Characterizing the role of the Pβ peptide: A potential mitochondria to nuclear signal in Mitochondrial quality control

by

Navdeep Deol

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Biochemistry
University of Toronto

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2016

Abstract

The PINK1/Parkin mediated mitophagy pathway is fundamental for maintaining a healthy mitochondrial network. We have previously identified the mitochondrial rhomboid protease PARL as a regulatory member of this pathway and have shown it to be required for recruitment of Parkin to damaged mitochondria, a key step during mitophagy. Self-regulated proteolysis of PARL at its N-terminus, known as β cleavage, results in two moieties, a 25 amino acid peptide (Pβ) and the rest of the PARL protease (β -PARL). β-cleavage is regulated by phosphorylation and we have previously shown that it might be required for efficient PINK1/Parkin mediated mitophagy. Based on previous studies on PARL and β-cleavage, we hypothesize that the Pβ-peptide, generated inside mitochondria after PARL senses mitochondrial damage, accumulates in the nucleus and plays a role in mitochondrial-nuclear communication. Using the neuroblastoma cell line SH-SY5Y, we have identified the endogenous Pβ peptide. We demonstrate that upon mitochondrial damage the Pβ peptide generated inside the mitochondria accumulates in the nucleus. Our data suggests that, in addition to its evolutionarily conserved role in regulating mitochondrial dynamics, PARL may execute a novel mitochondria-to-nucleus signaling pathway through regulated proteolysis of its N terminus and release of the Pβ peptide.
Firstly and foremost, I would like to extend my appreciation to my supervisor, Dr. Angus McQuibban, for his strong support and insightful guidance throughout my studies. He shared not only his vision and dedication on research, but also the experience of intellectual curiosity, life attitude, as well as career development all of which have benefited me enormously. I would also like to thank my supervisory committee members, Dr. David Williams and Dr. William Trimble, for their suggestions and guidance over the last two years. In addition, I would like to thank Dr. Lyndal Bayles (University, Australia) for providing me with the Pβ specific antibody and GFP-Pβ constructs for my project.

I would also like to thank the past and present members of the McQuibban lab for the many enjoyable conversations on research, academia and for their constant thoughtfulness, encouragement and support over the last two years. Specifically, I would like to thank Rediet for providing many helpful suggestions on my thesis. I would also especially like to thank Guang, who always had an answer for every question that I came up with and helped me with my various things on my project. I have had the pleasure of knowing many wonderful graduate students in the biochemistry department. I would like to thank my colleagues in the Department of Biochemistry for their help and advice in all aspects of my work.

One of the things that I have learned about being a researcher is that you get a front-row seat to the full spectrum of human emotion: the dizzying highs that come with an exciting new theoretical result, but also the dark lows when you just cannot push that proof any further or your experiments do not work out as you had hoped they would. Whenever I walked into my
supervisor’s office with fears and doubts, though, I always came out with a new way of looking at my problems and a renewed determination to solve them.

I would like to extend my deepest appreciation to my brothers and sister-in-laws for their love and support during my studies. Most importantly, I would like to thank my parents for their boundless love, support. Without their unconditional love and continuous encouragement, I will never achieve this far. Sincerest thanks to them from the bottom of my heart.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette transporter</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AR-JP</td>
<td>Autosomal recessive-juvenile Parkinsonism</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy-related gene</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Bcl2-L-13</td>
<td>Bcl-2-like protein 13</td>
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<td>bHLH-ZIP</td>
<td>basic Helix-loop-helix leucine zipper</td>
</tr>
<tr>
<td>BLI</td>
<td>Bio-Layer Interferometry</td>
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<tr>
<td>Cas</td>
<td>CRISPER- associated</td>
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<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrazone</td>
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<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<tr>
<td>CRISPERs</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<tr>
<td>CVα</td>
<td>Complex V subunit α</td>
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<td>DA</td>
<td>Dopaminergic</td>
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<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
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<tr>
<td>dbSNP</td>
<td>Single nucleotide polymorphism database</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
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<td>Full Form</td>
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<td>Dimethyl sulfoxide</td>
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<td>Dynamin related protein 1</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<td>Endoplasmic reticulum</td>
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<td>Electron transport chain</td>
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</tr>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GTPases</td>
<td>Guanosine triphosphatases</td>
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<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>IAPs</td>
<td>Inhibitor of apoptosis proteins</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
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<td>IMS</td>
<td>Intermembrane surface</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
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<td>Lewy Bodies</td>
</tr>
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<td>LC3</td>
<td>Microtubule associated Light chain 3</td>
</tr>
<tr>
<td>LN</td>
<td>Lewy Neurites</td>
</tr>
<tr>
<td>mAAA</td>
<td>mitochondrial ATPases associated with diverse cellular activities</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>MF</td>
<td>Mitochondrial fraction</td>
</tr>
<tr>
<td>Mfn 1/2</td>
<td>Mitofusin 1/2</td>
</tr>
<tr>
<td>MPP</td>
<td>Mitochondrial processing peptidase</td>
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<td>MPTP</td>
<td>1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>mtDNA</td>
<td>mitochondrial Deoxyribose nucleic acid</td>
</tr>
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<td>MTS</td>
<td>Mitochondrial targeting sequence</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NF</td>
<td>Nuclear fraction</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
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<td>NRF1/2</td>
<td>Nuclear respiratory factor 1/2</td>
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<tr>
<td>NTFs</td>
<td>Nuclear transcription factors</td>
</tr>
<tr>
<td>NCLX</td>
<td>Na(^+)-Ca(^{2+}) exchanger</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy 1</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PARL</td>
<td>Presenilin- associated rhomboid-like protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PDK2</td>
<td>Phospho dehydrogenase Kinase 2</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGAM5</td>
<td>Phosphoglycerate Mutase Family Member 5</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator-activated receptor gamma co-activator 1α</td>
</tr>
<tr>
<td>PIC</td>
<td>Protease inhibitor cocktail</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN- induced putative kinase 1</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RCR</td>
<td>Rhomboid cleavage region</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTG</td>
<td>Retrograde signalling pathway</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>Sequestosome 1</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TF</td>
<td>Total fraction</td>
</tr>
<tr>
<td>Tfam</td>
<td>mitochondrial transcriptional factor A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
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<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of the inner membrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the outer membrane</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer Ribonucleic acid</td>
</tr>
<tr>
<td>UCP 2</td>
<td>Uncoupling protein 2</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
Chapter 1
INTRODUCTION

1.1 MITOCHONDRIAL QUALITY CONTROL

The mitochondrion was first identified by scientists working during the mid-1800s. In 1857, Swiss anatomist Albert von Kölliker described what he called “granules” in muscle cells [1]. Other scientists of the era also noticed these “granules” in other cell types [1]. In 1886, cytologist Richard Altman was the first to recognize the ubiquitous occurrence of these structures and named them “bioblasts” and Altman postulated that these structures were the basic units of cellular activity carrying out various functions [2]. In 1898, Carl Benda coined the term “mitochondrion”, derived the term from the Greek words thread, mitos, and granule, chondros [3].

1.1.1 MITOCHONDRIA IN A NUT SHELL AND ITS SIGNIFICANCE

Since the discovery of the mitochondrion, various scientists have worked on elucidating its structure. Mitochondria are small cytoplasmic organelles that are thought to have evolved from an alpha-proteobacterium that developed a symbiotic relationship with a eukaryotic progenitor early in evolution after they were engulfed by the primitive eukaryotic cells (endosymbiosis) [4, 5]. This hypothesis has recently been substantiated by DNA sequence analysis, which revealed striking similarities between the genomes of mitochondria and that of
the bacterium *Rickettsia prowazekii*. *Rickettsia* are intracellular parasites which, like mitochondria, are only able to reproduce within eukaryotic cells. Consistent with their similar symbiotic lifestyles, the genomic DNA sequences of *Rickettsia* and mitochondria suggest that they share a common ancestor, from which the genetic system of present-day mitochondria evolved [6]. In this symbiotic relationship, the once independent aerobic and photosynthetic bacteria became the energy producing organelles of eukaryotic cells [6].

