Identifikacija fosfataze odgovorne za defosforilaciju mitohondrijskog receptora NIX

Škorput, Jadranka

University of Zagreb

Faculty of Science

Division of Biology

Jadranka Škorput

Identification of phosphatase responsible for dephosphorylation of mitochondrial protein NIX

Master Thesis

Zagreb, 2020.

The thesis was completed in the Laboratory for Cancer Research, University of Split, School of Medicine, under the mentorship of Assoc. Prof. Dr. Sc. Ivana Novak Nakir, and in collaboration with Leibniz Research Institute for Environmental Medicine, Germany, Duesseldorf, with the help of Assoc. Prof. Dr. Sc. Natascia Ventura. The thesis is submitted for the review to the Department of Biology of the Faculty of Science of the University of Zagreb (Zagreb, Croatia) in fulfillment of the requirements for the master's degree in Molecular Biology.

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek Diplomski rad

Identifikacija fosfataze odgovorne za defosforilaciju mitohondrijskog proteina NIX

Jadranka Škorput
Rooseveltov trg 6, 10 000 Zagreb, Hrvatska

Uklanjanje mitohondrija procesom mitofagije održava zdravu populaciju mitohondrija i pridonosi održavanju homeostaze stanice. Nedavna istraživanja ukazuju na važnost dimerizacije mitohondrijskog protein NIX. Dimerizacija proteina NIX prethodi indukciji mitofagije, a sama regulacija dimerizacije proteina nije poznata. Cilj ovog istraživanja je identificirati fosfatazu odgovornu za defosforilaciju mitohondrijskog proteina NIX i regulaciju dimerizacije. Proučavanjem znanstvene literature, odabrana je PP1 fosfataza. Interakcija PP1 fosfataze i proteina NIX, kao i njegovih mutanti, ispitana je *in vitro* u staničnoj liniji HEK293 *pull down* imunoprecipitacijom i *Western blot* imunodetekcijom. Utjecaj PP1 fosfataze na indukciju mitofagije, provjerio se metodom RNA interferencije *in vivo* u modelnom organizmu *Caenorhabditis elegans* u normalnim uvjetima, kao i u prisutnosti mitohondrijskog otrova – parakvat. Dobivenim rezultatima potvrđena je interakcija PP1 fosfataze i proteina NIX u staničnoj liniji HEK293 te je utvrđeno da PP1 fosfataza inhibira mitofagiju u normalnim uvjetima, ali ne i pri indukciji mitofagije.

(61 stranica, 14 slika, 2 tablice, 90 literaturnih navoda, jezik izvornika: engleski)
Rad je pohranjen u Središnjoj biološkoj knjižnici
Ključne riječi: autofagija, mitofagija, NIX, PP1, fosfataza
Voditelj: dr. sc. Ivana Novak Nakir, izv. prof.
Suvoditelj: dr. sc. Martina Šeruga Musić, izv. prof.
Ocjenitelji: dr. sc. Ivana Novak Nakir, izv. prof.
dr. sc. Martina Šeruga Musić, izv. prof.
X
X
Rad prihvaćen:

BASIC DOCUMENTATION CARD

University of Zagreb
Faculty of Science
Division of Biology

Master Thesis

Identification of phosphatase responsible for dephosphorylation of mitochondrial protein NIX

Jadranka Škorput
Rooseveltov trg 6, 10 000 Zagreb, Croatia

Removal of mitochondria, by the process of mitophagy, maintains a healthy population of mitochondria and contributes to maintenance of cell homeostasis. Recent research indicates the importance of mitochondrial protein NIX dimerization, whereas the regulation of protein dimerization remains unknown. The aim of this study was to identify the phosphatase responsible for dephosphorylation of the mitochondrial protein NIX, thus regulating the dimerization of protein NIX. Phosphatase PP1 was selected by searching the literature. Pull-down and Western blot analysis were used to investigate the interaction of PP1 phosphatase with NIX protein, as well as its mutants, *in vitro* in HEK293 cells. The effect of PP1 phosphatase in mitophagy induction was investigated with RNA interference assay *in vivo* in model organism *Caenorhabditis elegans*, both under normal conditions, as well as in the presence of mitochondrial toxin - paraquat. The results confirm the interaction between PP1 phosphatase and mitochondrial protein NIX. Moreover, the results show that PP1 inhibits mitophagy under normal conditions but show no impact after the induction of mitophagy.

(61 pages, 14 figures, 2 tables, 90 references, original in: english)
Thesis deposited in the Central Biological Library
Key words:
Supervisor: Dr. Sc. Ivana Novak Nakir Assoc. Prof.
Co-supervisor: Dr. Sc. Martina Šeruga Musić, Assoc. Prof.
Reviewers: Dr. Sc. Ivana Novak Nakir, Assoc. Prof.
Dr. Sc. Martina Šeruga Musić, Assoc. Prof.
x
x
Thesis accepted:

Table[D1] of contents:

List of abbreviations

ATG – autophagy-related genes

ATP – adenosine triphosphate

BH-3 – Bcl2-like domain 3

CCCP – carbonyl-cyanide 3-chlorophenyl hydrazone

CMA – chaperone-mediated autophagy

Drp1 – dynamin-related protein 1

FIS1 – fission protein 1

GABARAP – GABA type A receptor-associated protein

GTP – guanosine triphosphate

HIF1α – Hypoxia-inducible factor-1α

HRP – horseradish peroxidase

Hsc70 – heat shock cognate of the Hsp70 family

IMM – inner mitochondrial membrane

IMS – intermembrane space

LAMP2A - lysosomal-associated membrane[D2] glycoprotein 2A

LC3 – light chain 3

LIR – LC3-interacting region

Mfn1/2 – mitofusin 1/2

OMM – outer mitochondrial membrane

NIX/BNIP3L – BCL2/adenovirus E1B 19-kDa-interacting protein 3-like

OPA1 – optc atrophy 1-protein

PINK – PTEN-induced putative kinase

PKA – protein kinase A

ROS – reactive oxygen species

TCL – total cell lysate

TM – transmembrane domain

TOMM20 –mitochondrial import receptor subunit TOM20[D3] homolog

UPS – ubiquitin-proteasome system

1 Introduction

1.1 Mitochondria

Mitochondria are bacteria-derived compartmentalized organelles that generate energy in the form of adenosine triphosphate (ATP), a compound essential for numerous cellular process (Margulis, 1996). The structure of mitochondria is defined by their double membrane, which is composed of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM) which are separated by an intermembrane space (IMS) (see schematic depiction in *Figure 1*) (Alberts et al. 2002).

The outer mitochondrial membrane is the interface between mitochondria and the cytosol. The OMM contains a large number of integral membrane proteins and enzymes which are involved in diverse metabolic processes (Bayly, 2014). The intermembrane space (IMS) has a relatively low protein content that mostly contains enzymes included in the ATP transport (Manganas et al. 2017; Backes and Herrmann 2017),[D4] as well as a few effectors, such as cytochrome C which is one of the apoptosis effectors (Mijaljica et al., 2010). Disruption of the OMM can cause leaking of cytochrome C and other effectors from IMS to cytosol which can consequently lead to death of the whole cell (Garrido et al., 2006) (Chipuk et al., 2006). Any disruption of mitochondrial function, hence, negatively impacts the bio-energetic status of the whole cell itself (Manganas et al. 2017).

The inner mitochondrial membrane (IMM) is the frontier between the aforementioned intermembrane space and the mitochondrial inner matrix. It is the site of the chain respiratory complexes and ATP synthetase, and is nearly impermeable to all ions, including protons (H^+) (Alberts et al. 2007)[D5] . This impermeability allows the respiratory chain complexes to build up the proton gradient between membranes – the gradient that is required for the very ATP synthesis *via* oxidative phosphorylation. Reactive oxygen species (ROS) often occur as a byproduct of the electron transfer from

respiratory substrates all up to oxygen by a series of oxidation-reduction reactions and proton gradient (Alberts et al., 2007). The maintenance of the proton gradient and oxidative state of the cell is therefore of vital importance for cell bioenergetics (Quijano et al., 2016).

The matrix, on the other hand, is full of diverse proteins. There are, for example, many enzymes involved in Kreb's cycle, as well as mitochondrial ribosomes (O'Brien and Kalf, 1967). Mitochondria, together with the nucleus, are the only organelles of animal cell that contain their own DNA molecules, and machinery for RNA and protein synthesis (Wiedemann et al., 2003). Mitochondrial genome is a circular DNA molecule that encodes a small number of proteins. Those proteins are mostly respiratory chain proteins, all essential for its function (Nass and Nass, 1963)[D6]. Unlike nuclear DNA, mtDNA is not protected by histones, and because of that, it is much more prone to the ROS-induced mutations (Gilkerson et al., 2013). Nevertheless, mitochondrial biogenesis requires co-expression of both, mitochondrial and nuclear genes, since even 90% of the mitochondrial protein mass is encoded by the nuclear genome (Mijaljica et al. 2010).

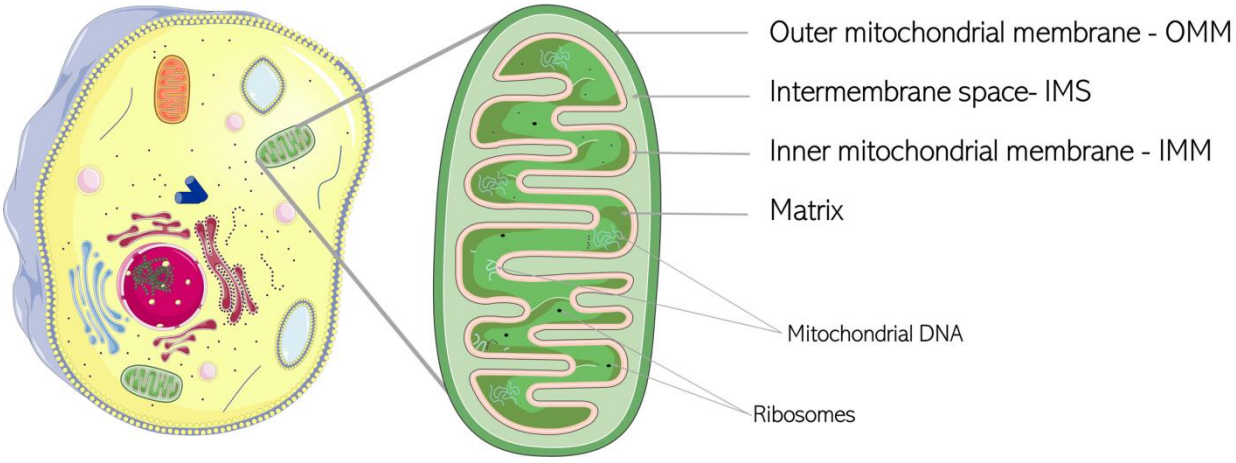


Figure 1. *Mitochondria.* All the eukaryotic cells have double-membrane ATP-producing organelles known as mitochondria. Their structure is defined by outer mitochondrial membrane – OMM and inner mitochondrial membrane – IMM between which there is an intermembrane space – IMS. Inside the IMM there is matrix, which contains different enzymes, ribosomes, as well as the circular molecules of mitochondrial DNA – mtDNA.

Other than the function in the bioenergetics homeostasis, mitochondria are also crucial in regulation of a diverse spectrum of cellular processes, such as iron and calcium

homeostasis, and biosynthetic pathways, respectively, such as lipid and heme synthesis (Spinelli and Haigis, 2018). Mitochondria also have an important role in regulation of programmed cell death pathways (Wang and Youle, 2009).

1.1.1 Mitochondrial dynamics

Mitochondria are highly dynamic organelles that may vary in size, number, and shape. All of this depends on type, developmental stage, and bio-energetic needs of the cell (Mishra and Chan, 2016). To regulate their reshaping and recycling, mitochondria undergo cycles of fusion and fission, known as mitochondrial dynamics. Mitochondrial dynamics regulates mitochondrial network connectivity (Liesa et al. 2009; Yoo and Jung 2018), thus determining overall mitochondrial morphology. In many organisms, as for example in fibroblast cells, mitochondria create an interconnected reticulum which act as an electrically united system (Mijaljica et al. 2010). The elimination of damaged or non-functioning mitochondria represents a quality control-check that keeps the whole mitochondrial network functional (Yang and Klionsky, 2010).

The events of mitochondrial dynamics are regulated by many intracellular, as well as extracellular signals. Examples of the signals are oxidative stress, disruption in membrane potential, mtDNA quality, and apoptosis (Kroemer et al. 2007). Cycles of fusion and fission are controlled by a specific set of proteins. Fusion is mediated by Mitofusins 1 and 2[D7] proteins (Mfn1, Mfn2) that enable fusion of outer mitochondrial membranes thorough their cytoplasm exposed GTPase domains (Züchner et al., 2004). The fusion of the inner mitochondrial membranes is mediated by Optic atrophy 1 (OPA1) (Alexander et al., 2000).

On the other hand, fission occurs when the GTPase dynamin protein 1 (Drp-1) is translocated to the mitochondrial outer membrane where it becomes associated with its receptor, Fission protein 1 (FIS1) where it forms a multimeric structure around the fission site of the mitochondrion (Marinković and Novak, 2015).

Though the regulation of proteins involved in the mitochondrial dynamics is not well understood, a regulatory role of phosphorylation has been suggested. Indeed, these fission events are regulated through phosphorylation and dephosphorylation events of Drp-1 by protein kinase A - PKA and Cfk1 phosphatase, but there are no known candidates suspected to regulate fusion events (Chang and Blackstone 2007; E. Smirnova et al. 2001; Smirnova et al. 1998) .

1.1.2 Mitochondrial quality control

Given the importance of mitochondria in the cell homeostasis, the number and the shape of mitochondria must be tightly regulated and balanced. Mitochondrial dynamics is also one of the mitochondrial quality-control mechanisms. Alongside the metabolic importance of the mitochondria in fatty acid oxidation, the Krebs cycle, and the oxidative phosphorylation, mitochondria are prone to release hazardous materials that can potentially damage cells. As aforementioned, mitochondrion is the site of the ROS production. Particularly dangerous are superoxide anion, hydrogen peroxide, and hydroxylradical (Adam-Vizi and Chinopoulos, 2006). They negatively impact lipid metabolism, damage mtDNA, as well as proteins, which leads to even bigger production of ROS resulting in ROS-induced mutation of the mtDNA (Ashrafi and Schwarz 2013; Quijano et al. 2016; Zhang et al. 2011).

Fusion of the mitochondria mediates (i) inter-exchange of mitochondrial genetic components (a way of recombination), (ii) protection of mitochondria from accumulating mutations, and (iii) support of optimal bio-energetic activity (Mijaljica et al. 2010; Sato et al. 2006). Thus, mitochondrial fusion contributes to the integrity of the mitochondrial network.

Fission, on the other hand, promotes the isolation of dysfunctional mitochondria from the mitochondrial network, promoting their selective removal (Tilokani et al., 2018). It has been observed, during the mitochondrial fission, that often one of the two daughter mitochondria have decreased membrane potential which makes them less likely to go

under fusion event, but rather to be selectively removed (Twig et al., 2008). Accumulation of impaired mitochondria by fusion causes the increase of the mitochondrial network fragmentation (Otsuga et al., 1998), which can lead to programmed cell death, apoptosis.

