figure 3. Coomassie Blue gel staining for sample pET23/6xHis-NIX wt after the protein purification technique. ***(A)*** *Protein visualization following protein purification steps such as protein resuspension, protein sonication and centrifugation for sample pET23/6xHis-NIX wt that was induced with IPTG for 3 hours.* ***(B)*** *Visualisation of proteins from sample pET23/6xHis-NIX wt which were induced with IPTG over night and purified.*

After each centrifugation, the supernatants were removed and wash buffer was added to the bacteria pellets of each sample.

In both samples for a 3 hour and overnight incubation for pET23/6xHis-NIX wt, a signal is strongly detected until the first centrifugation and slowly weakens as the centrifugation step is repeated.

Following protein purification, western blot was done again but there was no signal for any of the samples.

We decided we wanted to try these experiments with mutated BNIP3L/NIX sequences such as pET21/6xHis-NIX G204A, pET23/6xHis-NIX G204A and pGEX-4T1/GST-NIX G203A because there was not any major success with wild type variants. The plasmids were sent for sequencing before and their accuracies were confirmed.

In PCR, Taq polymerase was used in the beginning, but as the experiments continued to fail, a switch was made to use Pfu polymerase which brought success.

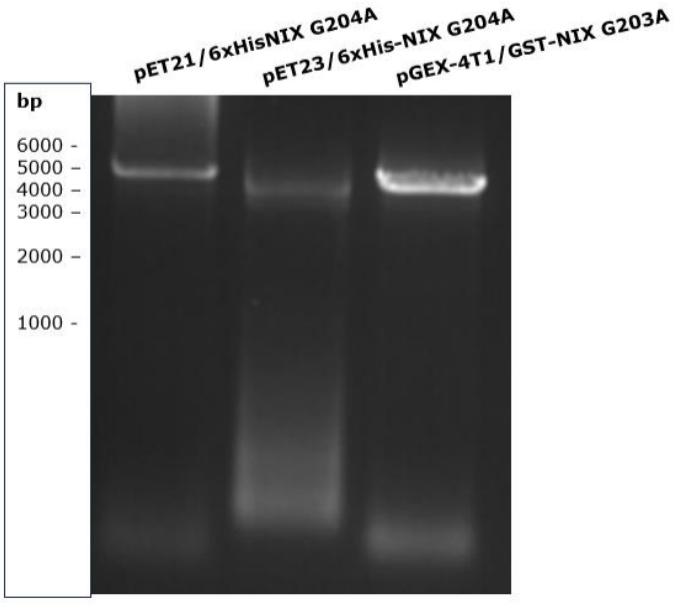


Figure 4. DNA electrophoresis gel for samples pET21/6xHis-NIX G204A, pET23/6xHis-NIX G204A and pGEX-4T1/GST-NIX G203A. *After performing PCR technique, samples pET21/6xHis-NIX G204A,*

pET23/6xHis-NIX G204A and pGEX-4T1/GST-NIX G203A were prepared for DNA electrophoresis. 1 kb DNA step ladder (Promega) was used as a DNA marker.

DNA bands for all three samples are detected at a similar size, at ~5000 bp (Fig.4). Sample pGEX-4T1/GST-NIX G203A shows the strongest signal compared to the other two samples.

Alongside the previously mutated GST-NIX strains, we have also experimented with pGEX-4T1/GST-NIX ΔTM. This plasmid sequence was not sent for sequencing as the rest of the mutated variants because it was already ready in the laboratory. After the protein purification and western blot with anti-NIX antibody, a signal was detected at ~68 kDa (Fig.5).