1.1.1.1 MITOCHONDRIAL ORGANISATION

Like their bacterial ancestor, mitochondria are comprised of two separate and functionally distinct outer and inner membranes that encapsulate the intermembrane space (IMS) and matrix compartments, respectively (Figure 1). The double membrane bound architecture of mitochondria was revealed in the 1950s by electron microscopy studies [7]. The mitochondrial outer membrane (MOM) separates the intermembrane space (IMS), the space between the outer and inner membranes, from the cytosol and allows the exchange of metabolites, adenine nucleotides, and cations between the mitochondria and the rest of the cell [8]. Communication between compartments is possible by the existence of many copies of a transport protein, porin, which forms aqueous channels that allow the passage of molecules smaller than 5 kD [9]. The outer membrane of the mitochondria also contains a large multiprotein translocase complex that recognizes mitochondrial signal sequences on larger proteins and permits their import [10]. The inner mitochondrial membrane contains all of the components of the electron transport system and the ATP synthase complex [8]. Additionally, it also has many invaginations, called cristae that expand the surface area of the inner mitochondrial membrane [11]. The intermembrane space has a high proton concentration due to the electron transport system of the inner
Figure 1: Mitochondrion organization

A. EM (electron microscope) tomography of the mitochondrion. Shown is the cross-sectional surface-rendered 3D image of an isolated rat-liver mitochondrion. Arrows point to tubular regions of cristae that connect them to the inner membrane and to each other. (This image is reprinted from C.A. Mannella (2006) *Biophysica et Biochimica Acta* 1762: 140-147, Copyright (2006), with permission from Elsevier.)

B. This schematic diagram shows components of the mitochondria.
mitochondrial membrane [8]. The core of the mitochondria is the matrix that harbors the mitochondrial genetic system, ribosomes as well as the enzymes responsible for the central reactions of oxidative metabolism. These include most of the enzymes that participate in the Tricarboxylic acid (TCA) cycle - which is also known as the Kreb’s Cycle [8].

1.1.1.2 THE MITOCHONDRIAL GENOME

Owing to their bacterial origin, mitochondria have their own DNA (mtDNA), and they divide by binary fission, similar to bacterial cell division [12]. The regulation of this division differs among eukaryotes. In many single-celled eukaryotes, their growth and division is linked to the cell cycle. For example, a single mitochondrion may divide synchronously with the nucleus. This division and segregation process must be tightly controlled so that each daughter cell receives at least one mitochondrion [12, 13]. In other eukaryotes (in mammals for example), mitochondria may replicate their DNA and divide mainly in response to the energy needs of the cell, rather than in phase with the cell cycle [12]. Mitochondrial DNA (mtDNA) is condensed into globular nucleoprotein structures called nucleoids and are shown to reside in the matrix tethered to the inner membrane [13, 14]. The proteins involved in the nucleoid packaging include non-histone, high mobility proteins such as mitochondrial transcription factor A (TFAM) in humans, and a number of bifunctional proteins that also have roles in mitochondrial metabolism and biogenesis. The mitochondrial genome is maternally inherited [13, 14]. In humans, when an ovum (egg) is fertilized by a sperm, the nuclei from both the ovum and sperm contribute equally to the genetic makeup of the zygote nucleus. In contrast, the mitochondria, and therefore the mitochondrial DNA, usually come from the ovum only. The paternal mitochondria are
marked with ubiquitin for their degradation inside the embryo, and, therefore they do not contribute towards the genetic makeup of the embryo [13, 14].

The human mitochondrial genome is composed of multiple copies of a 16,569 base pair, double-stranded circular mtDNA molecule encoding a total of 37 genes. There are 13 protein-coding genes, 22 tRNA genes, and 2 ribosomal genes, with the overall organization of the genome as shown in Figure 2 [15]. The protein-coding genes contribute to the ETC complexes I, III, IV, and V, with complex II being exclusively nuclear encoded. The tRNA- and rRNA-encoding genes are necessary for intra-mitochondrial protein synthesis [16, 17]. The mitochondrial genome is essential for respiratory function; however, the majority of mitochondrial proteins are encoded by the nuclear genome and are then imported into the mitochondria [18]. Since most mitochondrial proteins are nuclear-encoded and synthesized in the cytosol, translocases have evolved in both the outer and inner membranes to recognize and allow the import of proteins containing a mitochondrial targeting sequence (MTS), most commonly found at the N-terminus [19]. Proteins are transported through the outer membrane via the translocases of the outer membrane (TOMs), and through the inner membrane via the translocases of the inner membrane (TIMs). In addition, chaperones and proteases, found in the inter membrane space and particularly the matrix, are involved in protein maturation [19].

1.1.1.3 THE FUNCTIONS OF THE MITOCHONDRION

Mitochondria were long regarded as individual, “bean-shaped” organelles, nevertheless they are now recognised as a dynamic, inter-connected network, linked to other organelles and are important players in a myriad of cellular signaling pathways [20]. By regulating the connectivity
This schematic diagram shows the organization of human mitochondrial genome. The human mitochondrial genome is a circular DNA molecule of about 16 kb and encodes 37 genes. 13 genes encodes for subunits of respiratory complexes I, III, IV and V, 22 for mitochondrial tRNA and 2 for rRNA. One mitochondrion can contain two to ten copies of its DNA.
and the size of the mitochondrial network, the cell can regulate energy production and most other mitochondrial processes such as apoptosis and many catabolic and anabolic reactions, including the citric acid cycle, β-oxidation of fatty acids and the biosynthesis of haem, certain phospholipids and other metabolites. Indeed, mitochondria play a complex multi-factorial role in the cell. In addition to their primary role in ATP generation, the organelles sequester calcium, and play a role in intracellular signaling as well as in apoptosis that are briefly discussed below [22, 23, 24, 25]. All these functions are intimately inter-linked through the central bioenergetic parameter of the proton electrochemical gradient across the inner mitochondrial membrane. Hence, we will focus on mitochondrial bioenergetics and how generation of ROS leads to dysfunctional mitochondria.

CALCIUM BUFFERING BY MITOCHONDRIA: Mitochondrial Ca\(^{2+}\) uptake has been shown to control intracellular Ca\(^{2+}\) signaling, cell metabolism, cell survival and other cell-type specific functions by buffering cytosolic Ca\(^{2+}\) levels and regulating mitochondrial effectors [26, 27]. The mitochondrial ability to accumulate Ca\(^{2+}\) is an energy dependent process, both ATP (through reversal activity of the H\(^+\) ATPase) or the respiratory chain (through H\(^+\) pumping) can fuel mitochondrial Ca\(^{2+}\) uptake as they both generate a membrane potential (ΔΨ) across the inner mitochondrial membrane (IMM) that drives Ca\(^{2+}\) influx down its electrochemical gradient [28]. Ca\(^{2+}\) enters mitochondria mainly via a mitochondrial Ca\(^{2+}\) uniporter, a protein known as MCU [29]. Ca\(^{2+}\) accumulating within mitochondria in turn regulates cell metabolism as it activates three matrix dehydrogenases: pyruvate dehydrogenase is regulated by a Ca\(^{2+}\)-dependent phosphatase, and α-ketoglutarate- and isocitrate-dehydrogenases are regulated by direct binding of Ca\(^{2+}\) to these enzymes. Stimulation of Ca\(^{2+}\)-sensitive dehydrogenases increases NADH availability and hence the flow of electrons down the respiratory chain, thus adjusting ATP synthesis to the increased needs of a stimulated cell [28]. In addition to the action of Ca\(^{2+}\) within
mitochondria, Ca$^{2+}$ efflux mechanisms also exist, such as H$^+$ /Ca$^{2+}$ or Na$^+$ /Ca$^{2+}$ exchangers, that prevent reaching electrochemical equilibrium, something that is incompatible with cell physiology [28, 30]. Accumulating evidence suggests that the mitochondrial Ca$^{2+}$ release system is not only crucial in maintaining mitochondrial Ca$^{2+}$ homeostasis but also participates in the Ca$^{2+}$ crosstalk between mitochondria and the plasma membrane and between mitochondria and the endoplasmic reticulum/sarcoplasmic reticulum (ER/ SR) [30]. The close proximity of the mitochondria to the plasma membrane or the ER/SR within the cell is important for mitochondrial Ca$^{2+}$ uptake. For example, a mitochondrial Na$^+$–Ca$^{2+}$ exchanger (NCLX) has been shown to participate in insulin secretion in pancreatic β-cells, glutamate release in astrocytes and generation of the spontaneous rhythmicity of cardiomyocytes, via Ca$^{2+}$ crosstalk between mitochondria and the plasma membrane and/or between mitochondria and the ER/SR [30].