Besides the mitochondrial dynamics, there are different cellular pathways that prevent cellular damage. Unfolded and misfolded membrane proteins are degraded by a network of mitochondrial proteolytic enzymes - two AAA protease complexes anchored in IMM with catalytic subunit facing either side of the IMM (Glynn, 2017). Proteins on the OMM are regulated by cytosolic degradation enzymes of the proteasome (Karbowski and Youle, 2011). By degrading misfolded proteins, mitochondrial proteolytic system achieve a constant recycling of the mitochondrial proteome (Glynn, 2017).

Similar to the processing and sorting of any misfolded protein in Golgi apparatus (Hellerschmied et al., 2019), lysosomal pathway is characterized by vesicles of misfolded proteins budding from mitochondrial membrane that are directly sequestered to the lysosomes, keeping the whole organelle intact (Ashrafi and Schwarz, 2013). Even though lysosomal pathway is active under steady-state conditions, it is further stimulated by oxidative stress. Thus, it represents a pathway for the clearance of oxidized mitochondrial proteins.

If the mitochondrial damage reaches beyond damaged proteins, in that case damaged and superfluous mitochondria are selectively degraded in a process of selective autophagy of mitochondria, known as mitophagy (*Figure 2*). Recruitment of the mitophagic machinery leads to the removal of the impaired mitochondria and at the same time it prevents the release of pro-apoptotic proteins (Maiuri et al., 2007). Thus, it inhibits apoptosis by triggering mitophagy.

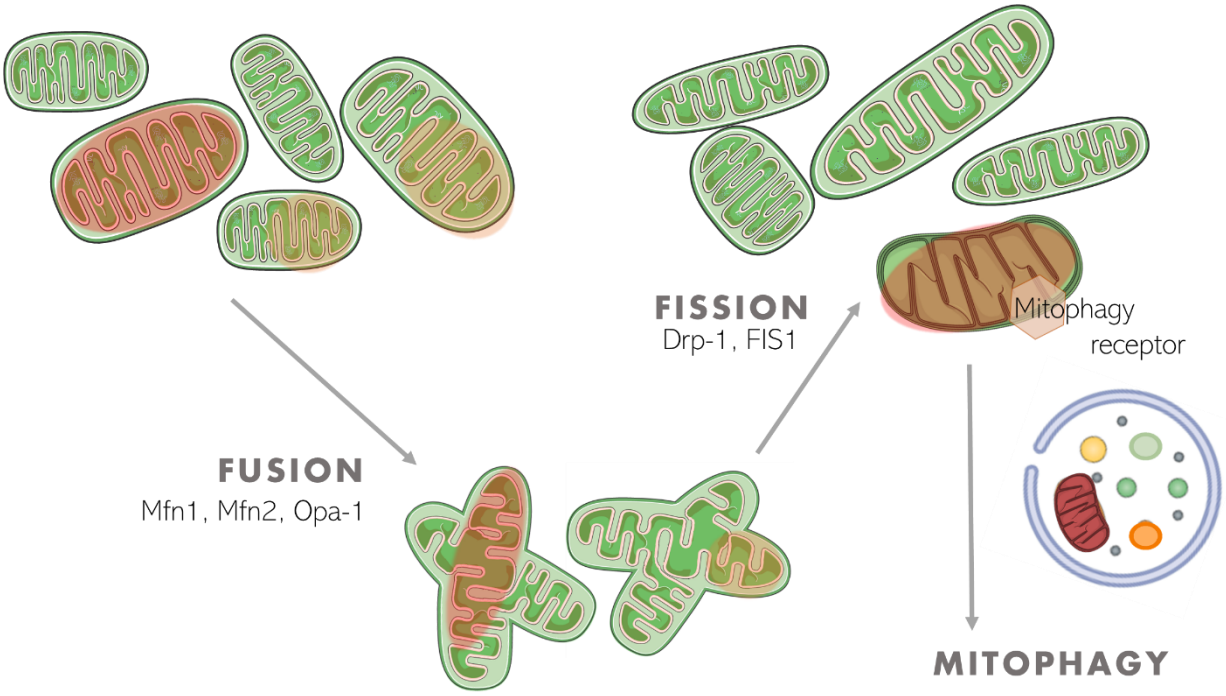


Figure 2 *Mitochondrial dynamics as quality control check.* Fusion protects mitochondria from accumulating mutations by interexchange of genetic material between mitochondria, while fission promotes isolation of dysfunctional mitochondria. Damaged mitochondria are selectively removed by recruiting the mitophagic machinery.

1.2 Autophagy

Metabolic homeostasis consists of an interplay between biosynthesis and degradation processes. Degradation of damaged, nonfunctional, and superfluous cellular parts is achieved by two different intracellular degradation mechanisms: the ubiquitin-proteasome degradation pathway – UPS, that is involved in degradation of single polypeptide chains, and the lysosome-mediated degradation pathway known as autophagy. Autophagy is involved in degradation of lipids, polysaccharides, DNA, RNA, proteins, and most importantly, even organelles.

Autophagy (from Greek, meaning “self-eating”) is an evolutionarily conserved process in eukaryotes by which cell degrades damaged compounds and organelles. Autophagic machinery can also be recruited as an adaptive response on exposure to various stresses and invading microorganisms. The initiation of autophagy is the sequestration of cytoplasmic cargo inside a double-membrane vesicle - autophagosome. Autophagosomes deliver the sequestered cargo to the lysosome for degradation, thus providing essential nutrients and energy (ATP) to the cell (Yang and Klionsky, 2010).

There are three distinct classes of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy – CMA (Ashrafi and Schwarz, 2013). They utilize different pathways to deliver cargoes to lysosome (*Figure 3*). In microautophagy, lysosomes directly engulf portions of the cytoplasm (Ashrafi and Schwarz, 2013), while CMA selectively recruits unfold substrate proteins with a KFERQ amino-acid motif into the lysosome with the help of chaperone proteins, but without autophagosome formation (Marinković and Novak, 2015). Macroautophagy is the major type of autophagy. The term “autophagy” is often referred to macroautophagic machinery, since it is the most studied mechanism of autophagy (Ashrafi and Schwarz, 2013). Depending on cargo, macroautophagy can selectively degrade unwanted or damaged organelles, and invading organisms (e.g. mitophagy, xenophagy). On the other hand, starvation-induced autophagy is rather non-selective mechanism by which degraded cargo is used to provide essential nutrients for the cell (Yang and Klionsky, 2009).

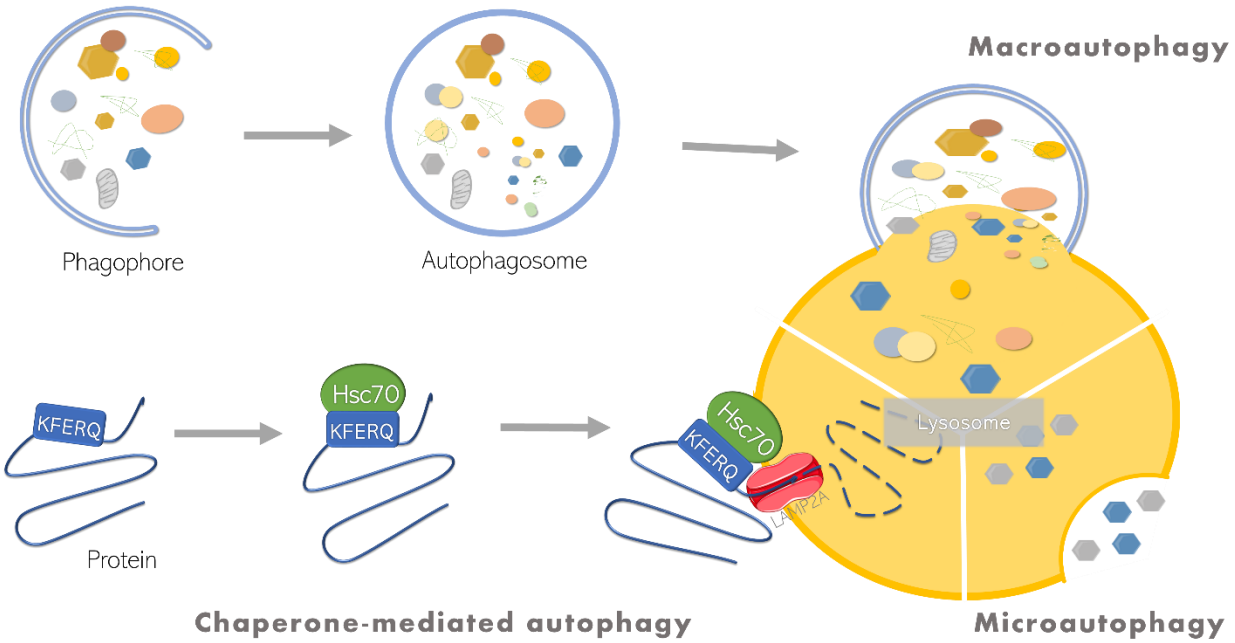


Figure 3 *Pathways of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMV).* Microautophagy degrades small portions of cytosol, while CMV degrades damaged proteins containing KFERQ motif with the help of Hsc70 chaperone. Macroautophagy is a selective type of autophagy which includes formation of autophagosome with cargo sequestered to lysosome for degradation.

1.3 Mitophagy

Cellular homeostasis depends on energy that is provided by mitochondria (Wilson, 2013). As mentioned, to meet metabolic requirement, differentiation status, and different physiological conditions of the cell, it is important to maintain healthy population of mitochondrial network. This includes selective removal of damaged mitochondrial proteins, repairing damaged, or destroying extremely impaired mitochondria (Novak, 2012). As in selective autophagy, mitochondria marked for degradation are engulfed by autophagosome and delivered to lysosome for degradation and recycling (Abada and Elazar, 2014). Mitophagy is sometimes also triggered to remove normal mitochondria during differentiation of individual cell lines and embryonal development (Sato and Sato, 2011). Recent study reveals an additional role of mitophagy in mitochondrial turnover for metabolic transitioning from carbohydrates to fatty acids in cardiomyocytes during the perinatal period (Yamaguchi et al., 2016).

.

Mitophagy utilize the same machinery as do other forms of selective autophagy, and can be induced by different intrinsic, and extrinsic factors (Mijaljica et al., 2014; García-Macia et al., 2019)[D8]. The molecular mechanisms of selective autophagy are still not well known, but it is known that autophagic machinery consists of autophagy receptors and adaptors that mediate the binding of the specific cargo and receptors on autophagosomal membrane. The mechanism of mitochondrial degradation is similar to selective autophagy. Selective autophagy consists of following stages: initiation of the isolation membrane, binding to specific cargo, closure of the isolation membrane and autophagosome formation, fusion of autophagosome and lysosome to autophagolysosome, and lysosomal degradation of the cargo (Ashrafi and Schwarz, 2013). Understanding the molecular basis of this process started with discovery of autophagy-related genes – ATG genes in yeast (Yamaguchi et al., 2016). ATG family of proteins receive signal from other signalling pathways to trigger autophagy and formation of isolation membrane (Ashrafi and Schwarz, 2013). Together with other regulatory proteins, they form the core machinery for initiation and elongation of autophagosome. Given the importance of this evolutionary-conserved pathway, homologues of ATG genes

can be found from yeast to mammals (Tsukada and Ohsumi 1993; Martens and Fracchiolla 2020). Of all, the most important ATG proteins of selective autophagy are ATG8 which are essential for autophagosome formation (Nakatogawa et al. 2007). In humans, ATG8 proteins are grouped in two sub-families: microtubule-associated proteins (LC3) and gamma-aminobutyric acid receptor-associated proteins (GABARAP) (Nguyen et al., 2016; Weidberg et al., 2011).

Even though, the origin of the isolation membrane is not known, it is proposed that the membrane is synthetized *de novo* (Ashrafi and Schwarz, 2013). However, different studies have implicated the endoplasmic reticulum - ER, Golgi (Tooze and Yoshimori, 2010), plasma membrane (Ravikumar et al., 2010), and mitochondria (Hailey et al., 2010) as possible membrane sources for the autophagosome. After the formation, double membrane autophagosome then fuses with lysosome through the outer autophagosomal membrane, allowing the acidic hydrolases to degrade the inner autophaosomal membrane (Yim and Mizushima, 2020). Autophagosome-lysosome fusion results in autolysosomes where the sequestered cargo is gradually degraded into smaller particles.

Specific set of proteins that mediate binding of specific cargo and members of ATG8 protein family on the isolation membrane, are known as autophagy receptors (Rogov et al., 2017). They are key factors in distinguishing cargo for removal in selective autophagy, thus stabilizing autophagosomal formation (Nishimura and Tooze, 2020). Discoveries of the new forms of selective autophagy, implicate that some autophagy receptors are highly specific so that a particular cargo can be recognized by several autophagy receptors (Marinković and Novak, 2015).

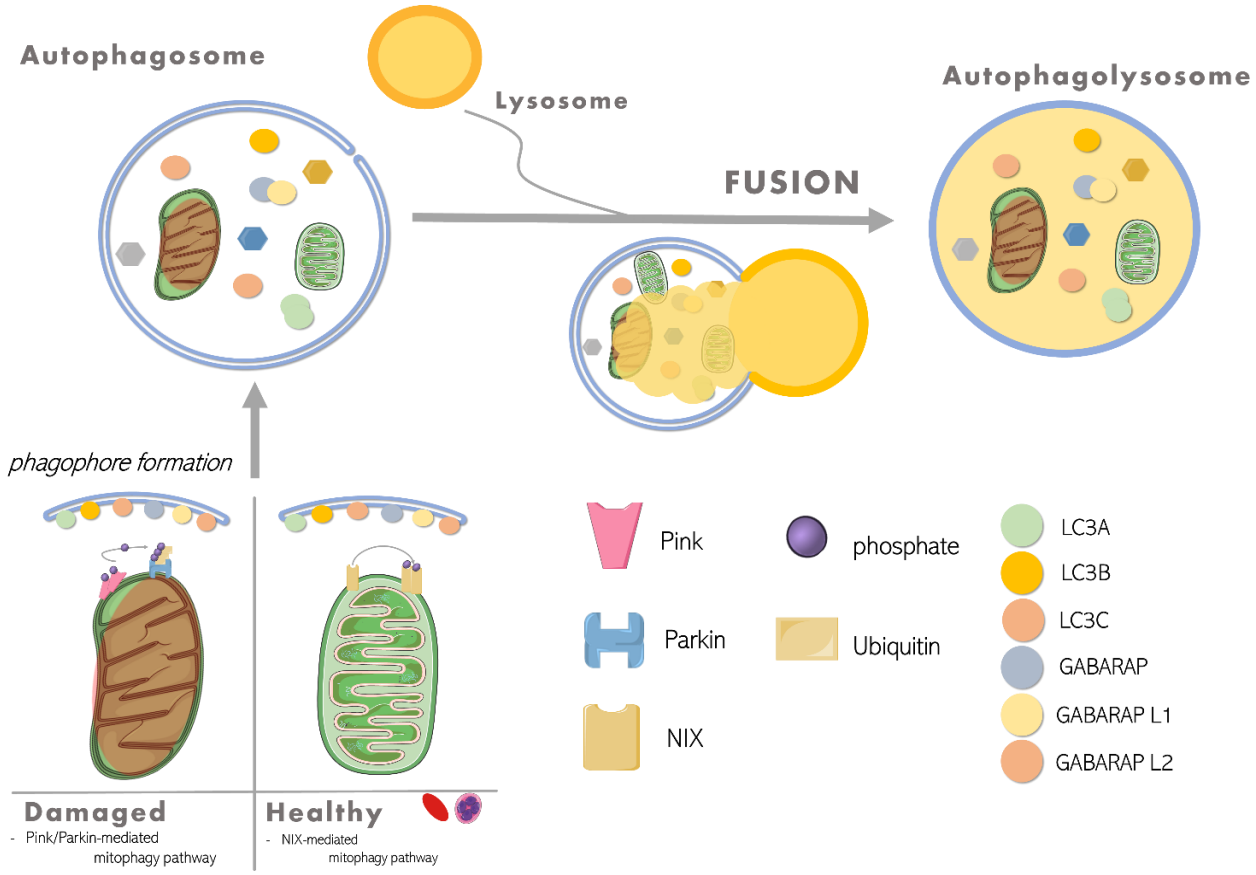


Figure 4 *Selective removal of damaged or healthy mitochondria.* Damaged mitochondria accumulate Pink protein on outer mitochondrial membrane (OMM). Autophosphorylated Pink phosphorylates ubiquitin which triggers the recruitment and activation of E3 ligase – Parkin, and autophagosome formation. Healthy mitochondria are selectively removed upon dimerization of NIX protein. Phosphorylation of LIR-region of NIX protein enhances its interaction with autophagosome.