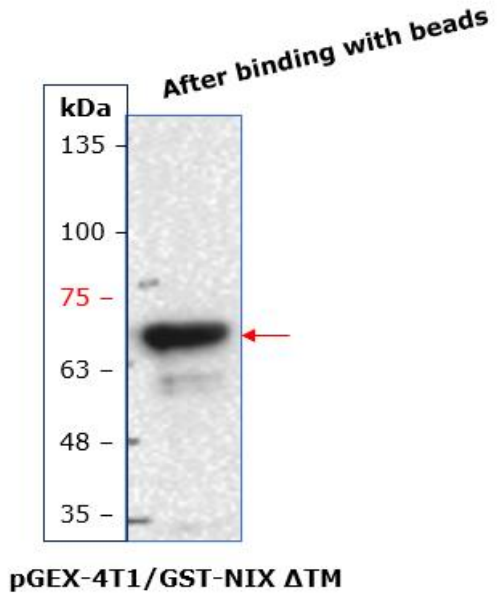


Figure 5. Western blot shows strong signal for sample PGEX-4T1/GST-NIX ΔTM. *Proteins binded to Glutathione Sepharose beads are visualised after the protein purification steps and blotting using anti-NIX antibody. Proteins are visualised with Image Lab software.*

After numerous experiments, we have managed to get a strong signal of detected proteins in the sample pGEX-4T1/GST-NIX wt. It was visualised at ~102 kDa (Fig.6A).

A) B)

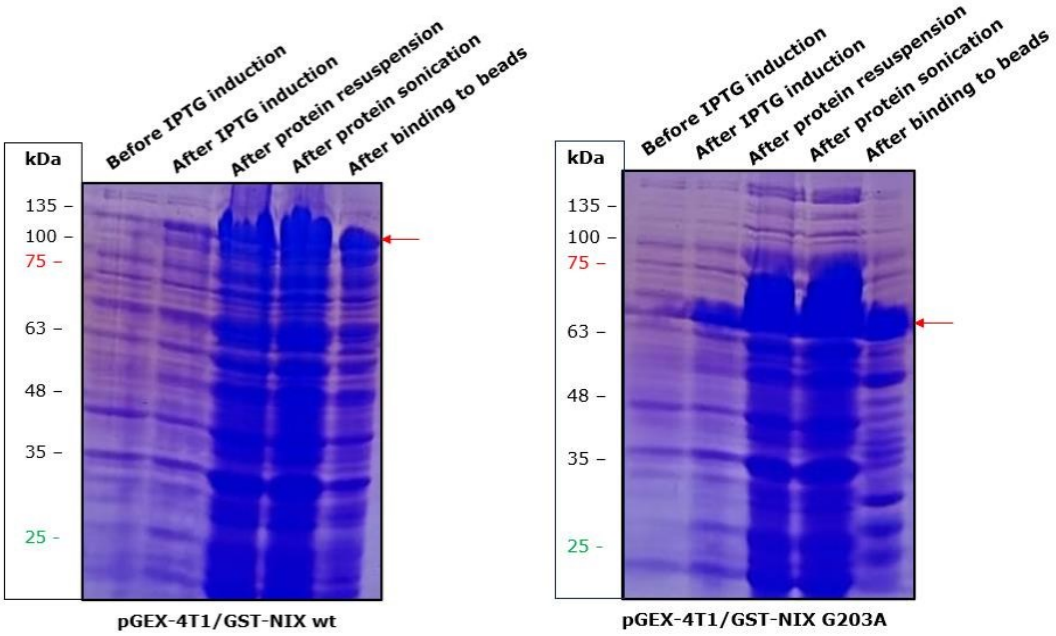


Figure 6. pGEX-4T1/GST-NIX wt and pGEX-4T1/GST-NIX G203A after the protein purification technique. ***(A)*** *Protein purification steps visualised by Coomassie Blue gel staining for sample pGEX-4T1/GST-NIX wt* ***(B)*** *Purified proteins from sample pGEX-4T1/GST-NIX G203A visualised with Coomassie Blue gel staining.*

As for sample pGEX-4T1/GST-NIX G203A, proteins were also visualised at at ~68 kDa (Fig.6B).

Western blot was done using anti-GST antibody, confirming that GST-tagged proteins are migrating at ~102 kDa for sample pGEX-4T1/GST-NIX wt (Fig. 7A) and at ~68 kDa for sample pGEX-4T1/GST-NIX G203A (Fig. 7B). Interestingly, in the sample pGEX-4T1/GST-NIX wt, there also visible protein bands at ~150 kDa.