APOPTOSIS AND MITOCHONDRIA: Apoptosis is fundamental to multi-cellular organisms during both development and homeostasis [31, 32]. The Bcl-2 family of proteins regulate apoptosis by controlling mitochondrial permeability [33]. It has been proposed that the anti-apoptotic proteins, such as Bcl-2, Bcl-XL, Bcl-w and Mcl-1, are found on the OMM where they act to inhibit cytochrome c release through interaction and inhibition of the pro-apoptotic proteins Bax and Bak [33]. The pro-apoptotic Bcl-2 proteins Bad, Bid, Bax, and Bim may reside in the cytosol but translocate to mitochondria in apoptotic cells, where they promote the release of cytochrome c. Bad translocates to mitochondria and forms a pro-apoptotic complex with Bcl-xL. This translocation is inhibited by survival factors that induce the phosphorylation of Bad, leading to its cytosolic sequestration [33]. Cytosolic Bid is cleaved by caspase-8 following signaling through Fas; its active fragment (tBid) translocates to mitochondria [34]. Bax and Bim translocate to mitochondria in response to death stimuli, including survival factor withdrawal. Upon release from mitochondria, cytochrome c binds to Apaf-1 and forms an activation complex
with caspase-9 [34]. Although the mechanism(s) regulating mitochondrial permeability and the release of cytochrome c during apoptosis are not fully understood, Bcl-xL, Bcl-2, and Bax may influence the voltage-dependent anion channel (VDAC), which may play a role in regulating cytochrome c release [33, 34].

MITOCHONDRIAL BIOENERGETICS: Mitochondria are often referred to as “the powerhouse of the cell” because mitochondria produce approximately 90% of the ATP required for numerous cellular processes [35]. Oxidative metabolism is fueled by pyruvate generated from carbohydrates in glycolysis and fatty acids produced from triglycerides [35, 36]. During oxidative phosphorylation (OXPHOS), sequential oxidation-reduction reactions at complexes I (NADH dehydrogenase), II (succinate dehydrogenase), III (coenzyme Q: cytochrome c-oxidoreductase), and IV (cytochrome c oxidase) are coupled to the translocation of protons across the inner mitochondrial membrane. The resulting electrochemical gradient is ultimately utilized by complex V (ATP synthase) for the generation of ATP from ADP and inorganic phosphate (Figure 3) [35, 36]. The proton gradient generated in the ETC is commonly referred to as the mitochondrial membrane potential (ΔΨm) and represents the energy available to drive changes in ATP/ADP ratio [37]. Membrane potential is harnessed for other essential mitochondrial functions, such as mitochondrial protein import [38], and to drive calcium accumulation into mitochondria [39]. The energy derived in the form of ATP by oxidative phosphorylation is more efficient than anaerobic glycolysis [40]. This suggests that organs with a high-energy demand are vulnerable to the depletion of mitochondrial energy production. In cell types that primarily depend on oxidative metabolism for ATP synthesis, e.g., neurons, red skeletal muscle fibers, or cardiac myocytes, mitochondria can account for up to 30% of the cytoplasmic volume. It is not surprising, therefore, that molecular defects in mitochondria primarily affect the brain and muscles.
Figure 3: Schematic diagram of mitochondrial electron transport chain (ETC)

Schematic diagram depicts the organization of the electron transport chain in the intermembrane space of mitochondria. Mitochondria are best known for their role in generation of the ATP from metabolic fuels through oxidative phosphorylation. NADH and FADH$_2$ are strong reducing agents that donate electrons to respiratory chain resulting in the establishment of an electrochemical gradient of protons across the mitochondrial inner membrane. Three multi-subunit respiratory complexes, I, III and IV, are embedded in the mitochondrial inner membrane and they serve as the sites of proton pumping from the matrix. In addition, complex V is of an ATPase coupled to an inner membrane proton channel that can dissipate the proton gradient in synthesis of the ATP or hydrolyze ATP to maintain the gradient. (Image taken from “Lehninger Principles of Biochemistry” David L. Nelson, Michael M. Cox.)
1.1.2 MITOCHONDRIAL DYSFUNCTION AND DISEASE

Mitochondrial dysfunction has emerged as a key factor in a myriad of diseases, including neurodegenerative diseases like Parkinson’s disease (PD), Alzheimer’s disease (AD) and metabolic disorders like type II diabetes [41, 42, 43]. Since mitochondria perform important cellular reactions which include the production of energy through the electron transport chain (ETC), it is not surprising that they are major sources of free radicals in the cell resulting in oxidative stress [44, 45, 46, 47]. Complexes I and III of the ETC generate reactive oxygen species (ROS), including oxygen radicals and hydrogen peroxide, which can damage key components of cells, including lipids, nucleic acids, and proteins [47, 48]. Mitochondria themselves are the predominant sites of damage from ROS. Exposure to oxidative stress promotes misfolding and aggregation of the mitochondrial proteins, leading to disassembly of protein complexes and eventual loss of mitochondrial integrity [47]. Impaired mitochondria further leads to increased oxidative stress and affects various cellular pathways, leading to the damage of cellular components and eventually results in cell death [49, 50, 51, 52]. ROS has been shown to contribute to diseases associated with mitochondrial dysfunction, including neurodegeneration [53]. Oxidative stress is also one of the pathogenic mechanisms of nigral dopamine cell death in PD [54, 55].
1.1.2.1 MITOCHONDRIAL DYSFUNCTION IN NEURONS

The requirement for effective mitochondrial quality control is most critical in neurons as they have very low glycolytic rates [56]; and therefore depend entirely on the mitochondria for energy production [57, 58, 59]. Remarkably, although the human brain consists of only 2% of the volume of the body, it is roughly responsible for 25% of net oxygen consumption in resting conditions [60] with neurons generating as much as 95% of their ATP exclusively from mitochondrial OXPHOS [61]. In neurons, synaptic transmission requires high levels of cellular ATP for numerous energy consuming processes, including the maintenance of synaptic membrane potential and reloading of synaptic vesicles with neurotransmitters [62]. The synaptic terminals require mitochondria for efficient sequestration of calcium ions (Ca$^{2+}$) that are released into the cytosol to elicit the fusion of synaptic vesicles with the plasma membrane [63]. The mitochondria also supply copious amounts of ATP as well as the TCA intermediates that serve as the building blocks for synthesis of gamma aminobutyric acid (GABA) and glutamate neurotransmitters [64, 65]. These additional metabolic functions of mitochondria depend, either directly or indirectly, on OXPHOS, and thus can be secondarily affected by changes in respiration and respiratory complex deficiency. Therefore, compromised oxidative metabolism may alter neurotransmitter levels and render the brain uniquely sensitive to oxidative energetic deficits. This mitochondrial dependence renders neurons especially vulnerable to mitochondrial damage, and, in turn, efficient and properly functioning mitochondrial quality control pathways are paramount to neuronal survival. This has been highlighted in previous genetic studies demonstrating the involvement of genes regulating mitochondrial quality control - i.e., AFG3L2, an ATP-dependent proteolytic complex of the mitochondrial inner membrane that degrades
misfolded proteins and regulates ribosome assembly, in Spinocerebellar Ataxia type 28, and PINK1 and Parkin in PD [66, 67]. Mitochondrial involvement in PD is of particular relevance to this thesis and will be discussed in the following section.

1.1.2.2 PARKINSON’S DISEASE (PD) AND THE MITOCHONDRION

Parkinson’s disease (PD) is a common neurodegenerative disease and is the most prominent, progressive movement disorder affecting the aging population [68]. It is characterised by the degeneration of dopaminergic neurons in the substantia nigra region of the brain. It is accompanied by the presence and accumulation of proteinaceous aggregates, referred to as Lewy Bodies (LB) or Lewy Neurites (LN), in the remaining neurons of affected individuals [69]. The characteristic symptoms of PD include postural instability, rigidity, resting tremor and bradykinesia. A significant proportion of individuals suffering from this disorder appear to have no known genetic cause, and they are typically referred to as sporadic or idiopathic patients. In contrast to this, 5–10% of individuals with the disease are familial patients because they carry heritable, disease-associated mutations in a series of genes [68].