Different signaling pathways can also regulate and affect mitophagy (*Figure 4*). Recent studies in the field on neurodegenerative diseases, show that mutations in outer mitochondrial membrane PTEN-induced putative kinase 1 – Pink1 and E3-ubiquitin ligase – Parkin are associated with defects in mitophagy, and might be involved in the pathogenesis of familial Parkin disease (Barazzuol et al., 2020). Upon disruption of mitochondrial membrane potential, Pink1 phosphorylates ubiquitin which activates Parkin and autophagosome formation. Parkin is translocated from cytoplasm to defective mitochondria, leaving the healthy mitochondria Parkin-free. Thus, recruiting the mitophagic machinery only to defective mitochondria and removing it. Since Parkin-mediated mitophagy is selective to removal of damaged mitochondria respectively, Parkin might have a role in mitochondrial quality control pathway (Novak, 2012).

1.4 Mitochondrial protein NIX

When it comes to mitophagy during developmental process, of special interest is the integral outer mitochondrial membrane protein NIX, an atypical BH3-only proapoptotic protein (Rogov et al., 2017). Unlike PINK1/Parkin-mediated mitophagy, NIX is known to have an essential role in mitochondrial removal during differentiation of reticulocytes (Sandoval et al., 2008; Schweers et al., 2007). Programmed elimination of mitochondrial population is necessary during erythrocyte maturation. Exposure to oxidative stress in mature erythrocytes, caused by hemoglobin-mediated oxygen transport, would increase mitochondrial ROS production and would lead to consequent cellular damage. To maximize the oxygen exchange between lungs and peripheral tissues, mature erythrocytes have no internal organelles, including mitochondria (Ashrafi and Schwarz, 2013). Even though, the role of NIX protein was firstly reported in erythrocyte maturation, NIX is also important for the activation of both apoptotic and necrotic cell death during cardiac hypertrophy and is a tumor suppressor.

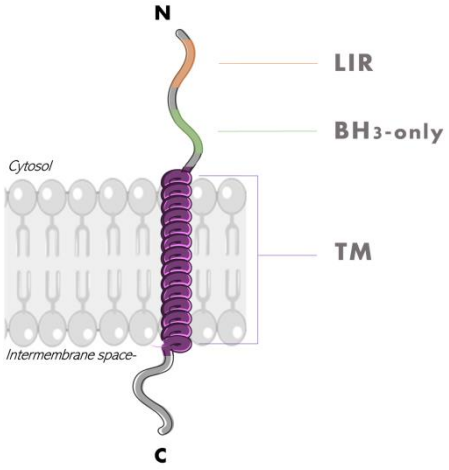


Figure 5 *Structure of protein NIX.* Mitophagy receptor NIX is anchored on outer mitochondrial membrane through its transmembrane domain – TM, with its C-terminal end in the intermembrane space. Upon mitophagy, NIX elongates autophagosomal formation via its LC3-interacting region – LIR. Furthermore, with Bcl-2 homologue domain – BH3-only, it has a role as proapoptotic protein.

NIX directly interacts with the autophagic adaptor ATG8/LC3 family-proteins, bringing the mitochondria to autophagosome. Direct interaction is achieved through the LC3-interacting region (LIR) - a tetrapeptide WxxL motif, located at the cytoplasmic N-terminal

end of NIX (Novak et al., 2010). The linear sequence motif is also found in other autophagic receptors. NIX is a protein that is an exclusive receptor for mitochondria, it is permanently anchored on the outer mitochondria membrane through its hydrophobic C-terminal transmembrane domain (TM) (*Figure 5*). Recent research showed the importance of TM domain in homodimerization of NIX protein. To recruit mitophagic machinery, NIX needs to undergo homodimerization. Recent research shows that mutations in TM domain decrease mitophagic response, showing the importance of the TM domain in NIX homodimerization, and mitophagy sequentially (Marinković et al. 2020).

The dimerization regulation is still unknown. However, 11 aminoacid residues on C-terminal end of protein NIX that are located in the IMS could be responsible for its regulation. Recent research showed that the NIX mutant with the substitution of 212. serine to glutamic acid (NIX-S212E) lacks the dimeric form suggesting phosphorylation-driven regulation as a mechanism for regulation of NIX dimerization (Marinković et al. 2020).

The orthologue of NIX protein in *Caenorhabditis elegans* is ceBNIP3 protein. Sequence analysis reveals that ceBNIP3 contains a conserved C-terminal TM domain important for its localization on the OMM and is also necessary for homodimerization. Just like NIX protein, it also contains a BH3-only domain suggesting its pro-apoptotic role in cell death (Cizeau et al., 2000).

1.4.1 Regulation of NIX mitochondrial protein

Mitochondrial dysfunction causes a wide variety of pathologies. To maintain the homeostasis, a cell must have an ability to regulate the activity of proteins involved in different signaling pathways.

Protein phosphorylation is the most common post-translational modification. It is a very important cellular regulatory mechanism, as many enzymes and receptors are

regulated by phosphorylation and dephosphorylation events. The mechanisms of phosphorylation consist of kinases, phosphatases, and phospho-binding receptor. Any stimuli (e.g. epigenetic modification) can trigger kinase activity, which causes the addition of a phosphate group by ATP hydrolysis. On the other hand, the removal of phosphate group is possible due to enzymatic activity of phosphatases. This post-translational switch modifies protein from hydrophobic (apolar, dephosphorylated) to hydrophilic (polar, phosphorylated), allowing the protein to change conformation when interacting with other cellular compounds, and consequently assemble of detach protein complexes. Protein kinases and phosphatases are involved in different signaling transduction pathways, and their malfunction can be found in several diseases, mostly tumor.

Phosphorylation-driven regulation of NIX protein can be observed in interaction with LC3/GABARAP proteins. As mentioned, direct NIX::LC3 interaction is faciliated through LIR-region, and phosphorylation of the serine amino acid of LIR stabilizes the NIX:LC3 complex. Its interaction enhances autophagosome recruitment to mitochondria and mitophagy, respectively (Rogov et al., 2017). It is proposed that both, LIR phosphorylation and receptor dimerization are essential for the proper mitophagy (Marinković et al. 2020).

1.5 Protein phosphatase – PP1

Enzymes that remove phosphate group are called phosphatases. Phosphatase candidate that was investigated in this thesis is a protein serine/threonine phosphatase Protein phosphatase 1 (PP1) that acts upon phosphorylated serine/threonine residues, and is known to be involved in the regulation of different cellular processes, such as, glycogen metabolism (Fong et al., 2000), cardiac function (Nicolaou and Kranias, 2009), mitosis (Tournebize et al., 1997), and mitochondrial dynamics (Kim et al., 2019). Each PP1 enzyme consist of a regulatory and catalytic subunit (Goldberg et al., 1995). Like most phosphatases, PP1 is not sequence-selective, and is known to dephosphorylate multiple substrates *in vivo* and *in vitro* (Pinna and Donella-Deana, 1994). Thus, re-localizating the PP1 phosphatase to specific subcellular compartments (Trinkle-Mulcahy et al., 2007), like IMS of mitochondria. To date, the number of known PP1 targeting subunits is rapidly increasing (Aggen et al. 2000), showing the importance of the PP1 in regulation of diverse cellular mechanisms.

2 The aim of the research

Recent research shows that mitophagic machinery is recruited upon dimerization of NIX protein on OMM. Furthermore, since mutation of 212. serine residue to glutamic acid inhibits dimerization of NIX, it is suggested that phosphorylation events of the 212. serine residue regulates the dimerization, hence, mitophagy.

The aim of the study is to identify phosphatase responsible for dephosphorylation of mitochondrial receptor NIX at serine 212.

Moreover, the role of the phosphatase candidate in mitophagy regulation will be investigated *in vivo* in *Caenorhabditis elegans* to investigate mitophagic events which occur upon the silencing of the expression of the phosphatase.

It is expected that the identification and the interaction analysis of this protein (NIX::phosphatase) may give new and interesting insights into the regulation of selective removal of mitochondria.

3 Materials and methods

3.1 Materials

3.1.1 Cell line

Human embryonic kidney - **HEK293** cells, a cell line that was cultured from kidney cells of a human embryo. HEK293 cell line is used in transfection experiments for over-expression of plasmid DNA with protein NIX, mutants of protein NIX, and phosphatase PP1α (*Table 1*).

The cells were maintained at 10-90% confluency in a 37 °C, 5% CO_2 tissue culture incubator. The cells were cultured in DMEM medium containing 10% FBS and 1% penicillin/streptomycin.

3.1.2 *Caenorhabditis elegans* Maupas, 1900 (Metazoa: Nematode)

The nematode *C. elegans* is a model organism widely used in research of fundamental cellular processes. It is a non-hazardous transparent organism ideal for *in vivo*, noninvasive, and real-time monitoring through fluorescence microscopy. Furthermore, the organism is defined by the invariant number of the organism's cells, and short lifespan, making it ideal for the study of processes related to programmed cell death and aging.

For monitoring mitophagy, the *C. elegans* strain (P_{myo3}::TOMM-20::Rosella) (Palikaras et al., 2015a) with **Rosella biosensor** was used (Rosado et al., 2008). Rosella is a dual fluorescent reporter, which combines a fast-maturing pH-insensitive RFP variant DsRed fused to a pH-sensitive GFP variant. This biosensor is fused with TOMM-20, an outer-mitochondrial-membrane protein which mediates import of proteins into mitochondria (Charmpilas et al., 2018). Upon mitochondria sequestering to lysosome, the GFP variant is degraded because of the low pH, whereas the red fluorescence will emit

regardless of its localization in the cell. Thus, providing the insight of the mitochondrial events.

The normal lifespan of *C. elegans* is depended on temperature and food concentration (Klass, 1977). After egg hatching, the animals pass through four larval stages before becoming fertile adults (*Figure 6*).

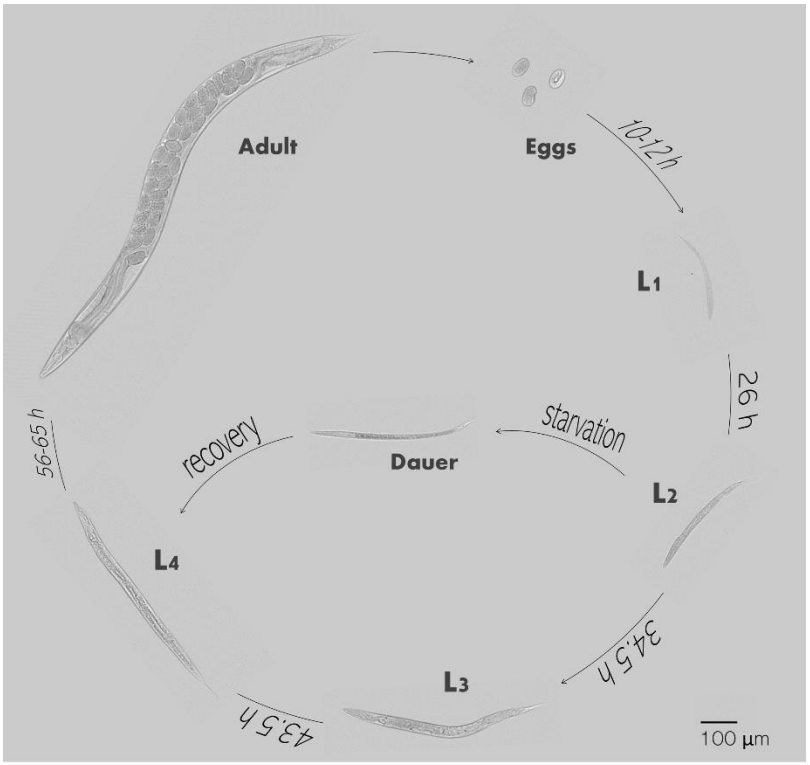


Figure 6. *Life cycle of Caenorhabditis elegans at figure °C.* The normal life cycle of animals, from eggs to fertile adults, grown on 20 °C is a 3-day process through which the animals go through four larval stages. Complete fertile adults can be recognized by fertilized eggs. Scale bar corresponds to 100 µm. Figure adapted from Link (2016)

Rosella transgenic animal strain was grown on OP50 *E. coli* seeded NGM plates. Nematodes were maintained in the HettCube 400 incubator at 20°C and were transferred to fresh *E. coli* (OP50) NGM plates twice per week.

3.1.3 Bacterial strains

Escherichia coli, strain **DH5α** – previously prepared calcium chloride competent strain of bacteria used for plasmid isolation

Escherichia coli, strain **OP50** – bacterial strain used as bacterial food for *C. elegans* (250 µl per dish)

Escherichia coli, strain **HT115** – bacterial strain used in RNAi assay for *C. elegans* (c: 0.9 mg/ml, 250 µl per dish)

3.1.4 Plasmids

To investigate the protein interactions, plasmids (*Table 1*) are used to overexpress proteins of interests or to target the phosphatase in RNA interference assay.

Table 1 Plasmids used in experiments, together with reference:

a. Plasmids used for investigating NIX-phosphatase interaction		
pEGFP-C1	GFP-tagged empty vector	AddGene
pEGFP-C1/NIX WT	GFP-tagged NIX protein	(Novak et al., 2010)
pEGFP-C1/NIX S212A	GFP-tagged NIX, serine 212. mutated to alanine	(Marinković et al. 2020)
pEGFP-C1/NIX S212E	GFP-tagged NIX, serine 212. mutated to glutamic acid	(Marinković et al. 2020)
pcDNA3.1(-)	mammalian expression vector r	AppliChem
pcDNA3.1(-)/MYC-PP1α	MYC-tagged human PP1α phosphatase	Not published[1]
b. Plasmids used in RNA interference		

[1] Cloned in the Laboratory for cancer research, School of Medicine, University of Split, Croatia

pL4440	Empty vector for expression in Nematode	AddGene[2]
pL4440/PP1β	dsRNA targeting PP1β phosphatase	(Kamath et al., 2003)[JŠ9]

3.1.5 Media

- **Bacterial culture**

LB broth media [D10][JŠ11](1.0% bacto. tryptone, 0.5% yeast extract, 0.5% NaCl in ddH_2O)

LB agar plates (1.0% bacto. tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% agar in ddH_2O);

With antibiotic selection when required: ampicillin (final concentration: 50 mg/ml) kanamycin (final concentration: 25 mg/ml), tetracycline (final concentration: 12.5 mg/ml).