A) B)

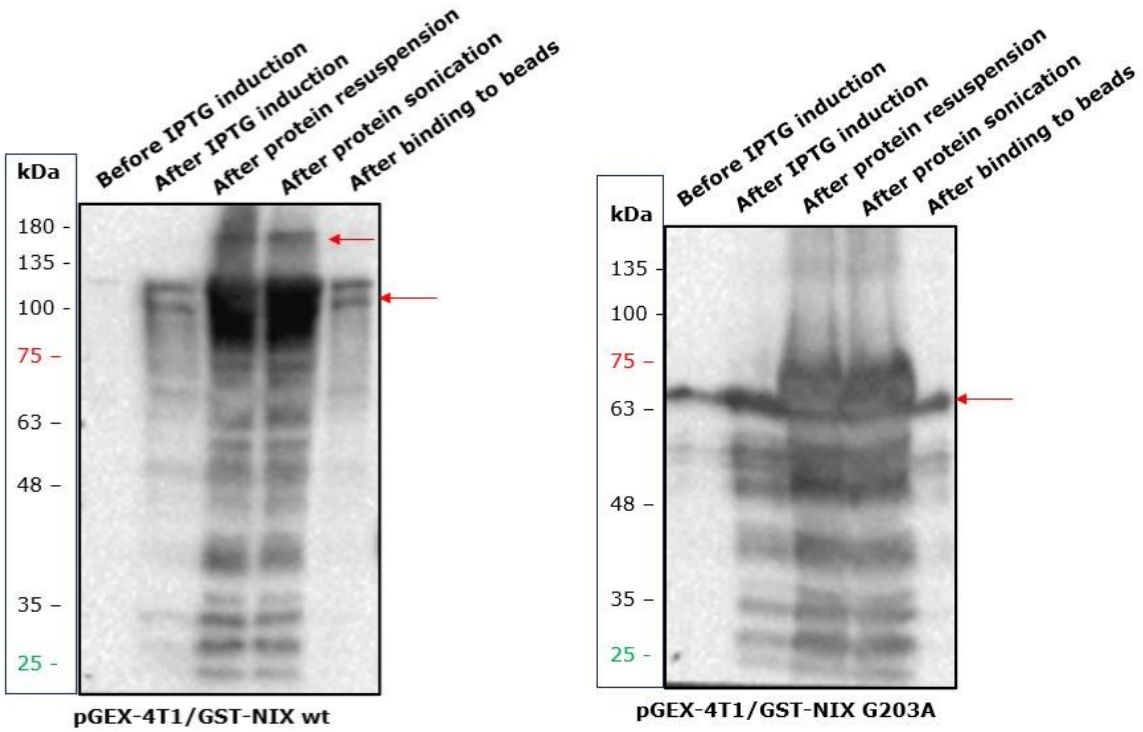
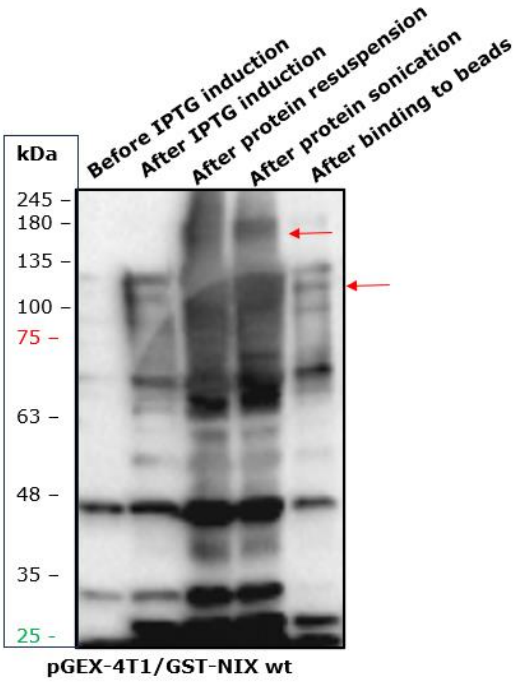