The dysregulation of mitochondrial quality control has emerged as a common theme for many neurodegenerative diseases including Parkinson’s disease (PD). Evidence of this involvement first emerged when abusive drug users were accidentally exposed to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), an inhibitor of mitochondrial complex I, that resulted in an acute and irreversible Parkinsonian syndrome almost indistinguishable from PD [69]. The drug users had ingested a compound produced during illicit synthesis of a narcotic related to
meperidine [69]. Studies afterwards exhibited that MPTP selectively kills dopaminergic neurons of the substantia nigra pars compacta (SNpc) [70]. Follow-up studies found that MPTP is oxidized to MPP+, which is selectively taken up into dopaminergic (DA) neurons causing an inhibition of complex I, a mitochondrial respiratory chain component of the oxidative phosphorylation (OXPHOS) machinery [71, 72]. Complex I deficiency was also found in the brain, skeletal muscle, and platelets of sporadic PD patients [73]. Pesticides and herbicides that selectively inhibit complex I, such as rotenone and paraquat, also cause Parkinsonism in animal models and possibly in humans [73, 74, 75]. These early observations indicated that DA neurons appear particularly sensitive to mitochondrial dysfunction. Other lines of evidence that implicate mitochondria in PD include reduced mitochondrial membrane potential (ΔΨm) accompanied with increased ROS production in PD cell models [76, 77], alterations in mitochondrial fission–fusion events [78, 79], defects in mitochondrial trafficking [80, 81] and the striking observation that all of the PD-related proteins either localize to, or can associate with the mitochondria [82-85]. A major breakthrough eventuated in a recent study in which researchers identified disease-causing mutations in PTEN-induced putative kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin in familial PD [82]. A series of studies involving these PD proteins, PINK1 and Parkin, have highlighted a potential role for both proteins in the clearance of mitochondria from cells via autophagy - a process known as mitophagy [86, 87]. This observation is intriguing because defects in mitophagy have been shown to recapitulate a series of reported PD features namely: impaired motor coordination, tremor and the accumulation of protein aggregates/ inclusion bodies in residual neurons [88, 89].

Since the accumulation of damaged mitochondria leads to degeneration of the neurons, their removal is critical to maintain healthy mitochondrial network in the cells. To this end, a distinct mitochondrial quality control mechanism for the elimination of whole mitochondria by
mitophagy functions in response to damaged mitochondria. In addition, after the efficient removal of damaged mitochondria, the cell replenishes and maintains a pool of healthy mitochondria via mitochondrial biogenesis. Hence, the coordination between the opposing processes of mitochondrial biogenesis and mitophagy enables cells to adjust their mitochondrial content in response to cellular metabolic state and mitochondrial stress signals. The loss of mitochondrial homeostasis reveals the vital role of mitophagy and mitochondrial biogenesis crosstalk for cellular survival in physiological and stressful conditions.

1.1.3 MITOCHONDRIAL BIOGENESIS

Mitochondrial biogenesis can be defined as the growth and division of pre-existing mitochondria. Mitochondrial proteins are encoded by both the nuclear and the mitochondrial genomes as shown in Figure 4. Correct mitochondrial biogenesis requires the co-ordinated synthesis and import of approx. 1000–1500 proteins encoded by the nuclear genome and synthesized on cytosolic ribosomes, of which some are assembled within phospholipid membranes of the inner and outer mitochondrial membranes [90]. In addition, mitochondrial DNA replication and mitochondrial fusion and fission mechanisms must also be coordinated (Figure 4). All of these processes have to be tightly regulated in order to meet the changing energy requirements of the cell during normal and stress conditions. Mitochondrial biogenesis is triggered by environmental stresses such as exercise, cold exposure, caloric restriction and oxidative stress, cell division and renewal, and differentiation [91]. The biogenesis of mitochondria is accompanied by variations in mitochondrial size, number, and mass [16].
Mitochondria adopt different shapes depending on the cell type and the metabolic demands of the cell. While the shape of the mitochondrial network is controlled by fusion and fission-specific GTPases, the size of the network is controlled by mitochondrial biogenesis and macroautophagy [22].

1.1.3.1 MITOCHONDRIAL FISSION/ FUSION: AN INTEGRAL PART OF MITOCHONDRIAL BIOGENESIS

Mitochondria in cells of most tissues are tubular, and dynamic changes in morphology are driven by the processes of fission and fusion [92]. The ability to undergo fission/fusion enables mitochondria to divide and helps ensure proper organization of the mitochondrial network during biogenesis [93]. The processes of fission/fusion are controlled by GTPases, most of which were identified in genetic screens in yeast [93, 94]. Mitochondrial fission is mediated by the dynamin-related protein 1 (DRP1) and mutations in the human DRP1 causes mitochondria to form perinuclear clusters. Optic atrophy 1 (OPA1) protein allows mitochondrial fusion of the inner mitochondrial membrane while outer mitochondrial membrane fusion is controlled by the mitofusins (Mfn1/2) [16, 94].

DRP1 is a soluble protein containing an N-terminal GTPase, a middle domain and a C-terminal GTPase effector domain that is involved in self-assembly. The function of the mitochondrial fission machinery is best understood in yeast. Current evidence indicates that in the nucleotide-free or GDP-bound state, DRPs form curved filaments, and in contrast, in the GTP-bound state, DRPs form spiral-like helical structures. Self-assembly of two membrane scission DRPs, dynamin and Dnm1, the mitochondrial division DRP, into helical structures in vitro drives the constriction of spherical artificial liposomes into tubules. Thus, helical DRP
Figure 4: Mitochondrial biogenesis: Co-ordination of mitochondrial and nuclear genome expression.

Shown here is the schematic representation of mitochondrial biogenesis. Peroxisome proliferator-activated receptor gamma co-activator (PGC-1α) activates nuclear transcription factors (NTFs) leading to transcription of nuclear-encoded genes and of the gene encoding mitochondrial transcription factor A (TFAM). TFAM activates transcription and replication of the mitochondrial genome. Nuclear-encoded proteins are imported into the mitochondria through the outer - (TOM) or inner (TIM) membrane transport machinery. Nuclear and mitochondrial encoded subunits of the respiratory chain are then assembled. Mitochondrial fission through the dynamin-related protein 1 (DRP1) for the outer membrane allows mitochondrial division while OPA1 for the inner membrane of mitochondria and mitofusins (Mfn) for the outer membrane of the mitochondria control mitochondrial fusion. Processes of fusion and fission lead to proper organization of the mitochondrial network. (Ventura-Clapier, R. et al. 2008) Reprinted, with permission, from the *Cardiovascular Research*, Volume 79, Copyright (2008).
structures are thought to be responsible in part for the ability of DRPs to remodel membranes (21). Assembly stimulated GTP hydrolysis is also essential for DRP function [93]. Mitochondrial fusion proteins are large GTPases that contain two transmembrane regions in the mitochondrial outer membrane, with a short loop in the intermembrane space and the major parts of the protein facing the cytosol [16, 94]. OPA1 is present in eight isoforms that are generated by alternative splicing and alternative processing at two cleavage sites that are located between the N-terminal transmembrane domain and the first heptad repeat. Several proteases have been implicated in alternative processing of mammalian OPA1, including the rhomboid-related protease presenilins-associated rhomboid-like (PARL) [94], AAA proteases in the matrix and the intermembrane space, and the inner membrane peptidase OMA1. These observations have been integrated into a hypothetical model of mitochondrial fusion where early during fusion two mitochondria approaching each other are tethered in a docking step. Consistently, the carboxyl-terminal heptad repeats of mammalian MFN1 have been shown to form an intermolecular antiparallel coiled coil that may tether adjacent mitochondria prior to fusion [94]. Coiled coil formation by mitofusins might then draw the membranes close together and initiate lipid bilayer mixing, and the GTPase could provide biomechanical energy for outer membrane fusion. After the completion of outer membrane fusion, Mgm1 is required in trans on both inner membranes of the fusion partners [94].

In addition to the control of the mitochondrial network, Mfn2 also stimulates the mitochondrial oxidation of substrates, cellular respiration, and maintenance of mitochondrial membrane potential, suggesting that this protein may play an important role in mitochondrial metabolism, and as a consequence, in energy balance [95]. OPA1, on the other hand, is involved in the remodelling of cristae. Mfn and DRP1 expression levels increase in parallel with
mitochondrial content and exercise capacity in human skeletal muscle, suggesting that fusion/fission processes are an integral part of mitochondrial biogenesis [96].

1.1.3.2 TRANSCRIPTIONAL CONTROL OF MITOCHONDRIAL BIOGENESIS

The peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) is a master regulator of mitochondrial biogenesis [97]. It is a transcriptional co-activator that stimulates mitochondrial biogenesis by activating downstream target genes that include additional transcription factors like the nuclear respiratory factors NRF-1/2 (Figure 4) [98]. Moreover, PGC-1α binds to and co-activates the transcriptional function of NRF-1 on the promoter for Transcription Factor A (TFAM), which drives the transcription and replication of mtDNA [98, 99]. Previous studies showed that PGC-1α stimulates mitochondrial biogenesis and respiration in muscle cells through induction of UCP 2 (uncoupling protein 2), NRF-1 and NRF-2 gene expression [98]. Transcription factors NRF1/2 are known to regulate the expression of multiple nuclear-encoded mitochondrial proteins, including subunits of the respiratory complexes, proteins involved in oxidative phosphorylation, Kreb's cycle and the regulation of mitochondrial fusion and fission [99 - 101]. A major adaptation to mitochondrial biogenesis results in altered mitochondrial number and function in response to external stimuli in eukaryotes [16, 98]. This further leads to increase in metabolic enzymes for glycolysis, oxidative phosphorylation and ultimately a greater mitochondrial metabolic capacity [104 – 106]. Therefore, the coordination between the opposing processes of mitochondrial biogenesis and
mitophagy enables cells to adjust their mitochondrial content in response to cellular metabolic state and mitochondrial stress signals.