- **Cell culture**

DMEM medium, supplemented with 10% FBS and 1% penicillin/streptomycin.

- **Maintaining *C. elegans***

NGM – nematode growth medium, specialized agar that supports the growth of nematodes and bacteria, made from standards protocol (*Table 2*). After the preparation of NGM, 7 ml of media was poured in 6 cm petri dishes and spotted with 250 µl of bacteria.

Table 2 The standard protocol for NGM preparation (600 ml)

NaCl	1.8 g
Bact peptone	1.5 g
Agar	12 g
ddH_2O	Up to 600 ml

[2] L4440 was a gift from Andrew Fire (Addgene plasmid # 1654 ; http://n2t.net/addgene:1654 ; RRID:Addgene_1654)

Autoclaved & cooled to 55 °C	
Cholesterol (5 mg/ml in ethanol)	600 µl
1M $CaCl_2$	600 µl
1M $MgSO_4$	600 µl
1M KH_2PO_4	15 ml

3.1.6 Chemicals

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid – HEPES (Carl Roth)

Acrylamide (AppliChem)

Agar (Carl Roth, Sigma Aldrich)

Agarose (Sigma Aldrich)

Ammonium persulfate – APS (Sigma Aldrich)

Ampicillin (Carl Roth)

Aprotinin (Sigma Aldrich)

Bacto peptone (Sigma Aldrich)

Bacto tryptone (Fluka)

Bovine serum albumin – BSA (Carl Roth)

Calcium chloride (Kemika)

Carbonyl cyanide 3-chlorophenylhydrazone – CCCP (Sigma Aldrich)

Cholesterol (Sigma Aldrich)

Dimethyl sulfoxide – DMSO (Carl Roth)

Ethanol (Kemika)

Ethylenediaminetetraacetic acid – EDTA (Carl Roth)

Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid - EGTA (Carl Roth)

Fetal bovine serum - FBS

GFP-Trap beads (ChromoTek)

Glycerol (Kemika)

Glycine (Carl Roth)

Loading dye for gel electrophoresis *Blue/Orange 6X Loading Dye* (Promega)

Phenylmethylsulphonyl fluoride – PMSF (Carl Roth)

Isopropanol (Kemika)

Isopropyl β-D-1-thiogalactopyranoside – IPTG (Carl Roth)

Kanamycin (Carl Roth)

Leupeptin (Sigma Aldrich)

Levamisole hydrochloride (Sigma Aldrich)

Medium for cell culture - *Dulbecco's Modified Eagle's Medium,* DMEM (Sigma Aldrich)

Methanol (Sigma Aldrich)

Nitrocellulose membrane for Western blot (GE Healthcare)

Nuclease-free water (Invitrogen)

Penicillin - streptomycin (GIBCO)

Potassium dihydrogen phosphate (Kemika)

Potassium chloride (Kemika)

Powdered milk (Carl Roth)

Protein Marker VI (10-245) prestained (AppliChem)

Sodium acetate (Carl Roth)

Sodium azide (Kemika)

Sodium chloride (Kemika)

Sodium dodecyl sulfate - SDS (Carl Roth)

Sodium fluoride (Fluka)

Sodium hydrogenphosphate heptahydrate (Kemika)

Sodium orthovanadate (Sigma Aldrich)

Tetramethyl ethylenediamine - TEMED (Carl Roth)

Tris (Sigma Aldrich)

Triton-X-100 detergent (Carl Roth)

Tween (Sigma Aldrich)

Yeast extract (Fluka)

β-mercaptoethanol (Fluka)

3.1.7 Buffer solutions

1X PBS - 10X stock solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 x 7 H_2O, 2.0 mM KH_2PO_4)

1X running buffer - 10X stock solution (250 mM Tris, 2 M glycine, 1% SDS in ddH_2O)

1X transfer buffer - 10X stock solution (250 mM Tris, 2 M glycine, 20% v/v methanol in ddH2O)

1X TAE buffer - 50X TAE stock buffer (2 M Tris, 5.7% ethanoic acid, 100 mM EDTA pH 8.0 in ddH_2O)

1X TBS buffer - 10X stock solution (500 Mm Tris, 1.5 M NaCl, pH 7.5 in ddH_2O)

2X Leammli sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 5,6% SDS, 0,01% bromphenol blue in ddH_2O)

4X Tris-HCl/SDS **buffer for running gel** pH 8.8 (1.5 M Tris, 0.4% SDS in ddH_2O)

4X Tris-HCl/SDS **buffer for stacking gel** pH 6.8 (0.5 M Tris, 0.4% SDS in ddH_2O)

TBS-BSA blocking buffer (5% BSA, 0.1% Na-azide, phenol red in 1X TBS-u, pH 7.4 in ddH_2O)

Washing buffer (1X TBS, 0.05% Tween, pH 7.4 in ddH_2O)

Cell lysis buffer, pH 7.5 (50 mM HEPES, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 25 mM NaF, 1% Triton x-100, 10 µM $ZnCl_2$ in ddH_2O)

M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$ in ddH_2O, sterilized by autoclaving)

3.1.8 Kits

GeneJET Plasmid Miniprep Kit (Fermentas)

jetPRIME Transfection Kit (Polyplus)

Western blotting luminol reagent (Santa Cruz Biotehnology)

3.1.9 Antibodies

b) Primary antibodies: monoclonal anti-GFP antibody from mice (Roche, 1:1000)

monoclonal anti-MYC antibody from mice (Santa Cruz Biotechnology, Inc., 1:1000)

c) Anti-mouse IgG antibody from goat conjugated with horse radish peroxidase - HRP (Bio-Rad, 1:5000)

3.1.10 Instruments and softwares

Electrophoresis apparatus Mini Protean 3 (Bio-Rad)

Automated cell counter Countess™ II Automated Cell Counter (Thermo Fisher Scientific)

Centrifuge Biofuge primo R (Heraeus)

Centrifuge Heraous Fresco 17 centrifuge (Thermo Fisher Scientific)

Fluorescence microscope Axioimager D1, Carl 165 Zeiss, Inc., (software: AxioVision software version 4.4; Carl Zeiss, Inc.) (Carl Zeiss)

GraphPad Prism software package (GraphPad Software Inc., San Diego, USA)

Image J (Fiji.app)

Incubator Economy Incubator with fan Size 1 (GALLENKAMP)

Incubator Hereaus Hera Cell 150 (Thermo Fisher Scientific)

Incubator HettCube 400 R (Hettich)

Microsoft Office 2011 Excel (Microsoft Corporation, Redmond, USA)

Nanodrop NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific)

Roteks General rotator STR4 (Stuart)

UV/VIS BioPhotometer (Eppendorf)

UV-B 236 light (Waldmann Medizintechnik)

Stereo microscope STEMI 508 (Carl Zeiss)

Transfer tank system Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad)

Thermoblock Thermo Shaker Incubator Thriller (PREQLAB)

Chemi Doc XRS (software: Image LabTM 6.0.1) (Bio Rad)

3.2 Methods

3.2.1 Protein-interaction assays

3.2.1.1 Phosphatase candidate selection

Possible phosphate candidate was selected by searching the literature. Since phosphatases are not highly selective, the criterion for phosphatase selection was that the active domain of phosphatase is located in the IMS of mitochondria, within which the C-terminal part of the NIX protein is located. PP1 phosphatase dephosphorylate a wide array of substrate proteins. It is found in the IMS of mitochondria which makes it a good phosphate candidate for removal of the phosphate on 212. serine residue of protein NIX.

Protein-interaction assays, done on HEK293 cell line, investigate the interaction between PP1α, one of the three catalytic subunits encoded by PP1CA gene that is found in *Homo sapiens* respectively.

On the other hand, RNA interference assay investigate the impact of PP1 homologue enzyme PP1β encoded by gsp-2 gene found in *Caenorhabditis elegans.*

3.2.1.2 Bacterial transformation using heat shock and DH5α competent bacteria

Bacterial transformation is a method of introducing foreign DNA (plasmid) into a bacterium which amplify the introduced DNA (Hotchkiss and Gabor, 1970). The competent DH5α bacteria were transformed with already available plasmid constructs listed in *Table 1*. Before heat shock, 100 ng of specific plasmid construct was added to 50 µl of competent bacteria and incubated for 30 min on ice. Afterwards, the bacteria were exposed to 42 °C for 2 min, and 1 mL of LB medium was added to regenerate bacteria. The bacteria were immediately incubated on 37 °C for 1 h with 450 rpm shaking.

This sudden increase in the temperature creates pores in the plasma membrane of the bacteria, allowing the plasmid DNA to enter the cell (Asif et al., 2017). Bacterial cells are harvested by centrifugation (5 min, 1500 x g). Bacteria transformed with pEGFP-C1 plasmid were spread on LB agar plates with kanamycin, while bacteria transformed with pcDNA3.1(-) were spread on LB agar plates with ampicillin. Transformed bacterial strains are grown overnight at 37 °C.

Single colony from LB agar plates were picked to inoculate ~6 mL LB media with kanamycin for pEGFP-C1 plasmid, or ampicillin for pcDNA3.1(-), overnight on 37 °C with 220 rpm shaking.

3.2.1.3 Plasmid DNA isolation

The basic steps for plasmid DNA isolation are disruption of the cellular structure, and separation of the plasmid from chromosomal DNA and other cellular material. The isolation of plasmid DNA was done as recommended by instructions of *GeneJET Plasmid Miniprep Kit* (GeneJET Plasmid Miniprep Kit instructions) which contains chemicals, and spin columns for plasmid DNA isolation. Isolated plasmid DNA was eluted in 50 µl of the nuclease-free water,

The concentration, yield, and purity of the isolated plasmids DNA was measured with NanoDrop 1000 Spectrophotometer. Absorbance at 260 nm and 280 nm is measured. The absorbance A_{260} is used to read the concentration of DNA, while the ratio of A_{260}/A_{280} shows the purity of DNA (A_{260}/A_{280} ratio = 1.8-2.0).

3.2.1.4 Transfection of HEK293 cells

Transfection is a method of introducing nucleic acids – plasmids, into eukaryotic cells. Over-expression of protein NIX, NIX-S212A, NIX-S212E mutants, and phosphatase candidate PP1α is achieved by co-transfection of plasmid DNA to HEK293 cells.

Transfection was done using *jetPRIME Kit* that mediate liposome delivery of plasmid DNA into the cell.

The HEK293 cells were seeded in two 6-well plates, concentration: 0.5 x 10^6 per 2 ml of DMEM (10% FBS, 1% penicillin/streptomycin) medium, and incubated in a 37 °C, 5% CO_2 tissue culture incubator. At the confluency of 60-80%, the cells were co-transfected with different plasmid constructs, as shown in the *Table 1*. Transfection reagents: 200 µl of the *jetPRIME* buffer i 2 µl of the *jetPRIME* reagent were mixed with 0.5 µg of each plasmid DNA (empty plasmid or with gene of interest). After vortexing and spin-down, the transfection mix is incubated on RT for 10 min. During incubation, reagent's lipid compounds associate with negatively charged DNA which allows the introduction of foreign DNA through the lipid layer of the cell membrane to the cytosol. Transfection mix was spotted on 60-80% confluent cells in the 6-well plates. The cells were incubated for 16 h in a 37 °C, 5% CO_2 tissue culture incubator.

The transfection of plasmid DNA with GFP reporter was evaluated after 16 h using fluorescence microscope. When excited by UV light, the GFP reporter emits green fluorescence light. Thus, evaluating the transfection efficiency of the GFP-tagged plasmids.

3.2.1.5 Treatment with CCCP

To induce mitophagy, transfected cells were treated with CCCP – mitochondrial poison that disrupt the mitochondrial membrane potential, thus, activating mitophagy. After removal of the old medium, new media was added to each well. One 6-well plate of transfected cells was treated with 1 ml DMEM media with addition of 1 µl of CCCP (final concentration: 10 µM) per each well for 2 h. Control 6-well plate of transfected cells were treated with DMSO (1 ml DMEM medium + 1 µl DMSO per well) (Figure *7*).

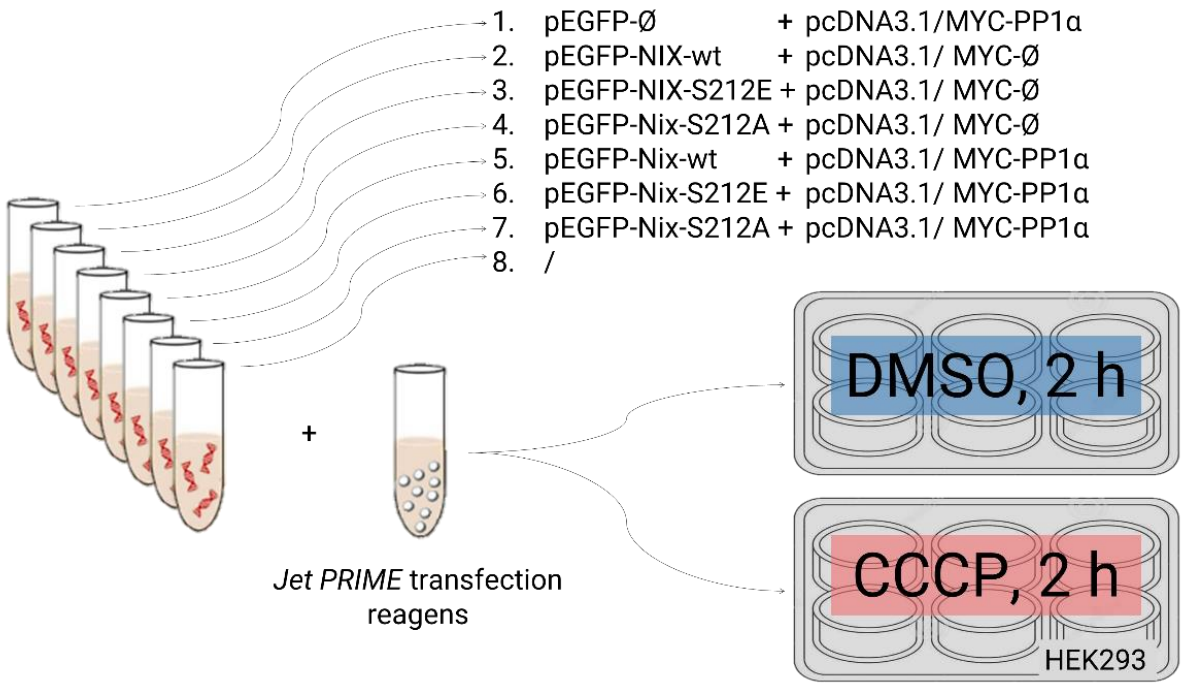


Figure 7 *The scheme of trasnfection and treatment protocol.* HEK293 cells were co-transfected with different variant of NIX proteins and PP1 phosphatase. Transfected cells were treated with mitochondrial poison CCCP which distrupts mitochondrial membrane potential. As negative control, transfected cells were treated with DMSO in the same manner.

3.2.1.6 Pull-Down Assay

Transfected cells have overexpressed proteins of interest: NIX, mutants of NIX, and phosphatase. To continue with analyzing the protein interactions, total cell lysates – TCL were prepared. First, after 2 h treatment with CCCP, medium was removed, and cells were collected with 500 µl of 1X PBS in 1.5 ml microcentrifuge tubes. Transfected cells were harvested by centrifugation on 4 ºC, for 20 min, 500x g. Cell pellets were mixed with 500 µl Cell lysis buffer (50 mM HEPES, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 25 mM NaF, 1% Triton x-100, 10 µM $ZnCl_2$ in ddH_2O, pH 7.5) supplemented with inhibitors: leupeptin (inhibits protases), aprotinin, PMSF (inhibit serine protease); and Dnase (degrades DNA) . It is important to keep TCLs 30 min on 4 ºC (ice) to keep proteins and protein interactions intact, while membrane and other cell debris is degraded. Soluble cell proteins were separated from insoluble cell content by centrifugation on 4 ºC for 20 min, 2500 x g. Total cell lysate was used in following steps for Pull-down assay.