Figure 7. Detection of GST-tagged proteins on samples pGEX-4T1/GST-NIX wt and pGEX-4T1/GST-NIX G203A. (A) *GST-tagged protein visualisation after blotting using anti-GST antibody for sample pGEX-4T1/GST-NIX wt. Visualised with Image Lab software.* ***(B)*** *Visualisation of GST-tagged proteins from sample pGEX-4T1/GST-NIX G203A after blotting using anti-GST antibody. Done by Image Lab software.*

Final confirmation of NIX dimer production in the sample pGEX-4T1/GST-NIX wt was made through western blot using anti-NIX antibody. Although a bit weak, a signal is detected at ~102 kDa (Fig. 8A). A signal was also detected at around ~150 kDa in the samples taken after protein resuspension and sonication. In the sample pGEX-4T1/GST-NIX G203A, a signal was detected at ~68 kDa (Fig. 8B).

A)



B)

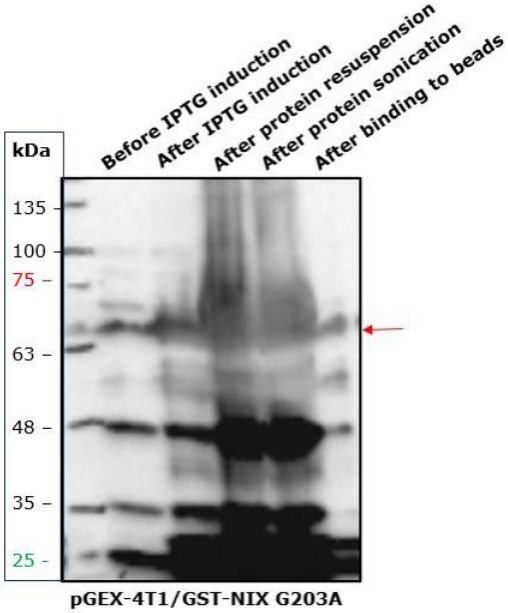


Figure 8. NIX dimer and monomer detected on membranes for samples pGEX-4T1/GST-NIX wt and pGEX-4T1/GST-NIX G203A respectively. ***(A)*** *Following Western blot using anti-NIX antibody, the membrane proteins from sample pGEX-4T1/GST-NIX wt were visualised by Image Lab software.* ***(B)*** *Proteins from sample pGEX-4T1/GST-NIX G203A were detected after blotting using anti-NIX antibody and visualised via Image Lab software.*

5. Discussion

BNIP3L/NIX was the first described receptor in the receptor-mediated mitophagy[26]. Firstly, it was mainly observed during terminal mammalian erythropoiesis but now, BNIP3L/NIX is gaining more attention as it is an important part behind the process of maintaining mitochondrial health and cellular homeostasis[4]. This protein is also interesting for its dual role in various diseases. In pancreatic cancer for example, it has a beneficial role where it delays cancer progression while in breast cancer it causes more damage than it should by delaying apoptosis in breast cancer cells[22,23].

BNIP3L/NIX dimerization plays a pivotal role in the initiation of mitophagy as its dimeric form is incredibly potent for autophagosome recruitment[4]. It is worth mentioning that, while BNIP3L/NIX monomers are capable of inducing mitophagy, their affinity for autophagosomal interactions is still noticeably low[27]. This is why we strived to produce and extract the dimeric form which is more capable of mediating this process.

Since BNIP3L/NIX dimer is a transmembrane protein, it is very difficult to extract it from the mitochondrial membrane. Using various methods and chemicals, we have tried to express and purify 6xHis and GST-tagged BNIP3L/NIX dimer in BL21 bacteria cultures, as well as experiment with the BNIP3L/NIX variants.

The first step in the experiment was to sequence various wild type BNIP3L/NIX plasmid sequences to confirm their accuracies. Following the BLAST analysis, we identified that human-derived pET21/NIX wt and pET23/NIX wt, and mice-derived pGEX-4T1/NIX wt sequences exhibited the highest similarity to the wild type BNIP3L/NIX sequences from both mice and humans.