1.1.4. MITOPHAGY

Autophagy, also called macroautophagy, is a biological catabolic process in all eukaryotic cells. It is recognized as an important intracellular quality control pathway that selectively eliminates damaged organelles, such as mitochondria, the endoplasmic reticulum (ER) and peroxisomes to regulate their number and maintain organellar fidelity [107 - 109]. It is generally induced under conditions of nutrient deprivation in order to sustain the energy requirements of the cell [110, 111]. In addition, autophagy also plays an important role in eliminating intracellular pathogens and protein aggregates, through their encapsulation by a double-membrane structure known as the autophagosome [107, 110].

The identification of the ATG (autophagy) genes using the yeast *Saccharomyces cerevisiae* provided the first insights into this process [108]. Although the importance of autophagy is now well recognized in mammalian systems, many of the mechanistic breakthroughs in delineating how autophagy is regulated and executed at the molecular level have been made in yeast (*S. cerevisiae*) [108, 109]. Currently, 32 different autophagy-related genes (ATG) have been identified by genetic screening in yeast and, significantly, many of these genes are conserved in slime mould, plants, worms, flies and mammals, emphasizing the importance of the autophagic process in responses to starvation across phylogeny [107]. Among these Atg proteins, one subset is essential for autophagosome formation, and is referred to as the “core” molecular machinery [108]. These core Atg proteins are composed of four subgroups: (1)
The Atg1/unc-51-like kinase (ULK) complex; (2) two ubiquitin-like protein (Atg12 and Atg8/LC3) conjugation systems; (3) the class III phosphatidylinositol 3-kinase (PtdIns3K)/Vps34 complex I; and (4) two transmembrane proteins, Atg9/mAtg9 (and associated proteins involved in its movement such as Atg18/WIPI-1) and VMP1. The proposed site for autophagosome formation, to which most of the core Atg proteins are recruited, is termed the phagophore assembly site (PAS) [108, 109]. In general, autophagy begins with an isolation membrane, also known as a phagophore that is likely derived from lipid bilayer contributed by the endoplasmic reticulum and/or the trans-Golgi and endosomes [110]. Concomitantly, a cytosolic form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is processed and conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is inserted into the extending phagophore membrane. This phagophore expands to engulf intracellular cargo, such as protein aggregates, organelles and ribosomes, thereby sequestering the cargo in a double-membraned autophagosome [111]. The loaded autophagosome matures through fusion with the lysosome, promoting the degradation of autophagosomal contents by lysosomal acid proteases. At the same time, LC3-II in the autolysosomal lumen is degraded [111].

Mitophagy, the selective elimination of mitochondria, has been identified in yeast and it is mediated by autophagy-related gene 32 (Atg32) [109, 112]. The Atg32 protein localizes on mitochondria. Following the induction of mitophagy, Atg32 binds Atg11, an adaptor protein for selective types of autophagy, and is then recruited to and imported into the vacuole along with mitochondria [109, 113]. Therefore, Atg32 confers selectivity for mitochondrial sequestration as a cargo and is necessary for recruitment of this organelle by the autophagy machinery for mitophagy [109, 114]. The presence of mitochondria within autophagosomes was initially observed in mammalian cells by scanning electron microscopy in 1957 [115]. Mitophagy is
thought to play a role in mitochondrial quality control by maintaining a healthy mitochondrial population via removal of dysfunctional mitochondria. Beyond quality control, mitophagy has been shown to be required for steady-state turnover of mitochondria, the adjustment of mitochondrion numbers to changing metabolic requirements [112] and specialized developmental stages in mammalian cells, such as during red blood cell differentiation [116]. In mammals, mitophagy occurs during red blood cell differentiation via NIP3-like protein X (NIX; also known as BNIP3L) [116]. Mitophagy, in both yeast and mammalian cells, is preceded by mitochondrial fission, which divides elongated mitochondria and results in predictive and asymmetric changes in mitochondrial membrane potential of the two daughter mitochondria [117, 118]. Bcl-2-like protein 13 (Bcl2-L-13) is a mammalian Atg32 homologue that binds to LC3 to mediate mitophagy and mitochondrial fragmentation [118]. But the most recently identified pathway that mediates mitophagy in mammalian cells, involves the kinase PTEN-induced putative kinase protein 1 (PINK1) and the E3 ubiquitin ligase Parkin (neither of which have yeast homologues) [79, 80].

Recent studies on PINK1 and Parkin have demonstrated that they play a key role in a mitochondrial quality control by facilitating the degradation of depolarized mitochondria through mitophagy. Importantly, these studies have identified mutations in PINK1 and Parkin in autosomal-recessive juvenile parkinsonism (AR-JP) in humans [79]. Detailed genetic analysis done on patients with AR-JP revealed that chromosome 6q25.2-27 harboured an unidentified gene responsible for AR-JP. It was later confirmed by the Shimizu group and other studies that mutations in the PARK2 gene are found in patients of various ethnicities with early-onset PD [119, 120, 121]. PARK2 contains 12 exons that encode the 465 amino acid protein Parkin [122]. The second gene identified in early-onset recessive PD cases was found in a study within a large Italian pedigree on chromosome 1 at the PARK6 locus [82]. These PARK6 mutation patients had
symptoms clinically identical to those of patients with sporadic forms of PD [82, 123]. The 8 exon PARK6 gene encodes the 581 amino acid protein phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) [124]. The protein sequence of PINK1 reveals a predicted C-terminal kinase domain and a mitochondrial targeting sequence at the N-terminus, suggesting that it is imported into mitochondria, which is consistent with its mitochondrial localization in cells [124]. This mitochondrial localization supported prior evidence of an involvement of mitochondrial dysfunction in the pathophysiology of PD.

As mitophagy is a mitochondrial quality control system, its failure is thought to trigger the degeneration of neurons, which is considered a hallmark of PD [78]. As already discussed, neurons critically depend upon mitochondrial respiration for energy production and they cannot switch to glycolytic metabolism (as an ATP-generating mechanism) during acute mitochondrial stress [56-60]. Failure to eliminate mitochondria, which have respiration defects and/or generate increased levels of damaging ROS, will impose a significant oxidative burden over time. In several models of PD, enlarged or swollen mitochondria have been reported [125-127]. This observation potentially highlights a significant role for mitophagy in PD pathogenesis. Under conditions of normal mitochondrial membrane potential, PINK1 is cleaved by mitochondrial proteases such as mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL) in the inter-membrane space [128, 129]. However, PINK1 accumulates on the OMM in response to dissipation of the mitochondrial membrane potential triggered by mitochondrial damage [130]. PINK1, once stabilized on the outer membrane of damaged mitochondria, activates Parkin’s E3 ubiquitin ligase activity, and recruits Parkin to the dysfunctional mitochondrion (Figure 5) [131, 132]. Recent studies have shown that PINK1 phosphorylates S65 on the ubiquitin chains linked to OMM proteins that recruit Parkin to mitochondria and activates it [133, 134]. Then, Parkin mediates ubiquitination and proteasomal
Figure 5: The model of PINK1/Parkin-induced mitophagy pathway

The PINK1/Parkin pathway mediates mitochondrial elimination via mitophagy. Accumulation of ROS can damage mitochondria, resulting in the loss of mitochondrial membrane potential. Loss of mitochondrial membrane potential triggers the accumulation of PTEN-induced kinase 1 (PINK1) on the mitochondrial outer membrane (OMM). The accumulation of PINK1 recruits the cytoplasmic E3 ubiquitin ligase, Parkin, to the damaged mitochondria. Additionally, PINK1 phosphorylates ubiquitin and Parkin to activate Parkin’s E3 ligase activity. Parkin further ubiquitinates various OMM proteins for two disparate processes: autophagosome recruitment and ubiquitin proteasome degradation of ubiquitinated mitochondrial substrates. Fis1 is a receptor on the outer membrane that governs the developing LC3 isolation membrane to generate the autophagosome around the damaged mitochondria. The autophagosome is then delivered to the lysosome for degradation. (Pickrell, A.M; Youle, R.J. 2015) Reprinted, with permission, from Neuron, Volume 85, Copyright (2015)
degradation of several OMM proteins. The ubiquitination of mitochondrial proteins by Parkin has been suggested to recruit ubiquitin-binding adaptor proteins, such as p62/SQSTM1, to depolarized mitochondria [130, 134]. Adaptor proteins further recruit LC3-II in nascent autophagosomes and they have been shown to induce mitochondrial clustering around the nucleus, possibly facilitating the autophagy of mitochondria by increasing their proximity to the endoplasmic reticulum, a possible source of autophagic membranes [134].