To analyze interaction between NIX protein and its mutants with phosphatase, 200 µl of TCL was separated in fresh 1.5 ml tube. Since, protein NIX and its mutants are fused with GFP reporter, 2 µl of GFP-Trap beads was added to TCL. The GFP magnetic beads have a “bait” protein that binds GFP-tagged proteins, NIX protein and its mutant, together with its interacting proteins. Interaction between GFP and GFP magnetic beads facilitate one-step isolation of GFP-tagged proteins and their interacting partners. After 4 h incubation on a rotator (4 ºC), the samples were centrifugated for 3 min on 4 ºC, 400 x g. This ensured that GFP-tagged NIX protein (and its possible interactor) are separated from the rest of the cell lysate that is removed with syringe needle to avoid discarding of proteins bind to beads. Unbound proteins, that are not discarded, are washed with addition of 400 µl of Cell lysis buffer and centrifugation for 3 min on 4 ºC, , 400 x g. This step was repeated twice. After the final removal of the unbound proteins in the supernatant, the rest of the magnetic beads are mixed with 20 µl of 2X Laemmli buffer incubated 5 min on 95 ºC. This step causes breakage of covalent bonds, including separation of proteins and magnetic beads. Additional 50 µl of TCL was mixed with 12.5 µl of 5X Laemmli buffer and incubated 5 min on 95 ºC.

3.2.1.7 Western blot

The Western blot analysis is widely used technique of detection specific proteins based on specific antibody recognition. The proteins were separated by SDS-PAGE electrophoresis (sodium dodecyl-sulphate – polyacrylamide gel electrophoresis). Sodium dodecyl-sulfate is detergent that eliminates the influence of structure and charge of the protein, separating the proteins solely on the mass. The proteins were separated with 12% separating gel (12% acrylamide (40%), 25% buffer pH 8.8, 0.5% APS, 0.1% TEMED) and 4% stacking gel (4% acrylamide (40%), 24% buffer pH 6.8, 0.5% APS, 0.1% TEMED). The samples (denatured with 2X Laemmli buffer) were loaded on gels V in 1X runner buffer. Electrophoresis is done 15 min under 80 V, and 60 min under 120 V.

Next step was to transfer proteins separated in the gels on a solid support matrix – nitrocellulose membrane that facilitates the detection of proteins using antibodies agais the protein. The gel was placed in the transfer sandwich: filter paper-gel-nitrocellulose membrane-filter paper, cushioned by pads and pressed together in the tank filled with 1X transfer buffer. The transfer of proteins from gel to nitrocellulose membrane relies on the electrophoretic mobility of proteins upon electric field. The proteins (negatively charged) bind to nitrocellulose membrane (positively charged), creating a copy of the protein separation pattern from the original gel. The constant amperage of 200 mA was applied to transfer tank for 60 min. Membranes with transferred proteins were incubated in TBS-BSA blocking buffer for 1 h, on RT. This step is important to block unspecific binding of primary proteins on membrane (*"background"*). After blocking, the membranes were incubated over-night on 4°C with primary antibodies that are highly specific for reporter tags fused with proteins of interest. The primary antibodies were diluted in TBS-BSA buffer. For detection of protein NIX, monoclonal anti-GFP antibody from mice was used, and for detection of phosphatase, anti-MYC antibody from mice was used. The membranes were washed 5x in *Washing* buffer to remove the un-binded antibodies.

In order to visualize the proteins, the membranes were incubated 60 min with secondary antibody diluted in 5%-powder milk solution. Since the primary antibodies are isolated from mice, the secondary antibodies used were anti-mice antibodies that are conjugated with horse radish peroxidase (HRP). After the incubation with secondary antibody, the membranes were again washed 5x in *Washing* buffer, also to remove unbound antibodies. Hence, lowering the background.

For protein detection, HRP conjugated secondary antibodies were activated with Western blotting luminol reagent. The chemiluminescence was analyzed with *Chemi Dox XRS* (*Figure 8)*.

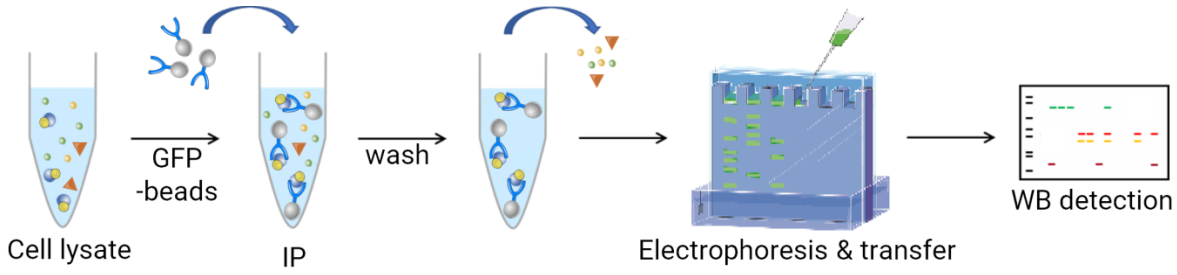


Figure 8. *Pull down assay scheme.* Transfected HEK293 cells were lysated and proteins were collected as total cell lysates. After the addition of GFP-magnetic beads, the GFP-tagged NIX proteins will be bounded to the beads, together with its interactors. After washing the non-interacting proteins from the cell lysate, the proteins that were bounded to the beads, are analyzed using Western blot analysis.

3.2.2 Monitoring mitophagy *in vivo*

3.2.2.1 Growth of nematode population

The *C. elegans* strain, which express mitochondria-targeted Rosella (mtRosella) reporter, will be referred as Rosella strain. Adult transgenic animals were transferred on three 10 cm *E. coli* (OP50) NGM plate and incubated at 20 °C until the worms laid at least 100 eggs per plate. Adult worms were removed, and plates, with eggs, were incubated on 20 °C in HettCube 400 incubator.

3.2.2.2 Preparation of NGM plates for RNA interference

RNA interference – RNAi is a method to determine gene function. The method utilizes biological processes of inhibiting translation of messenger RNA – mRNA to protein. Hence, inhibiting gene function. In *C. elegans,* RNAi was achieved by feeding Rosella transgenic animals with *E. coli* (HT115) bacteria carrying a plasmid expressing doube-stranded RNA (dsRNA) against the gene of interest – PP1 phosphatase.

The preparation of RNAi plates is similar to preparation of standard NGM plates, but with addition of antibiotics (ampicillin and tetracycline) and IPTG after autoclaving.

The *E. coli* bacterial strain used in RNAi is HT115 containing pL4440 plasmid (with or without gene of interest – PP1).

Previously transformed HT115 bacteria were defrosted from glycerol stock and were streaked on LB agar plate with antibiotics (ampicillin and tetracycline). The bacteria grew overnight on 37 °C for 16 h. After the incubation, single colony of transformed bacteria were transferred in 12 ml of LB media with antibiotics and incubated for 16 h on 37 °C while 180 rpm shaking. For efficient RNAi, it is important to have the same concentration of bacteria during the whole experiment as the concentration of bacteria is proportional to concentration of dsRNA delivered to the animals. After the incubation, the optical densities of bacterial cultures were measured with spectrophotometer and diluted to standardized concentration of 0.9 mg/ml. The bacteria were diluted with LB media with addition of ampicillin and tetracycline and were spotted on RNAi agar plates in volume of 250 µl per plate.

In order to investigate mitophagic events under mitochondrial stress, RNAi plates were treated with paraquat, a pesticide which is toxic to animals and humans due to its redox activity. Similar to CCCP treatment done on HEK293 cells, paraquat causes a disruption of mitochondrial membrane potential, thus, causing ROS-induced stress. Since the bacteria can metabolize paraquat, RNAi plates were exposed to UV light for 25 min in order to kill bacteria. Paraquat was added to RNAi plates at the final concentration of 8 mM in the total agar volume respectively. For negative control, RNAi plates without addition of paraquat were used.

3.2.2.3 RNA interference

Before each experiment, fresh RNAi plates with paraquat were made. At least 20 transgenic L4 larvae animals were transferred from 2- and 4-days-old *E.coli* (OP50) plates to three RNAi plates per condition - with and without paraquat, and with bacteria expressing empty or vector with phosphatase PP1. Transferred animals were incubated 2 days at 20 °C.

3.2.2.4 Imaging of transgenic animals on fluorescence microscope

Upon 2 days of paraquat exposure, the animals were collected with a platinum pick and imobillized in 12 µl of 20 mM M9-Levamisole solution on a microscope slide and gently covered with a coverslip (*Figure 9*).

As prepared, the animals were observed under a fluorescent microscope. For each animal, the GFP and DsRed channel images were acquired using the same conditions throughout the experiment (x10 magnification, exposure times: GFP – 180 ms, DsRed – 70 ms).

3.2.2.5 Analysis

Mitophagy events can be quantified by analyzing the fraction of green fluorescence which has quenched as a consequence of fusion of mitochondria-tagged Rosella with lysosomes. Previously acquired images were analyzed using ImageJ software to measure the average pixel intensity value. To avoid intestinal autofluorescence, free-hand tool was used to analyze the head region of animals, respectively.

Pixel intensity was normalized by subtracting the background fluorescence for both channels using Microsoft Office Excel software. The ratio of GFP to DsRed fluorescence was calculated, and normalized by dividing the values of each condition with values of control group without interference.

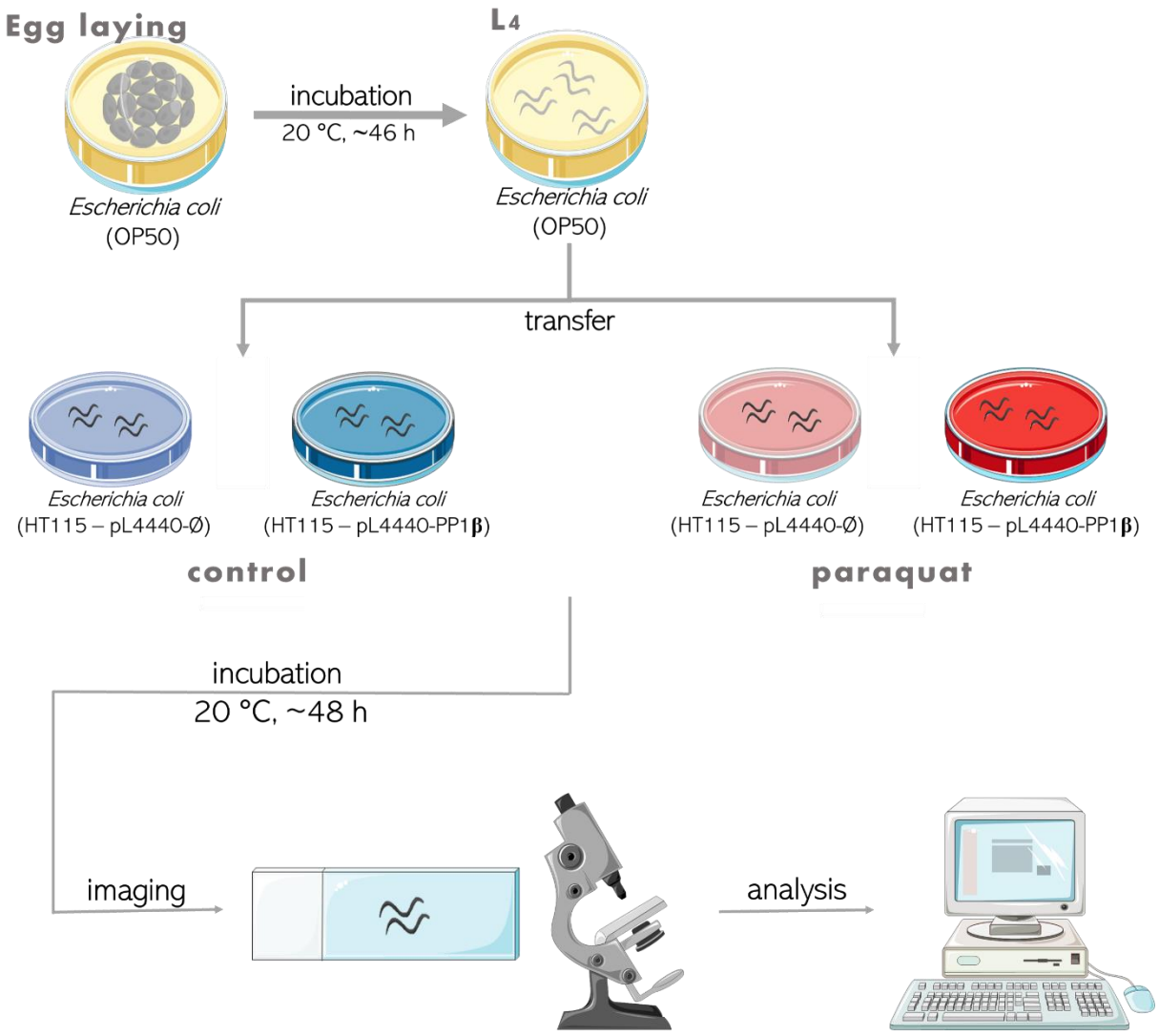


Figure 9 *The scheme of the experiment for monitoring mitophagy in vivo.* Transgenic *Caenorhabditis elegans* animals were grown on agar plates seeded with *Escherichia coli* (OP50) bacteria. After incubation, the L4 adult animals were transferred on agar plates containing mitophagy inducer – paraquat seeded with *E. coli* (HT115) bacteria expressing dsRNA for PP1β phosphatase. Images are acquired 2 days after the transfer, and analysed using software tools for fluorescence intensity analysis.

Statistical analysis

Statistical analysis was done using software tools *GraphPad Prism 8* and *Microsoft Excel*. Difference between two conditions was analyzed using student's t-test with a significance cutoff level of ***$p<0.01$. To determine whether there are any statistically significant differences between all conditions of the experiment, the one-way analysis of

variance – ANOVA was performed. Protein interaction assays were repeated at least 3 times, whereas RNA interference assay was repeated also 3 times with at least 30 individual animals per condition.