Next, we conducted investigations to find the duration required for IPTG-induced BNIP3L/NIX protein expression. Multiple samples were made for each of the wild type variants, such as a 3-hour incubation sample and an overnight one at 37°C. The results suggest that the proteins were produced

similarily in both samples as strong protein bands signals were visualized on the gels, suggesting the possible presence of BNIP3L/NIX monomers and dimers (Fig.1).

Based on this result, we have concluded that BNIP3L/NIX could be expressed in a 3-hour incubation. We have decided to use this in future experiments as a convenient and faster way to get results.

In the early experiments, there were problems regarding the protein purification protocol. The ending results of these experiments were unsatisfactory as no signals were detected on the membranes. To investigate why this was happening, a variation of the protein purification protocol was made. During the protocol, it was found that BNIP3L/NIX is most likely accumulated within the bacterial pellet rather than the supernatant. The results support this as the gels demonstrate weakening signals as the centrifugations steps are carried out (Fig. 3). It can be assumed that, as the centrifugations continue, the protein becomes entrapped even further in to the bacterial debris.

The focus turned to the GST-tagged BNIP3L/NIX variants as the 6xHis-tagged protein demonstrated disappointing results. Experiments involving the mutated variants showed more easily achived and satisfying results. The accuracies for the mutated plasmid sequences were confirmed by NCBI „BLAST" program. Both GST-NIX G203A and GST-NIX ΔTM exhibited instant results after western blot analysis. GST-NIX G203A is a mutated variant that comes in a monomeric form that is still positioned on the OMM. On the other hand, GST-NIX ΔTM is characterised with the deletion of transmembrane domain. It is formed as a monomer and is located in the mitochondrial cytosol. These observations suggest that variants incapable of producing dimers are much easier to purify from bacteria and their localization on the OMM does not pose as a problem during purification.

In the final experiment, the BNIP3L/NIX dimer was succesfully produced and purified. To reach this final result, it took making variations of the

protein purification protocols and ensuring that the used chemicals were freshly prepared. Only the GST-tagged dimer was succesfully produced and purified whereas the 6xHis-tagged variant brought unsatisafactory results.

Furthermore, a possible BNIP3L/NIX oligomer was also detected. The protein bands were visualized in the protein resuspension and sonication samples collected during protein purification. This large molecular size at ~150 kDa could indicate a production of such BNIP3L/NIX form (Fig. 8). This hypothesis is not entirely impossible as BNIP3L/NIX was initially described as one of the pro-apoptotic mitochondrial membrane proteins that have a tendency to form oligomers[4]. The signal is lost following the addition of Glutathione Sepharose beads. This could imply that the oligomer was too substantial in molecular weight to be extracted from the bacterial pellet and bind to the beads.

With the successful expression and purification of the BNIP3L/NIX dimer, various other experiments can be conducted to further research the underlying mechanisms behind BNIP3L/NIX-mediated mitophagy. This includes experiments such as researching interactions between the dimer and autophagosomal proteins, exploration of the triggers that initiate BNIP3L/NIX dimerization and researching more thoroughly the phosphorylation process of the BNIP3L/NIX dimer that enhances mitophagy. Moreover, the regulation system of BNIP3L/NIX-mediated mitophagy needs research as well as it is still unclear what specific environmental signals affect BNIP3L/NIX and its performance in mitophagy[16]. As previously noted, mitophagy is vital for mitochondrial clearance during terminal mammalian erythropoiesis but still, its exact role in the maturation of erythrocytes is not understood completely[4]. With the purified BNIP3L/NIX dimer, many experiments could be conducted to research this more thoroughly.

All of the methods, particularly the protein purification method, need more work. During the experiments, bacterial supernatants were mostly very viscous and hard to work with. I would suggest using some type of

detergent that would help this issue and provide a more efficient protein extraction from the bacterial pellet. Moreover, I would suggest further research into why the 6xHis-tagged variants of BNIP3L/NIX were unsuccesful throughout the course of the experiments. Perhaps the protein purification method needs more perfecting, or alternatively, the root of the issue is in the bacteria that are incompetent in producing the 6xHis-tagged BNIP3L/NIX protein. For that matter, I suggest using different bacterial strains that are competent for protein production.