Since it has been proposed that the inhibition of the proteolysis of PINK1 could be a mechanism for selective PINK1 mitochondrial accumulation, this has directly implicated PARL in regulating PINK1/Parkin mediated mitophagy. Using mouse models, previous studies have shown that PINK1 undergoes PARL dependent proteolysis leading to release of its 55-kDa C-terminal fragment into the cytoplasm where it is targeted for proteasomal degradation (Figure 6) [135 - 137]. Additionally, a recent study has exhibited that both Parkin recruitment and mitophagy are impaired in PARL−/−MEFs upon CCCP-induced mitochondrial depolarization [138]. These studies strongly suggest that PARL could be a regulatory member of the PINK1/Parkin mitophagy pathway.

1.2 THE MITOCHONDRIAL RHOMBOID PROTEASE PARL

The Rhomboid family of serine proteases is one of the major classes of the intramembrane proteases. The identification of the first rhomboid in a genetic screen performed in *Drosophila melanogaster* led to the name “rhomboid” for the gene because the fly embryos with the mutant phenotype had a mis-shapened rhombus-like head skeleton [139]. Additionally, rhomboid proteases have been shown to be indispensable regulators of EGF signaling in *D.*
Figure 6: Proposed model for how PARL regulates PINK1 localization under normal conditions and during stress.

In a healthy mitochondrion, newly synthesized PINK1 is imported by the TOM/TIM complex. Once it is tethered to the inner mitochondrial membrane (IMM), PINK1 is effectively cleaved by the mitochondrial rhomboid protease PARL, which associates with the TOM/TIM protein-importing machinery. This PARL-dependent cleavage releases PINK1 into the intramembrane space (IMS). The cleaved PINK1 is then retrotranslocated to the cytoplasm, where it undergoes rapid proteasomal degradation. (Image taken from Shanbhag, R. et al. (2012) Parkinson’s disease 2012, 9.)

melanogaster. Selective activation of the epidermal growth factor (EGF) is dependent upon the spatial localization of rhomboid [140]. However, the presence of rhomboids in organisms that lack EGF signaling led to speculation that they might have additional conserved functions [141].
1.2.1 THE DISCOVERY OF THE MITOCHONDRIAL RHOMBOID PROTEASE

The mammalian mitochondrial rhomboid protease, presenilins associated rhomboid-like (PARL), was originally identified in a yeast two-hybrid screen as a putative metalloprotease that interacted with the presenilins, which are implicated in Alzheimer’s disease [142]. It was later discovered that PARL is not a functional interacting partner of the presenilins. The original identification of PARL as an interactor of the presenilins could be a caveat of the classical yeast two-hybrid system, which is poorly suited for membrane proteins. PARL was later discovered to be a mitochondria-localized rhomboid protease in yeast, where it cleaves mitochondrial membrane fusion protein Mgm1 and is implicated as a critical regulator of mitochondrial membrane fusion [149].

1.2.2 THE STRUCTURE AND CATALYSIS OF MITOCHONDRIAL RHOMBOID PROTEASES

All active prokaryotic and eukaryotic rhomboids share a catalytic domain composed of six-transmembrane helixes (TMHs). In eukaryotes, two distinct and ancient lateral gene transfers gave rise to two rhomboid protein families, PARL and RHO [141]. For this study, we will focus on PARL family members, of which the mitochondrial PARL protease is the prototype. They share a ‘1+6’ structure, consisting of a TMH appended at the N terminus of the 6-TMHs that form the catalytic rhomboid domain (Figure 7A) [141]. In all rhomboids, the protease activity is attributed to the invariant Serine and Histidine residues that, akin to many soluble serine
proteases, form a catalytic dyad. In PARL, these residues are S277 in TMH-4 and H335 in TMH-6 and are poised for catalysis similar to other soluble serine proteases that contain a catalytic dyad [147]. Catalytic activity of PARL requires proper positioning of S277 and H335 on TMH-4 and TMH-6 respectively, which can only be achieved by proper spatial ordering and orientation of these TMHs (Figure 7B) [146]. Studies have shown that the positioning of these TMHs appears to depend on Gly residues, consistent with the known role of these residues in mediating helix–helix interactions, often through GxxxG-like motifs [147]. In TMH-4, G278 sits next to S277 and forms a GxxxG-like motif that is positioned directly opposite the catalytic H335 on TMH-6, allowing a close approach of these two TMHs and of their catalytic residues. G278A substitution has been reported to affect the expression of the PARL protein, but not its mRNA, which suggests that the proximity of these TMHs is also important for PARL folding and/or stability in vivo. In addition, the PARL-specific Asp319 position in TMD 5 is critical for PARL expression and activity, and might serve as a catalytic residue [139]. Whether D319 participates in catalysis still has to be resolved with high-resolution structures. In addition, the loop between TMDs 1 and 2 is highly conserved in vertebrates [139].

The rhomboid domain of mammalian PARL shares <19% sequence identity with GlpG, a bacterial rhomboid protease [148]. Homology modeling of the six core PARL TMDs based on the bacterial rhomboid protease GlpG suggests that disrupting the “1 + 6” PARL structural motif might alter the orientation of TMD 5, increasing the distance between the PARL catalytic site and the proposed catalytic aspartate in TMD 5, and consequently reducing PARL catalytic activity [149].

The substrate specificity for the mitochondrial rhomboid proteases is not well characterized. A recent study indicated that the yeast and human mitochondrial rhomboid
**Figure 7: Structure and mechanistic basis of mammalian rhomboid protease PARL.**

**A.** Overall topology of the PARL. The topology of the (1+6)-type mitochondrial rhomboid. It consists of a TMH, labeled as TMH$_A$, at the N-terminus of the 6-TMHs that form the catalytic rhomboid domain. The membrane-spanning TMH1-6 are depicted as cylinders and sequentially labeled. Catalytic residues are highlighted in green.

**B.** Catalytic activity requires close apposition of TMH-4 and -6. (a) The catalytic S277 and H335 residues lie on TMH-4 and -6, respectively. The close packing of TMH-4/-6 is mediated by small Gly and Ala residues, which allow proper positioning of the catalytic S277 and H335 residues. TMH-4/-6 are depicted as ribbons, interfacial residues are depicted with CPK space-filling models. (Jeyaraju, D.V. et al. 2011) Reprinted, with permission from *Cell Death Differ.* Volume 18, copyright (2011).
proteases are not selective in the sequence they cleave. The sequence can be highly variable although hydrophobicity is one of the required characteristics for cleavage. This has been demonstrated in a study where the entire rhomboid cleavage region (RCR) of the mitochondrial fusion protein Mgm1, dynamin-related GTPase, was replaced with two different hydrophobic sequences that are not physiologically cleaved by the yeast mitochondrial rhomboid protease Rbd1, but did not alter the efficiency of Rbd1-dependent cleavage of Mgm1. However, this cleavage was shown to be dependent on a 13 amino acid stretch of negatively charged residues C-terminal to the RCR. Mutating these residues resulted in impaired Mgm1 cleavage by both the yeast and mammalian mitochondrial rhomboid proteases, demonstrating possible conservation of substrate recognition [149]. PARL showed properties very similar to those of Rbd1 regarding recognition of Mgm1. This is in agreement with the observed ability of PARL to functionally complement Rbd1 in yeast [149]. The latter study further demonstrated that the human ortholog of Mgm1, OPA1, is processed neither by Rbd1 nor by PARL, but can be processed in yeast by the m-AAA (mitochondrial-ATPases associated with diverse cellular activities) protease. Taken together, the mitochondrial rhomboid proteases Rbd1 and PARL share conserved properties for recognition of substrates that are apparently distinct to non-mitochondrial rhomboid proteases [149]. In contrast, Mgm1 and OPA1 have apparently diverged such that only Mgm1 is still recognized as a substrate for intramembrane proteolysis by mitochondrial rhomboid proteases [149].
1.3 THE FUNCTION AND REGULATION OF THE MAMMALIAN RHOMBOID PROTEASE, PARL