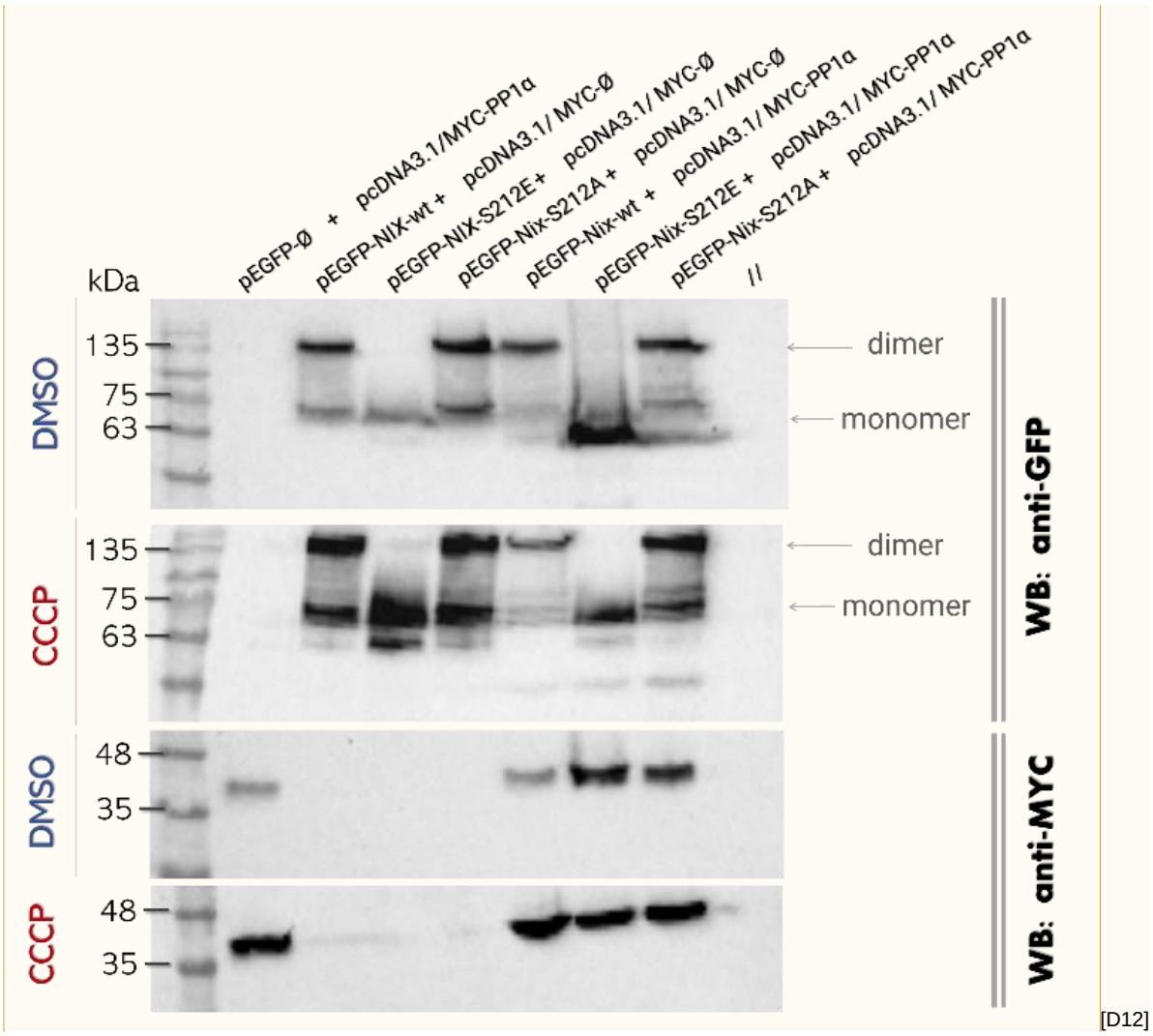
4 Results

4.1 Protein-interaction assays

4.1.1 Over-expression of NIX protein and PP1 phosphatase

Overexpressed proteins were isolated from HEK293 transfected cells. Total cell lysates were analysed by Western blot immuno-detection using primary antibody against GFP reporter to detect NIX protein, and its mutants – NIX-S212A, NIX-S212E. Proteins NIX-wt and NIX-S212A are detected in monomer and/or dimer form, whereas NIX-S212E lack the dimer form because the negative (-) charge of phosphomimetic glutamic acid inhibits the dimerization of the protein. In order to analyze over-expression of PP1 phosphatase, primary antibody against MYC was used.

Analysis of the total cell lysates shows that the proteins of interest were successfully expressed and isolated from HEK293 transfected cells, both in control – DMSO and treated – CCCP group (*Figure 10*).



[D12]

Figure 10 *Western blot imunodetection of proteins isolated from HEK293 transfected cells.* Total cell lysates were analyzed using anti-GFP antibody to detect NIX-wt, NIX-S212A, and NIX-S212E. Mutation of serine 212 to glutamic acid inhibits dimerization, hence, NIX-S212E is detected only in monomer form. Identification of PP1α was done using anti-MYC antibody. Expression of NIX-wt, NIX-S212A, NIX-S212E, and PP1α phosphatase was successful in both control and cells treated with CCCP.

4.1.2 PP1α phosphatase interacts with NIX protein *in vitro*

Elution of GFP-tagged proteins, and its interactors, was done using GFP-Trap beads that were added to TCL. Eluted proteins were detected by Western blot analysis using aforementioned antibodies.

Membranes incubated with primary antibody against GFP show that the pull down of GFP-fused proteins was successful (*Figure 11*.).

On the other hand, membranes incubated with primary antibody against MYC-reporter show that there is an interaction between PP1α phosphatase and NIX-S212E mutant *in vitro*, both in control and cells treated with CCCP (*Figure 11*). Since this NIX protein has artificial substituted serine 212 residue with glutamic acid, which has phosphomimetic effect, the binding of phosphatase was as expected.

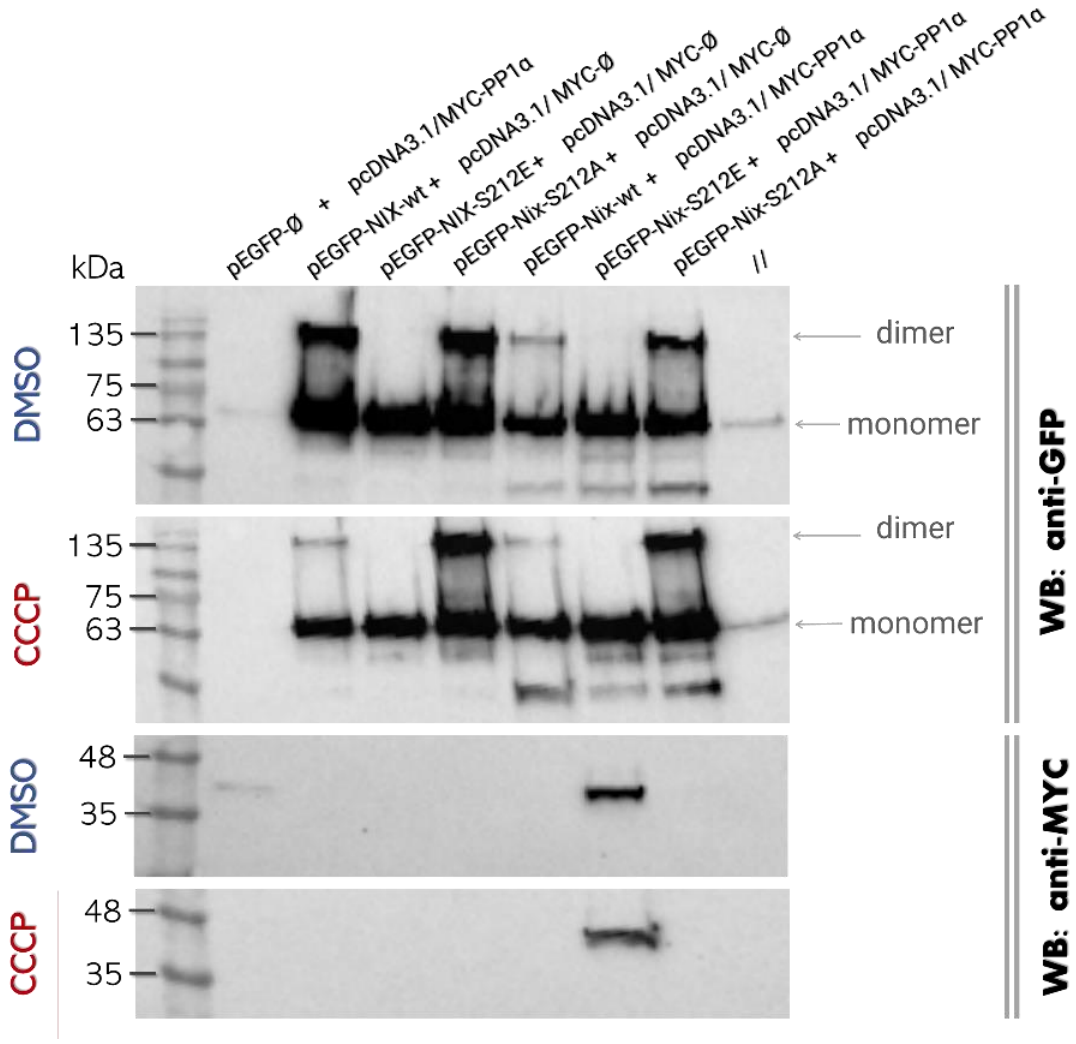


Figure 11 *Western blot imunodetection of proteins eluted from total cell lysates by GFP pull down method*. Detection of proteins fused with GFP-reporter (NIX-wt, NIX-S212A, NIX-S212E) is done with anti-GFP antibody. Interacting protein, PP1α is detected with .anti-MYC antibody.

4.2 RNA interference assay

The role of PP1 phosphatase in selective removal of mitochondria was investigated *in vivo* in nematode *C. elegans*. Since the PP1α phosphatase, used in pull down assay, is not found in nematodes, the homologue PP1β (encoded by gsp-2 gene) was targeted.

4.2.1 PP1β inhibits mitophagy under basal conditions

Transgenic *C. elegans* animals (P_{myo3}::TOMM-20::Rosella) were grown on plates containing mitophagy inducer – paraquat. Animals were fed with *E. coli* bacterial strain expressing dsRNA against PP1β. Since the Rosella biosensor has pH-sensitive GFP, and pH-insensitive RFP, the ratio of GFP to RFP (dsRed) should drop down upon lysosomal degradation of GFP fluorescence. Thus, lower GFP/dsRed ratio represent higher mitophagic turnover. The occurrence of mitophagy events were measured by dividing the fluorescence intensity of GFP and RFP (dsRed) subunits of Rosella reporter anchored to TOMM20 mitochondrial protein. The results of repeated experiments were then normalized by dividing the values of each condition with values of control group without RNA interference.

Upon silencing of expression of PP1β phosphatase, the GFP/dsRed ratio of control group with the interference dropped almost as the value of the group treated with paraquat, but without RNA interference. Or in other words, silencing of the PP1β phosphatase expression induces the mitophagy even if there is no mitophagy inducer. Whereas the knock-down of phosphatase with paraquat treatment, shows no significant difference in comparison to paraquat treatment without silencing phosphatase expression. On the other hand, each condition showed significant difference (***p>0.01) in comparison to, untreated, control group without the interference of PP1β phosphatase. (*Figure 12*).

Since the RNA interference assay targets the expression of gene of interest, in this case PP1β phosphatase, the results are representing the mitophagy events in the

absence of the phosphatase. Upon silencing of PP1β phosphatase expression, the pixel intensity GFP/dsRed ratio dropped down significantly, both in control and treated group, in comparison to control group without the interference of PP1β phosphatase expression (***p<0.01). Hence, results suggest that PP1β phosphatase could inhibit mitophagy under basal conditions. Moreover, after the induction of mitophagy, PP1β phosphatase shows no effect on inhibiting, nor promoting mitophagy.

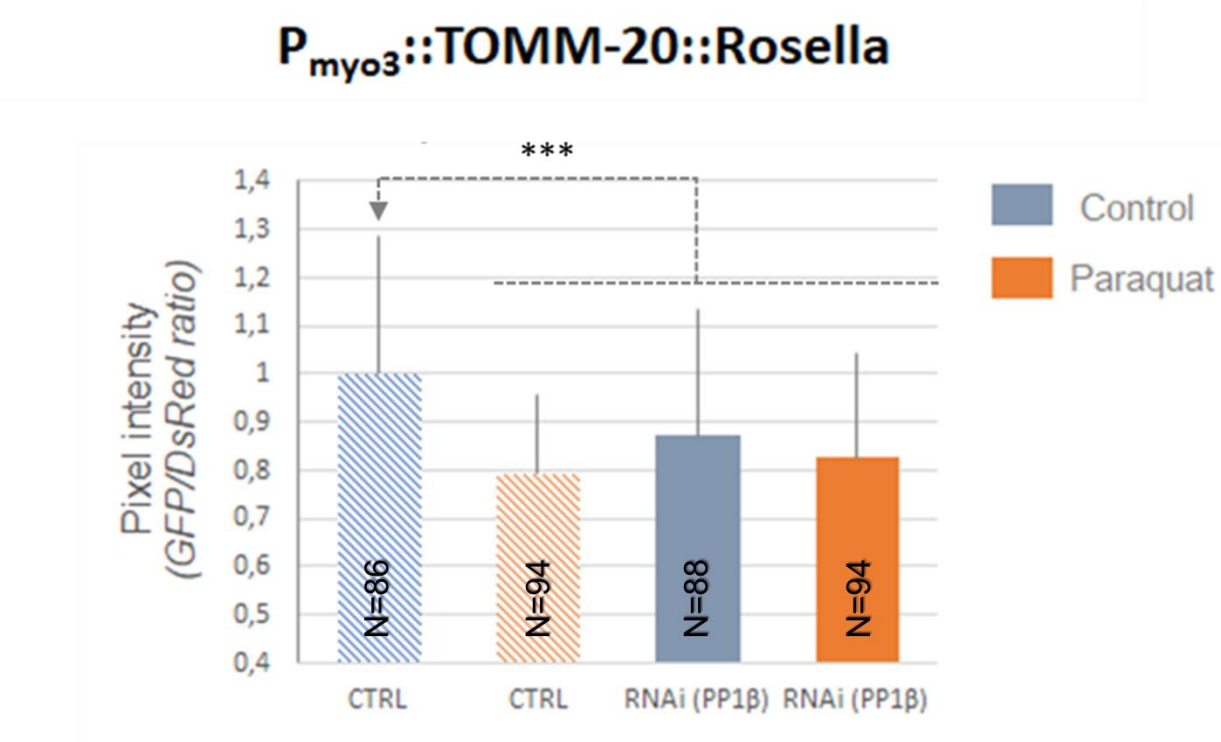


Figure 12 *Results of RNA interference of PP1β phosphatase*. The graph shows the pixel intensity ratio of GFP/DsRed reporters. Animals treated with paraquat are marked with orange, whereas animals without the treatment are marked with blue color. Standard deviation is showed on each bar, together with the statistical significance cut off (***p<0.01). Number of analyzed transgenic animals is shown as the N value.

The statistical difference between each condition (group) was analyzed using student t-test. The difference between control and treated group, but without the interference, shows that there is a significant statistical difference, which means that the experiments were properly conducted.

5 Discussion

Autophagy is, without doubt, a necessary intracellular process important in maintaining the homeostasis within eukaryotic cells. Importance of autophagy is evident by the fact that since the discovery of the basic mechanisms of autophagy in the 1990s, the scientific interest in the field autophagy is rapidly emerging. According to the PubMed database, the number of publications related to autophagy increased from less than 100 to more than 6,000 publications per year (Kirkin, 2020). Moreover, the disturbance of the autophagy mechanism is related to a variety of diseases. Hence, the understanding of the molecular basis of the autophagy mechanism is important for, not only better understanding of the processes itself, but also as a possible therapeutic cure for certain diseases related to autophagy (Villanueva Paz et al., 2016). Since the discovery of selective autophagy receptors, there has been an emerging interest in the field of mitophagy. Selective removal of mitochondria is important in the regulation of healthy mitochondrial network. It includes removal of redundant, damaged or dysfunctional mitochondria, and is important in different physiological processes, such as embryonal development, differentiation of certain cell lineages, and neurodegenerative diseases.