As previously mentioned, BNIP3L/NIX is involved in many diseases and its defectiveness causes dysfunctional mitophagy[13]. Defective mitochondria are associated with neurological diseases and it has a inconsistent role in tumorigenesis[16]. The outcomes of these experiments hold the potential to contribute to studies that are aimed at finding the solutions or treatments for these disorders. If not the treatment, it could at least bring a better comprehension of the diseases.

6. Conclusion

BNIP3L/NIX dimerization remains a complex process that little is known about. It is a crucial step in the BNIP3L/NIX mitophagy, and deceiphering it would broaden understanding of this vital homeostatic mechanism. During these experiments, we have managed to come closer to a better comprehension of BNIP3L/NIX and its dimer form.

The results obtained from the experiments have led to several key conclusions. Firstly, we have concluded that BNIP3L/NIX can be expressed within a 3 hour protein induction period at 37°C. Secondly, BNIP3L/NIX is entrapped in the bacterial pellet rather than the supernatant. Lastly, we have concluded that the wild type variant of BNIP3L/NIX is the hardest to purify whereas the G203A and ΔTM variants of the same protein are much easier to produce and extract. We can assume that the main obstacle in purifying this protein is not the fact that it is on the OMM but that its dimer form causes problems.

The question that remains is the expression and purification of a 6xHis-NIX dimer. Throughout the experiments, there was not significant success while working with those samples. It is unclear what could have possibly prevented the production of the dimer. The protocols for all of the methods used definitely need perfecting which might help this problem during future experiments.

On the other hand, we have achieved successful expression and purification of the GST-NIX wt dimer. The findings of this thesis have a potential to a better comprehension of BNIP3L/NIX and its role in BNIP3L/NIX-mediated mitophagy. They can aid in researching BNIP3L/NIX's involvement in various diseases and potentially lead us to the identification of therapeutic targets for diseases linked to dysfunctional mitochondrial degradation.

7. Literature

1. Billman GE. Homeostasis: The Underappreciated and Far Too Often Ignored Central Organizing Principle of Physiology. Frontiers in Physiology. 2020;11. Available from: https://www.frontiersin.org/articles/10.3389/fphys.2020.00200

2. Asarian L, Gloy V, Geary N. Homeostasis. In: Ramachandran VS, editor. Encyclopedia of Human Behavior (Second Edition). San Diego: Academic Press; 2012. p. 324–33. Available from: https://www.sciencedirect.com/science/article/pii/B9780123750006001919

3. Libretti S, Puckett Y. Physiology, Homeostasis. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2023. Available from: http://www.ncbi.nlm.nih.gov/books/NBK559138/

4. Marinković M, Novak I. A brief overview of BNIP3L/NIX receptor-mediated mitophagy. FEBS Open Bio. 2021 Dec;11(12):3230–6.

5. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol. 2010 May;221(1):3–12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2990190/

6. Harnett MM, Pineda MA, Latré de Laté P, Eason RJ, Besteiro S, Harnett W, et al. From Christian de Duve to Yoshinori Ohsumi: More to autophagy than just dining at home. Biomedical Journal. 2017 Feb 1;40(1):9–22. Available from: https://www.sciencedirect.com/science/article/pii/S2319417016303274

7. Yang Z, Klionsky DJ. An Overview of the Molecular Mechanism of Autophagy. Curr Top Microbiol Immunol. 2009;335:1–32. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2832191/

8. Tran M, Reddy PH. Defective Autophagy and Mitophagy in Aging and Alzheimer's Disease. Frontiers in Neuroscience. 2021;14. Available from: https://www.frontiersin.org/articles/10.3389/fnins.2020.612757

9. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell Res. 2014 Jan;24(1):24–41. Available from: https://www.nature.com/articles/cr2013168

10. Molecular mechanisms and physiological functions of mitophagy | The EMBO Journal. Available from: https://www.embopress.org/doi/full/10.15252/embj.2020104705