1.3.1 THE FUNCTIONS OF PARL

1.3.1.1 PARL PLAYS A ROLE IN APOPTOSIS

In mammals, PARL regulates mitochondrial cristae remodeling and cytochrome c release during apoptosis. Using mouse embryonic fibroblasts (MEFs) derived from PARL−/− mice, studies have shown that changes in mitochondrial cristae structure that supported cytochrome c release during apoptosis were observed in PARL−/− MEFs compared to PARL WT MEFs. This indicated that PARL plays an anti-apoptotic role in mammalian cells. In support of this, it has been shown that PARL−/− mice have a reduced lifespan and increased muscle wasting due to increased apoptosis [145]. Additionally, PARL regulates mitochondrial adaptation to heat shock by protecting cells from apoptosis. Sensitivity to thermal stress increases for cells lacking PARL and they release cytochrome c more quickly than WT cells [145, 151]. PARL has also been shown to regulate mitochondrial morphology as overexpression of PARL in mammalian cells results in mitochondrial fragmentation [135, 144]. However, studies have shown that PARL is not involved in the maintenance of proper mitochondrial morphology [145, 152]. Additionally, PARL has been shown to cleave mitochondrial Ser/Thr protein phosphatase phosphoglycerate mutase 5 (PGAM5) [153]. Interestingly, a recent study reported that a cleaved C-terminal fragment of PGAM5 (after amino acid 24, Δ24 PGAM5) accumulates in the cytosol as a substrate of the inhibitor of apoptosis proteins (IAPs). This was concomitant with an increase in the expression of active caspase 3, promoting apoptosis [154]. Hence, PARL is suggested to play
a crucial role in the cellular response to mitochondrial damage as it cleaves PGAM5 upon mitochondrial damage, promoting caspase dependent apoptosis [153, 154]. Impaired PARL function is associated with impaired mitochondrial function and quality control that are proposed to contribute to type 2 diabetes [155, 156] and Parkinson's disease [135, 157].

1.3.1.2 PARL CLEAVES PINK1 AND PLAYS A ROLE IN MITOPHAGY

Although the exact role of PARL in mitochondrial dynamics remains to be determined, recent research has uncovered another role of PARL in mitochondrial quality control that is becoming the focus of intense research. Similar to the Drosophila orthologs, mammalian PINK1 is also processed by PARL. PINK1 is a mitochondrial kinase that senses mitochondrial fidelity and recruits Parkin selectively to depolarized mitochondria. It is cleaved by PARL at A103 and this cleavage of PINK1 is required for its proper localization [135, 136]. Upon import into mitochondria, the mitochondrial targeting sequence (MTS) of PINK1 is removed by the matrix processing peptidase, resulting in the formation of a ~ 60-kD fragment [137, 158]. Subsequent cleavage by PARL results in the formation of a ~ 52-kD fragment that is released back into the cytoplasm by some unknown mechanism [136 - 138]. PINK1 is primarily localized to outer membrane of mitochondria in the absence of catalytically active PARL. The cleavage of PINK1 and its cytoplasmic localization can only be detected in the presence of WT PARL and not the catalytically inactive S277G PARL mutant [135 - 138]. More importantly, it is proposed that PARL-dependent cleavage of PINK1 is required for its downstream function of Parkin recruitment to damaged mitochondria to initiate mitophagy [135, 159].
1.3.1.3 THE ROLE OF PARL IN PARKINSON’S DISEASE

Previous work on PARL reported that PINK1-dependent Parkin recruitment to damaged mitochondria is impaired when cells express the S277G catalytically inactive PARL mutant. It has been proposed that dysfunctional mitophagy could contribute to the pathogenesis of PD [160]. Since PARL’s catalytic activity is required for Parkin recruitment during mitophagy, it strongly suggests that PARL might be a key player in the prevention of PD. In support of this, a recent study identified a mutation in the N-terminus of PARL in PD patients, implicating PARL as a PD-linked gene. This study investigated the PARL gene as a functional candidate gene for PD and sequenced the PARL gene in ~2700 PD patients from various ethnic groups which revealed a novel mutation that was not present in 999 control samples [135]. The mutation results in a substitution of a serine to an asparagine at codon 77 in an individual with no family history of PD. Strikingly, this position is highly conserved (Figure 8) and represents a critical site where auto-catalytic processing, β-cleavage, is known to occur. This substitution of a serine to asparagine at position 77 has been shown to abolish β-cleavage. Additionally, any variant affecting amino acid 77 in PARL in the single nucleotide polymorphism database (dbSNP) or 1000 genomes was not identified [135]. Overall, the p.Ser77Asn variant was detected in the PD population at a frequency of 1 in 1291 (95% confidence range of 1 in 357 to 1 in 10,000) [135].

In addition, the PARL S77N mutant was unable to induce PARL-dependent mitochondrial fragmentation. PINK1-dependent Parkin recruitment upon mitochondrial damage was also impaired in PARL−/− MEFs expressing the S77N PARL mutant [135]. This was the first indication that PARL is a PD-linked gene. Furthermore, a recent study reported that pathogenic PINK1 mutants which are not cleaved by PARL affect PINK1 kinase activity and the ability to
induce PARK2-mediated mitophagy [157]. This study suggests that PARL is an important intrinsic player in mitochondrial quality control, a system substantially impaired in Parkinson disease as indicated by reduced removal of damaged mitochondria. Since PARL has been shown to be a key player in mitochondrial quality control, the regulation of PARL seems to be critical to maintain a healthy mitochondrial pool in the cell [157].

Figure 8. The amino acid sequence in the region of the β-cleavage site of PARL is highly conserved.

Alignment of orthologous amino acid sequences of PARL using CLUSTALW demonstrates the high degree of sequence similarity in mammals. The position of the mutated amino acid is highlighted in red. Those amino acids required for β-cleavage are in the rectangle and the cleavage site is indicated by the arrow. The alignment also shows the equivalent sequence of the Drosophila rhomboid-7 sequence which indicates that the overall conservation of the PARL N-terminus is restricted to mammals. (Image taken from Shi, G. et al. (2011) *Human Molecular Genetics* **20**, 10:1966–1974.)

### 1.3.2 THE REGULATION OF PARL ACTIVITY BY β-CLEAVAGE

The PARL protein, encoded by the nuclear genome, contains an N-terminal mitochondrial targeting sequence (MTS). PARL undergoes α-cleavage to remove the MTS [147] followed by the self-regulated proteolysis at the N-terminus known as β-cleavage [144, 161,
PARL β-cleavage occurs between amino acids S77 and A78, N-terminal to the first TMD (transmembrane domain). β-cleavage of PARL results in two moieties: a small peptide, Pβ, and the rest of PARL, termed as β-PARL as shown in Figure 9A. The Pβ peptide has been hypothesized to translocate from mitochondria to the nucleus, possibly mediating mitochondria-nucleus signaling [144]. Overexpression of β-cleaved PARL in HeLa cells results in fragmented mitochondria, similar to cells expressing WT PARL [162]. Mutating the serine at position 77 or the residues around it abolishes β-cleavage and has been shown to be associated with impaired PARL-mediated mitophagy [135, 144, 162]. This strongly implies that β-cleavage is a very important regulatory mechanism of PARL.

β-cleavage of PARL has also been demonstrated to be developmentally regulated. Studies have shown that an antibody targeted against the N-terminus of PARL was mitochondrially-localized in mature neurons compared to immature neurons, where its staining was primarily nuclear. This result strongly suggested that β-cleavage occurs during cellular differentiation, likely regulating neuronal maturation [144]. Additionally, recent work by our group demonstrated that β-cleavage is strongly induced during three types of mitochondrial stress: membrane depolarization, increase in ROS, and depletion of ATP. Since both membrane depolarization and increased ROS result in depleted ATP, this work suggests that β-cleavage is triggered by excessive damage that reduces ATP levels (unpublished data).

In addition to β-cleavage, PARL has been proposed to undergo γ-cleavage. PARL’s γ-cleavage occurs between the first and second TMDs of PARL and removes first transmembrane domain, TMA, from the rest of the protein, resulting in a PARL protein with only the six core conserved rhomboid TMDs. The functional significance of γ-cleavage has not been well established. Cells overexpressing γ-cleaved PARL resulted in elongated mitochondria similar to untransfected cells [161]. This phenotype is in distinct contrast to the fragmented mitochondrial
morphology observed when cells expressed WT or β-cleaved PARL and suggested that γ-cleavage abolishes PARL activity [161, 162]. Additionally, studies have shown that γ-cleavage is severely diminished if the β-cleavage of PARL is abolished [161].