Mitochondrial protein NIX is the first described receptor for the selective removal of mitochondria (Novak et al., 2010). Its structure is characterized with a BH3-only and TM domain, both important for the induction of programmed cell death, apoptosis (Ohi et al., 1999). Other than pro-apoptotic function, NIX is also a tumor suppressor that enables the cell to survive under stress conditions (Fei et al., 2004). This dual role of the receptor makes NIX protein interesting for future research in neurodegenerative diseases, aging, erythropoiesis, and many others. However, more detailed molecular mechanisms of regulation of NIX-mediated mitophagy remain unknown.

Given the importance of the mitochondrial protein NIX in selective removal of mitochondria, but also in maintaining the overall homeostasis of the organism, more research of its molecular activity should be examined.

Even though, it was previously known that NIX protein forms stable homodimers of ~80 kDa size (Novak et al., 2010), Marinković et al. (2020) showed the importance of the TM domain of NIX protein for the successful formation of dimers, as well as, for their proper placement in the OMM. Regulation of the dimerization is still unknown.

The C-terminal end of the NIX protein is located in the IMS and is composed of 11 aminoacid residues. As mentioned, IMS contains a number of different regulators, and could take place for phosphorylation-driven regulation of pro-apoptotic and autophagic roles of the NIX protein. Recent research showed that the NIX mutant with the substitution of serine 212 to phosphomimetic glutamic acid (NIX-S212E) lacks the dimeric form. Hence, it is hypothesized that the removal of the negative phosphate group on the C-terminal end of the NIX protein allows the dimerization of the protein, thus, activating the mitophagy (Marinković et al. 2020) (*Figure 13*).

The first aim of the research was to decipher the phosphatase responsible for dephosphorylation of the serine 212 in NIX. Indeed, pull down assay and Western blot immunodetection show that there is an interaction between NIX protein and investigated phosphatase candidate PP1 *in vitro*. However, to confirm the interaction, additional experiments should be conducted. The protein-phosphatase interaction should be furthermore investigated by creating a constitutively active and inactive phosphatase dead mutant, colometric and radioactive assays for serine/threonine phosphatase. The functional analysis of the PP1 phosphatase role in mitophagy induction should be analyzed by silencing the phosphatase expression in HEK293 cell line, and also by monitoring co-localization of the fluorescence labeled-proteins, NIX protein and PP1. Interestingly, PP1 inactive mutant D95N has been already described by several groups (Gallego et al. 2006; Hutchinson et al. 2011; Luo et al. 2007), and PP1 constitutively active mutant T320A has also been created (Berndt et al. 1997). The functional analysis of the PP1 phosphatase role in mitophagy induction and progession should be analyzed by silencing the phosphatase expression in cell line and monitoring the NIX-dependent mitochondrial removal in this condition and system.

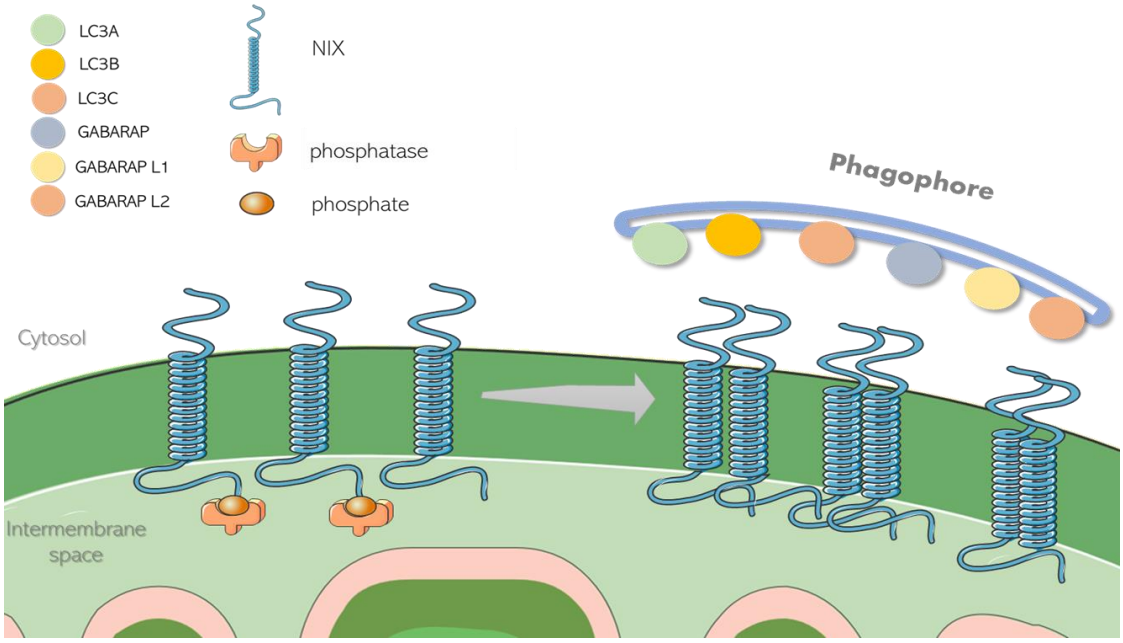


Figure 13 *Hypothesized regulation of dimerization of mitochondrial receptor NIX.* Dimerization of NIX protein is essential for recruitment of mitophagic machinery. Mutation of C-terminal 212.serine residue to negatively charged glutamic acid inhibits the dimerization. Hence, it is hypothesized that the dephosphorylation (removal of negatively charged phosphate group) of C-terminal end allows the dimerization and consequently induces the autophagosome formation. Thus, activating the mitophagy.

Impairment of mitophagy is often investigated *in vivo* in nematode *Caenorhabditis elegans* as a model organism. This transparent organism is ideal for noninvasive and real-time monitoring of different cellular mechanisms, including mitophagy.

However, not until recently, there was little known about the mitochondrial receptor responsible for mitochondrial clearance in *C. elegans.* In 2015, Palikaras et al. (2015) showed the importance of ceBNIP3, orthologue of the protein NIX in *C. elegans*, encoded by dct-1 gene, as a key-mediator of mitophagy,. Sequence analysis of the ceBNIP3 shows that ceBNIP3 contains a C-terminal TM domain, and BH3-only domain. These shared domains have been conserved throughout evolution. Just as the NIX protein, these domains have an important role in localization, dimerization and inducing apoptosis. However, the amino acid residues on the C-terminal end differ from the one of the NIX protein. Hence, the mechanisms of ceBNIP3 regulation are still unknown and require further research.

Functional analysis of the PP1 phosphatase impact on mitophagy events was performed by silencing the expression of the phosphatase in RNAi assay. Intriguingly, the results of RNAi showed that PP1 phosphatase inhibits mitophagy, suggesting a multiple role of the PP1 phosphatase in the process of mitophagy. In addition, research of hypoxia-induced autophagy in cardiomyocytes showed similar results. Song et al. (2015) showed that PP1 dephosphorylated the 139.serine residue of ATG16L1, an autophagy-related protein that controls the elongation of autophagosomal membrane, and phagophore assembly. Thus, inhibiting autophagy.

These results show a possible multiple role of the PP1 phosphatase in different steps of mitophagy process. In contrast to increasing knowledge of autophagy-related kinases, the understanding of the molecular basis of phosphatases function, and substrate interaction involved in different steps of autophagy are still quite limited.

However, for the future references, the protein interaction between PP1 and *C. elegans* should also be investigated in order to decipher if there is a role of PP1 in, not only inhibiting the phagophore formation, but also in direct interaction with ceBNIP3 orthologue. Furthermore, it would be interesting to investigate the impact of silencing of PP1 expression in the *C. elegans* strain with integrated human NIX protein fused with Rosella biosensor, and also, to conduct the co-localization assays with fluorescence-tagged NIX protein and fluorescence-tagged LGG – an autophagosomal adaptor in *C. elegans*, homologue of LC3 adaptor in mammalian cells.

6 Conclusions

The results show the interaction between phosphate candidate PP1 and NIX, but to confirm the interaction additional research need to be conducted.

Upon silencing of the expression of PP1 phosphatase, the mitopaghy increased in normal conditions, whereas upon mitophagy induction, PP1 phosphatase show no effect. Hence, PP1 phosphatase inhibits mitophagy under basal conditions. This result suggest that PP1 phosphatase has multiple roles in mitophagy. Not only that it interacts with mitochondrial protein NIX, it probably interacts with proteins involved in the phagophore formation, thus, inhibiting mitophagy prior its interaction with the mitophagy receptor.

However, given the importance of the mitophagy as an essential intracellular process in maintaining the homeostasis of the cell, and its relation with a number of diseases. The regulation of mitophagic events should be investigated further.

7 References

Abada, A., Elazar, Z., 2014. Getting ready for building: signaling and autophagosome biogenesis. EMBO Rep. **15,** 839–852.

Adam-Vizi, V., Chinopoulos, C., 2006. Bioenergetics and the formation of mitochondrial reactive oxygen species. Trends Pharmacol. Sci. **27**(12), 639-645.

Aggen, J.B., Nairn, A.C., Chamberlin, R., 2000. Regulation of protein phosphatase-1. Chem Biol. **7**(1), R13-R23

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2007. Molecular Biology of the Cell, 6th ed, Molecular Biology of the Cell. Garland Science, New York.

Alexander, C., Votruba, M., Pesch, U.E.A., Thiselton, D.L., Mayer, S., Moore, A., Rodriguez, M., Kellner, U., Leo-Kottler, B., Auburger, G., Bhattacharya, S.S., Wissinger, B., 2000. OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nat. Genet. **26**, 211–215.

Ashrafi, G., Schwarz, T.L., 2013. The pathways of mitophagy for quality control and clearance of mitochondria. Cell Death Differ. **20**, 31–42.

Asif, A., Mohsin, H., Tanvir, R., Rehman, Y., 2017. Revisiting the mechanisms involved in calcium chloride induced bacterial transformation. Front. Microbiol. **8**, 2169.

Backes, S., Herrmann, J.M., 2017. Protein Translocation into the Intermembrane Space and Matrix of Mitochondria: Mechanisms and Driving Forces. Front Mol Biosci. **4**, 83. [JŠ13]

Barazzuol, L., Giamogante, F., Brini, M., Calì, T., 2020. PINK1/Parkin Mediated Mitophagy, Ca2+ Signalling, and ER-Mitochondria Contacts in Parkinson's Disease. Int. J. Mol. Sci. **21**(5), 1772.

Bayly, G.R., 2014. Lipids and disorders of lipoprotein metabolism, in: Clinical

Biochemistry: Metabolic and Clinical Aspects: Third Edition. Elsevier Inc., pp. 702–736.

Berndt, N., Dohadwala, M., Liu, C.W.Y., 1997. Constitutively active protein phosphatase 1α causes Rb-dependent G1 arrest in human cancer cells. Curr. Biol. **7**, 375–386.

Chang, C.R., Blackstone, C., 2007. Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J. Biol. Chem. **282**, 21583–21587.

Charmpilas, N., Kounakis, K., Tavernarakis, N., 2018. Monitoring mitophagy during aging in Caenorhabditis elegans. Methods Mol Biol. **1759**, 151-160.

Chipuk, J.E., Bouchier-Hayes, L., Green, D.R., 2006. Mitochondrial outer membrane permeabilization during apoptosis: The innocent bystander scenario. Cell Death Differ. **13**(8), 1396-1402

Cizeau, J., Ray, R., Chen, G., Gietz, R.D., Greenberg, A.H., 2000. The C. elegans orthologue ceBNIP3 interacts with CED-9 and CED-3 but kills through a BH3- and caspase-independent mechanism. Oncogene **19**, 5453–5463.

Fei, P., Wang, W., Kim, S.H., Wang, S., Burns, T.F., Sax, J.K., Buzzai, M., Dicker, D.T., McKenna, W.G., Bernhard, E.J., El-Deiry, W.S., 2004. Bnip3L is induced by p53 under hypoxia, and its knockdown promotes tumor growth. Cancer Cell **6**, 597–609.

Fong, N.M., Jensen, T.C., Shah, A.S., Parekh, N.N., Saltiel, A.R., Brady, M.J., 2000. Identification of binding sites on protein targeting to glycogen for enzymes glycogen metabolism. J. Biol. Chem. **275**, 35034–35039.

Gallego, M., Kang, H., Virshup, D.M., 2006. Protein phosphatase 1 regulates the stability of the circadian protein PER2. Biochem. J. **399**, 169–175.

García-Macia, M., Santos-Ledo, A., Caballero, B., Rubio-González, A., de Luxán-Delgado, B., Potes, Y., Rodríguez-González, S.M., Boga, J.A., Coto-Montes, A., 2019. Selective autophagy, lipophagy and mitophagy, in the Harderian gland along the oestrous cycle: a potential retrieval effect of melatonin. Sci. Rep. **9**, 1–11.

Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C., Kroemer, G., 2006. Mechanisms of cytochrome c release from mitochondria. Cell Death Differ. **13,** 1423–1433.

Gilkerson, R., Bravo, L., Garcia, I., Gaytan, N., Herrera, A., Maldonado, A., Quintanilla, B., 2013. The mitochondrial nucleoid: Integrating mitochondrial DNA into cellular homeostasis. Cold Spring Harb Perspect Biol. **5**(5), a011080.

Glynn, S.E., 2017. Multifunctional mitochondrial AAA proteases. Front Mol Biosci **4,** 34.

Goldberg, J., Huang, H. Bin, Kwon, Y.G., Greengard, P., Nairn, A.C., Kuriyan, J., 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature **376**, 745–753.

Hailey, D.W., Rambold, A.S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P.K., Lippincott-Schwartz, J., 2010. Mitochondria Supply Membranes for Autophagosome Biogenesis during Starvation. Cell **141,** 656–667.

Hellerschmied, D., Serebrenik, Y. V., Shao, L., Burslem, G.M., Crews, C.M., 2019. Protein folding state-dependent sorting at the Golgi apparatus. Mol. Biol. Cell **30**, 2296–2308.

Hotchkiss, R.D., Gabor, M., 1970. Bacterial transformation, with special reference to recombination process. Annu. Rev. Genet. **4**, 193–224.

Hutchinson, J.A., Shanware, N.P., Chang, H., Tibbetts, R.S., 2011. Regulation of ribosomal protein S6 phosphorylation by casein kinase 1 and protein phosphatase 1. J. Biol. Chem. **286**, 8688–8696.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A.,

Kamath, Ravi S., Andrew G. Fraser, Yan Dong, Gino Poulin, Richard Durbin, Monica Gotta, Alexander Kanapin, Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D.P., Zipperlen, P., Ahringer, J., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature **421**(6920), 231–37. [JŠ14]

Karbowski, M., Youle, R.J., 2011. Regulating mitochondrial outer membrane proteins by

ubiquitination and proteasomal degradation. Curr Opin Cell Biol. **23**(4), 476–482.

Kim, J.E., Choi, H.C., Song, H.K., Kang, T.C., 2019. Blockade of AMPA receptor regulates mitochondrial dynamics by modulating ERK1/2 and PP1/PP2A-Mediated DRP1-S616 phosphorylations in the normal rat hippocampus. Front. Cell. Neurosci. **13**, 179

Kirkin, V., 2020. History of the Selective Autophagy Research: How Did It Begin and Where Does It Stand Today? J. Mol. Biol. **432**, 3–27.

Klass, M.R., 1977. Aging in the nematode Caenorhabditis elegans: Major biological and environmental factors influencing life span. Mech. Ageing Dev. **6**, 413–429.