11. Kanki T. Nix, a receptor protein for mitophagy in mammals. Autophagy. 2010 Apr;6(3):433–5.

12. Ma K, Chen G, Li W, Kepp O, Zhu Y, Chen Q. Mitophagy, Mitochondrial Homeostasis, and Cell Fate. Frontiers in Cell and Developmental Biology. 2020;8. Available from: https://www.frontiersin.org/articles/10.3389/fcell.2020.00467

13. Ney PA. Mitochondrial autophagy: Origins, significance, and role of BNIP3 and NIX. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2015 Oct;1853(10):2775–83. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0167488915000725

14. Fritsch LE, Moore ME, Sarraf SA, Pickrell AM. Ubiquitin and Receptor Dependent Mitophagy Pathways and Their Implication in Neurodegeneration. J Mol Biol. 2020 Apr 3;432(8):2510–24. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7195237/

15. Zhang J, Ney PA. ROLE OF BNIP3 AND NIX IN CELL DEATH, AUTOPHAGY, AND MITOPHAGY. Cell Death Differ. 2009 Jul;16(7):939–46. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2768230/

16. Li Y, Zheng W, Lu Y, Zheng Y, Pan L, Wu X, et al. BNIP3L/NIX-mediated mitophagy: molecular mechanisms and implications for human disease. Cell Death Dis. 2021 Dec 20;13(1):14.

17. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, et al. NIX is required for programmed mitochondrial clearance during

reticulocyte maturation. Proc Natl Acad Sci U S A. 2007 Dec 4;104(49):19500–5.

18. Sandoval H, Thiagarajan P, Dasgupta SK, Schumacher A, Prchal JT, Chen M, et al. Essential role for Nix in autophagic maturation of erythroid cells. Nature. 2008 Jul 10;454(7201):232–5.

19. Esteban-Martínez L, Boya P. BNIP3L/NIX-dependent mitophagy regulates cell differentiation via metabolic reprogramming. Autophagy. 2018 Feb 9;14(5):915–7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6070009/

20. Wang W jia, Wang Y, Chen H zi, Xing Y zhen, Li F wei, Zhang Q, et al. Orphan nuclear receptor TR3 acts in autophagic cell death via mitochondrial signaling pathway. Nat Chem Biol. 2014 Feb;10(2):133–40.

21. Meyer N, Zielke S, Michaelis JB, Linder B, Warnsmann V, Rakel S, et al. AT 101 induces early mitochondrial dysfunction and HMOX1 (heme oxygenase 1) to trigger mitophagic cell death in glioma cells. Autophagy. 2018;14(10):1693–709.

22. Humpton TJ, Alagesan B, DeNicola GM, Lu D, Yordanov GN, Leonhardt CS, et al. Oncogenic KRAS Induces NIX-Mediated Mitophagy to Promote Pancreatic Cancer. Cancer Discov. 2019 Sep;9(9):1268–87.

23. Moussay E, Kaoma T, Baginska J, Muller A, Van Moer K, Nicot N, et al. The acquisition of resistance to TNFα in breast cancer cells is associated with constitutive activation of autophagy as revealed by a transcriptome analysis using a custom microarray. Autophagy. 2011 Jul;7(7):760–70.

24. Goldberg MS, Fleming SM, Palacino JJ, Cepeda C, Lam HA, Bhatnagar A, et al. Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. J Biol Chem. 2003 Oct 31;278(44):43628–35.

25. Yuan Y, Zheng Y, Zhang X, Chen Y, Wu X, Wu J, et al. BNIP3L/NIX-mediated mitophagy protects against ischemic brain injury independent of PARK2. Autophagy. 2017 Oct 3;13(10):1754–66.

26. Marinković M. Regulacija aktivnosti receptora autofagije proteina NIX u selektivnom uklanjanju mitohondrija [Disertacija]. Split: Sveučilište u Splitu, Medicinski fakultet; 2020 Dostupno na: https://urn.nsk.hr/urn:nbn:hr:171:836758

27. Marinković M, Šprung M, Novak I. Dimerization of mitophagy receptor BNIP3L/NIX is essential for recruitment of autophagic machinery. Autophagy. 2021 May;17(5):1232–43.