Kroemer, G., Galluzzi, L., Brenner, C., 2007. Mitochondrial membrane permeabilization in cell death. Physiol. Rev. **87:1**, 99-163.

Labbadia, J., Morimoto, R.I., 2015. The biology of proteostasis in aging and disease. Annu. Rev. Biochem **84**, 435-64.

Liesa, M., Palacín, M., Zorzano, A., 2009. Mitochondrial dynamics in mammalian health and disease. Physiol. Rev. **89**, 799–845.

Luo, W., Peterson, A., Garcia, B.A., Coombs, G., Kofahl, B., Heinrich, R., Shabanowitz, J., Hunt, D.F., Yost, H.J., Virshup, D.M., 2007. Protein phosphatase 1 regulates assembly and function of the β-catenin degradation complex. EMBO J. **26**, 1511–1521.

Maiuri, M.C., Criollo, A., Tasdemir, E., Vicencio, J.M., Tajeddine, N., Hickman, J.A., Geneste, O., Kroemer, G., 2007. BH3-only proteins and BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2/Bcl-XL. Autophagy **3**, 374–376.

Manganas, P., MacPherson, L., Tokatlidis, K., 2017. Oxidative protein biogenesis and redox regulation in the mitochondrial intermembrane space. Cell Tissue Res. **367**(1), 43–57.

Margulis, L., 1996. Archaeal-eubacterial mergers in the origin of Eukarya: Phylogenetic classification of life. Proc. Natl. Acad. Sci. U.S.A. **93**, 1071–1076.

Marinković, M., Novak, I., 2015. The Role of Autophagy Receptors in Mitophagy. In: Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging. Elsevier Inc., pp. 243–256.

Marinković, M., Šprung, M., Novak, I., 2020. Dimerization of mitophagy receptor BNIP3L/NIX is essential for recruitment of autophagic machinery. Autophagy 1–12.

Martens, S., Fracchiolla, D., 2020. Activation and targeting of ATG8 protein lipidation. Cell Discov**. 6,** 23

Mijaljica, D., Prescott, M., Devenish, R.J., 2014. Mitophagy: An Overview. In: Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging. Elsevier Inc., pp. 103–116.

Mijaljica, D., Prescott, M., Devenish, R.J., 2010. Mitophagy and mitoptosis in disease processes. In: Methods in Molecular Biology. Humana Press Inc., pp. 93–106.

Mishra, P., Chan, D.C., 2016. Metabolic regulation of mitochondrial dynamics. J. Cell Biol. **212,** 379–387

Nakatogawa, H., Ichimura, Y., Ohsumi, Y., 2007. Atg8, a Ubiquitin-like Protein Required for Autophagosome Formation, Mediates Membrane Tethering and Hemifusion. Cell **130**, 165–178

Nass, M.M., Nass, S., 1963. Intramitochondrial Fibers With Dna Characteristics. I. Fixation and. J. Cell Biol. **19**, 593–611.

Nguyen, T.N., Padman, B.S., Usher, J., Oorschot, V., Ramm, G., Lazarou, M., 2016. Atg8 family LC3/GAB ARAP proteins are crucial for autophagosome-lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. J. Cell Biol**. 215**, 857–874. h

Nicolaou, P., Kranias, E.G., 2009. Role of PP1 in the regulation of Ca cycling in cardiac

physiology and pathophysiology. Front. Biosci. **14**, 3571–3585.

Nishimura, T., Tooze, S.A., 2020. Emerging roles of ATG proteins and membrane lipids in autophagosome formation. Cell Discov. **6,** 32

Novak, I., 2012. Mitophagy: A complex mechanism of mitochondrial removal. Antioxidants Redox Signal **17**(5), 794-802

Novak, I., Kirkin, V., McEwan, D.G., Zhang, J., Wild, P., Rozenknop, A., Rogov, V., Löhr, F., Popovic, D., Occhipinti, A., Reichert, A.S., Terzic, J., Dötsch, V., Ney, P.A., Dikic, I., 2010. Nix is a selective autophagy receptor for mitochondrial clearance. EMBO Rep. **11**, 45–51.

Ohi, N., Tokunaga, A., Tsunoda, H., Nakano, K., Haraguchi, K., Oda, K., Motoyama, N., Nakajima, T., 1999. A novel adenovirus E1B19K-binding protein B5 inhibits apoptosis induced by Nip3 by forming a heterodimer through the C-terminal hydrophobic region. Cell Death Differ. **6**, 314–325.

Otsuga, D., Keegan, B.R., Brisch, E., Thatcher, J.W., Hermann, G.J., Bleazard, W., Shaw, J.M., 1998. The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast, J. Cell. Biol. **143**(2), 333-49

Palikaras, K., Lionaki, E., Tavernarakis, N., 2015a. Coupling mitogenesis and mitophagy for longevity. Autophagy **11**, 1428–1430.

Palikaras, K., Lionaki, E., Tavernarakis, N., 2015b. Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. Nature **521,** 525–528.

Pinna, L.A., Donella-Deana, A., 1994. Phosphorylated synthetic peptides as tools for studying protein phosphatases. Biochim Biophys Acta **1222**(3), 415-31.

Plasmid, G., Kit, M., 2008. GeneJET TM Plasmid Miniprep Kit. Cell. URL https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012655_GeneJET_Plasmid_Miniprep_UG.pdf (accessed 9.1.20).

Quijano, C., Trujillo, M., Castro, L., Trostchansky, A., 2016. Interplay between oxidant species and energy metabolism. Redox Biol. **8**, 28–42.

Ravikumar, B., Moreau, K., Jahreiss, L., Puri, C., Rubinsztein, D.C., 2010. Plasma membrane contributes to the formation of pre-autophagosomal structures. Nat. Cell Biol. **12**, 747–757.

Rogov, V. V., Suzuki, H., Marinković, M., Lang, V., Kato, R., Kawasaki, M., Buljubašić, M., Šprung, M., Rogova, N., Wakatsuki, S., Hamacher-Brady, A., Dötsch, V., Dikic, I., Brady, N.R., Novak, I., 2017. Phosphorylation of the mitochondrial autophagy receptor Nix enhances its interaction with LC3 proteins. Sci. Rep. **7**, 1131

Rosado, C.J., Mijaljica, D., Hatzinisiriou, I., Prescott, M., Devenish, R.J., 2008. Rosella: A fluorescent pH-biosensor for reporting vacuolar turnover of cytosol and organelles in yeast. Autophagy **4**, 205–213.

Sandoval, H., Thiagarajan, P., Dasgupta, S.K., Schumacher, A., Prchal, J.T., Chen, M., Wang, J., 2008. Essential role for Nix in autophagic maturation of erythroid cells. Nature **454,** 232–235.

Sato, A., Nakada, K., Hayashi, J.I., 2006. Mitochondrial dynamics and aging: Mitochondrial interaction preventing individuals from expression of respiratory deficiency caused by mutant mtDNA. Biochimica et Biophysica Acta **1763(5-6),** 473-81

Sato, M., Sato, K., 2011. Degradation of paternal mitochondria by fertilization-triggered autophagy in C. elegans embryos. Science **334**(6059), 1141-4.

Schweers, R.L., Zhang, J., Randall, M.S., Loyd, M.R., Li, W., Dorsey, F.C., Kundu, M., Opferman, J.T., Cleveland, J.L., Miller, J.L., Ney, P.A., 2007. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc. Natl. Acad. Sci. U.S.A. **104**, 19500–19505.

Smirnova, E., Griparic, L., Shurland, D.L., Van der Bliek, A.M., 2001. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol. Biol. Cell

12, 2245–2256.

Smirnova, E., Shurland, D.L., Ryazantsev, S.N., Van Der Bliek, A.M., 1998. A human dynamin-related protein controls the distribution of mitochondria. J. Cell Biol. **143**, 351–358

Song, H., Pu, J., Wang, L., Wu, L., Xiao, J., Liu, Q., Chen, J., Zhang, M., Liu, Y., Ni, M., Mo, J., Zheng, Y., Wan, D., Cai, X., Cao, Y., Xiao, W., Ye, L., Tu, E., Lin, Z., Wen, J., Lu, X., He, J., Peng, Y., Su, J., Zhang, H., Zhao, Y., Lin, M., Zhang, Z., 2015. ATG16L1 phosphorylation is oppositely regulated by CSNK2/casein kinase 2 and PPP1/protein phosphatase 1 which determines the fate of cardiomyocytes during hypoxia/reoxygenation. Autophagy **11**, 1308–1325.

Spinelli, J.B., Haigis, M.C., 2018. The multifaceted contributions of mitochondria to cellular metabolism. Nat. Cell Biol. **20**(7), 745-754

Tilokani, L., Nagashima, S., Paupe, V., Prudent, J., 2018. Mitochondrial dynamics: Overview of molecular mechanisms. Essays Biochem. **62**(3), 341-360.

Tooze, S.A., Yoshimori, T., 2010. The origin of the autophagosomal membrane. Nat. Cell Biol. **(9),** 831-5

Tournebize, R., Andersen, S.S.L., Verde, F., Dorée, M., Karsenti, E., Hyman, A.A., 1997. Distinct roles of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis. EMBO J. **16,** 5537–5549.

Trinkle-Mulcahy, L., Chusainow, J., Lam, Y.W., Swift, S., Lamond, A., 2007. Visualization of intracellular PP1 targeting through transiently and stably expressed fluorescent protein fusions. Methods Mol. Biol. **365,** 133–154. https://doi.org/10.1385/1-59745-267-X:133

Tsukada, M., Ohsumi, Y., 1993. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. FEBS Lett. 333, 169–174. https://doi.org/10.1016/0014-5793(93)80398-E

Twig, G., Elorza, A., Molina, A.J.A., Mohamed, H., Wikstrom, J.D., Walzer, G., Stiles, L.,

Haigh, S.E., Katz, S., Las, G., Alroy, J., Wu, M., Py, B.F., Yuan, J., Deeney, J.T., Corkey, B.E., Shirihai, O.S., 2008. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 27, 433–446.

Villanueva Paz, M., Cotán, D., Garrido-Maraver, J., Cordero, M.D., Oropesa-Ávila, M., De La Mata, M., Delgado Pavón, A., De Lavera, I., Alcocer-Gómez, E., Sánchez-Alcázar, J.A., 2016. Targeting autophagy and mitophagy for mitochondrial diseases treatment. Expert Opin. Ther. Targets **20**, 487–500.

Wang, C., Youle, R.J., 2009. The role of mitochondria in apoptosis. Annu. Rev. Genet. **43**, 95–118.

Weidberg, H., Shpilka, T., Shvets, E., Abada, A., Shimron, F., Elazar, Z., 2011. LC3 and GATE-16 N Termini Mediate Membrane Fusion Processes Required for Autophagosome Biogenesis. Dev. Cell **20**, 444–454.

Wetterauer, P., 2018. Natural products against neurodegenerative diseases: effects in the model organism Caenorhabditis elegans. University of Heidelberg, Germany.

Wiedemann, N., Kozjak, V., Chacinska, A., Schönfisch, B., Rospert, S., Ryan, M.T., Pfanner, N., Meisinger, C., 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. Nature **424**, 565–571.

Wilson, D.F., 2013. Regulation of cellular metabolism: Programming and maintaining metabolic homeostasis. J. Appl. Physiol. **115**, 1583–1588.

Yamaguchi, O., Murakawa, T., Nishida, K., Otsu, K., 2016. Receptor-mediated mitophagy. J. Mol. Cell. Cardiol. **95**, 50-6

Yang, Z., Klionsky, D.J., 2009. An Overview of the Molecular Mechanism of Autophagy. Curr Top Micro[D15]biol Immunol. **335**, 1–32

Yang, Z., Klionsky, D.J., 2010. Eaten alive: A history of macroautophagy. Nat. Cell Biol. **12**, 814–822.

Yim, W.W.Y., Mizushima, N., 2020. Lysosome biology in autophagy. Cell Discov. **6**, 6.

Yoo, S.M., Jung, Y.K., 2018. A molecular approach to mitophagy and dynamics. Mol. Cells. **41**(1):18-26.

Zhang, W., Liu, Y., An, Z., Huang, D., Qi, Y., Zhang, Y., 2011. Mediating effect of ROS on mtDNA damage and low ATP content induced by arsenic trioxide in mouse oocytes. Toxicol. Vitr. **25**, 979–984.

Züchner, S., Mersiyanova, I. V., Muglia, M., Bissar-Tadmouri, N., Rochelle, J., Dadali, E.L., Zappia, M., Nelis, E., Patitucci, A., Senderek, J., Parman, Y., Evgrafov, O., De Jonghe, P., Takahashi, Y., Tsuji, S., Pericak-Vance, M.A., Quattrone, A., Battologlu, E., Polyakov, A. V., Timmerman, V., Schröder, J.M., Vance, J.M., 2004. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat. Genet. **36**, 449–451.

8 Resume

Jadranka Škorput

Nationality: Croatian

Date of birth: 29/03/1996

Gender: Female

Email address: jadranka.skorput.96@gmail.com

Address : 21000 Split (Croatia)

EDUCATION AND TRAINING

Elementary school
Skalice [2002 – 2010]

Address: Put Skalica 18, 21000 Split

High School
IV. Gymnasium Marko Marulić [2010 – 2014]

Address: Zagrebačka ulica 2, 21000 Split (Croatia)

Music High School
Josip Hatze [2010 – 2014]

Address: Trg Hrvatske bratske zajednice 3, 21000 Split

Piano professor: Najda Čapalija

Bachelor's degree in Nutrition
Faculty of Science, University of Split [2014 – 2017]

Address: Ruđera Boškovića 33, 21000 Split (Croatia)
Thesis : Human microbiome - the role in health and disease, mentor: doc. Viljemka Bučević Popović, dr. sc.

Dean's award of the Faculty of Science, Split for academic year 2016-17.

Master's degree in Molecular biology
Faculty of Science, University of Zagreb [2017 – Current]

Address: Rooseveltov trg 6, 10000 Zagreb (Croatia)

Internship
The Ruđer Bošković Institute, Laboratory of cell biophysics [03/2018 – 07/2018]

Address: Bijenička cesta 54 10000, Zagreb Zagreb (Croatia)

mentor: Vedrana Filić Mileta, PhD.

Internship
School of Medicine, University of Split, Laboratory for cancer research [07/2019 – 01/2020]

Address: Šoltanska 2, 10000 Split (Croatia)

mentor: Assoc. Prof. Ivana Novak Nakir, PhD.

Co-supervisor: Mija Marinković, PhD.

Internship
Leibniz Research Institute for Environmental Medicine [02/2020 – 08/2020]

Address: Auf'm Hennekamp 50, 40225 Dusseldorf

mentor: Assoc. Prof. Natascia Ventura, PhD.

Co-supervisor: Alfonso Schiavi, PhD.

LANGUAGE SKILLS

Mother tongue(s):

Croatian

English

LISTENING: C1 **READING:** C1 **UNDERSTANDING:** C1
SPOKEN PRODUCTION: C1
SPOKEN INTERACTION: C1

Italian

LISTENING: A2 **READING:** A2 **UNDERSTANDING:** A2
SPOKEN PRODUCTION: A2 **SPOKEN INTERACTION:** A2

DIGITAL SKILLS

Inkscape / **Basic skills in Adobe Photoshop** / **Fiji-ImageJ**

Tools for statistical analysis
GraphPad Prism / **R (R-studio)** / **MS Office (Access, Excel, PowerPoint, Word)**