1.3.2.1 THE REGULATION OF PARL β-CLEAVAGE BY PHOSPHORYLATION

β-cleavage is regulated by phosphorylation at amino acids S65, T69 and S70, a mechanism that inhibits β-cleavage [140]. A previous study has demonstrated that β-cleavage of PARL is severely diminished after mutating these residues which mimic the phosphorylation state (Figure 9B). As phosphorylation of these residues inhibits PARL activity, PARL-induced mitochondrial fragmentation could not be observed in cells expressing the S65D/T69D/S70D triple phosphomimetic mutants. Interestingly, the inhibitory effect on β-cleavage was most pronounced with the S70 phosphomimetic mutant [162]. Since S70 is in closest proximity to S77 where β-cleavage occurs, it is possible that the presence of a phosphate group at S70 presents a steric hindrance, preventing β-cleavage. As previously discussed, β-cleavage has been proposed to regulate PARL’s activity [144, 162]. Thus, phosphorylation is yet another level at which PARL’s activity could be regulated. PARL mutants with reduced β-cleavage still retain their catalytic activity, although impaired β-cleavage is associated with impaired PARL activity [162].
During stress, such as accumulation of ROS and the depolarization of mitochondrial membrane potential, mitochondrial ATP levels drop significantly as a result of an inhibition of the electron transport chain (ETC). Reduced ATP levels result in the dephosphorylation of PARL’s N terminus which leads to β-cleavage. β-cleavage generates a short nuclear-targeted Pβ peptide. When β-cleavage is inhibited as with S77N mutant, PARL retains its uncleaved form even when the mitochondrial ATP levels are low. (Image adapted from Shanbhag, R. et al. (2012) Parkinson’s disease 2012, 9.)
1.3.2.2 PYRUVATE DEHYDROGENASE KINASE 2 (PDK2): A POTENTIAL KINASE THAT REGULATES PARL β-CLEAVAGE.

Recent work by our group has identified pyruvate dehydrogenase kinase isoform 2 (PDK2) as a potential kinase that regulates PARL β-cleavage (unpublished data). Pyruvate dehydrogenase kinase 2 (PDK2) directly phosphorylates PARL to prevent cleavage, and its inhibition has been shown to promote β-cleavage. Furthermore, this study showed that the β-cleavage is enhanced after the cells were treated with a small molecule inhibitor, called dichloroacetate (DCA) (unpublished data). Dichloroacetate (DCA) inhibits PDK and has recently been proposed as a novel and relatively non-toxic anti-cancer agent [163, 164]. DCA is a well-established activator of pyruvate dehydrogenase (PDH) with a potential to play a key role in anticancer therapeutics as it stimulates mitochondrial activity [165 – 168]. Pharmacological studies indicate that DCA-facilitated increase in PDH activity have beneficial effects in several metabolic and cardiovascular disorders, including diabetes, myocardial ischemia, lactic acidosis, and premature muscle fatigue [169]. The activation of PDH by DCA results from DCA inhibiting PDK activity by binding at the pyruvate site [163, 164]. Pyruvate dehydrogenase (PDH) governs the conversion of pyruvate to acetyl Co-A through TCA cycle. Therefore, PDH can control the flow of metabolites from glycolysis to the citric acid cycle and the generation of ATP by mitochondria. PDH is regulated by pyruvate dehydrogenase kinase (PDK) that phosphorylates and inactivates it [163, 164]. Therefore, PDK negatively regulates PDH and leads to conversion of pyruvate to acetyl-CoA, promoting oxidative phosphorylation [167]. By utilizing this metabolic switch, DCA reverses cancer cell abnormal metabolism from aerobic glycolysis to oxidative phosphorylation by reducing the activity of mitochondrial pyruvate dehydrogenase kinases (PDK) [169]. Hence, PDK inhibition by DCA has been shown to reverse
the glycolytic phenotype in a number of cancer cell lines, depolarizing the hyperpolarized inner mitochondrial membrane potential to normal levels and increasing mitochondrial metabolism [164, 170]. The recent structural and mutational analyses showed that DCA binds to a hydrophobic pocket in the N-terminal domain of PDK2 [170, 171]. Given the beneficial effects of DCA, development of inhibitors of PDK has become a focus of the pharmaceutical industry. Additionally, a recent study has highlighted effects of DCA treatment on mitochondrial morphology in the neuronal-like SH-SY5Y cells. This study reported remodeling of the mitochondrial network and induction of partial mitophagy in the SH-SY5Y cells upon treatment with DCA [172]. DCA caused decrease in cell viability and profound morphological changes in the mitochondrial network resulting in shorter and more fragmented mitochondrial fragments [172]. A recent study by our group demonstrates induction of PARL β-cleavage in the SH-SY5Y cells upon treatment with DCA. This suggests that partial mitophagy induced by the DCA treatment in the SH-SY5Y cells may lead to induction of β-cleavage of PARL as we have previously reported that β-cleavage might be a response to overcome mitochondrial damage.

1.3.3 Pβ PEPTIDE: THE PRODUCT OF β-CLEAVAGE OF PARL

PARL β-cleavage liberates a 25-aa peptide termed the Pβ peptide. Studies done on the topology of PARL showed that this peptide is generated in the mitochondrial matrix [144]. The Pβ peptide contains three closely spaced doublets of positively charged amino acids (spanning amino acids 54-65), the first and second of which are conserved in vertebrates, whereas the third one is mammalian-specific [144]. Previous studies have shown that overexpressed GFP-tagged Pβ peptide goes to the nucleus compared to the Pβ in which two of the conserved doublets of
positively charged amino acids was mutated to a pair of small residues [144]. In addition, this study reported that the nuclear/cytosolic distribution of these mutants did not change when cells were treated with a nuclear export machinery inhibitor, leptomycin B, indicating that these mutations affected the rate of nuclear import rather than the export of the GFP fusion proteins [144]. This suggests that the Pβ peptide does not harbor typical monopartite and bipartite nuclear localization signals (NLS), found both at the N- and C-termini of nuclear proteins. However, the three closely spaced doublets of positively charged amino acids it contains serve as a putative nuclear localization signal (NLS).

Intriguingly, the N-terminal region of PARL, the Pβ peptide, shows no detectable similarity to any other available protein sequences as demonstrated using iterative BLAST searches (Figure 8). This region of PARL is conserved among mammals and it is vertebrate-specific [144]. Indeed, there is no evident insertion or deletion in 58 of the 62 residues of the Pβ domain emphasizing the invariant status of the Pβ domain [144]. This suggests that the N-terminal region of mammalian PARL could have a distinct and important function. Previous studies suggest that the N-terminal region of PARL was subject to strong purifying selection at least during mammalian evolution, which can be explained by functional constraints of the mitochondrial rhomboid protease within higher vertebrates [144]. Hence, the biological relevance of the Pβ domain is evident from its sequence conservation although its biological function remains unknown.

1.3.3.1 MITOCHONDRIAL- NUCLEAR CROSSTALK: RETROGRADE RESPONSE

Cells can monitor and respond to changes in the state of their organelles. For instance,
there is a stress-related signal transduction pathway in the ER that responds to the accumulation of unfolded proteins in the lumen of the ER and activates expression of genes encoding some ER-resident proteins, such as the chaperone BiP [173]. Likewise, alterations in mitochondrial function can result in modulation of expression of nuclear genes, termed retrograde regulation [174, 175]. In animal cells, for example, deficits in mitochondrial function or certain external cues can lead to increased expression of genes encoding components of the mitochondrial oxidative phosphorylation apparatus and, in some instances, lead to a general increase in the biogenesis of mitochondria [22, 99, 176 -179]. These events are controlled, in part, by transcriptional activators and coactivators whose targets include nuclear genes encoding mitochondrial proteins. The best-characterized mitochondria-to-nuclear signal transduction pathway, retrograde response (RTG), is thoroughly characterized in yeast. During mitochondrial dysfunction, the RTG pathway is activated to increase the activity of numerous metabolic pathways that compensate for the lack of mitochondrial activity. The RTG pathway senses the functional state of mitochondria via the level of glutamate [157, 180, 181]. Because glutamate is a potent repressor of RTG-dependent gene expression, the retrograde pathway is likely to be important for glutamate homeostasis. The RTG response is mediated by RTG1 and RTG3, two basic helix-loop-helix leucine zipper (bHLH/Zip) transcription factors [181, 182]. When mitochondria are functional, RTG3 is hyperphosphorylated and sequesters both itself and RTG1 in the cytoplasm. However, mitochondrial dysfunction causes RTG3 to be partially dephosphorylated allowing nuclear translocation of the RTG1/3 complex, and the induction of the compensatory response which is maintenance of intracellular glutamate supplies in yeast cells [181]. In cells with compromised or dysfunctional mitochondria, the expression of CIT1, ACO1, IDH1, and IDH2 (genes encoding the enzymes catalyzing the first three steps in
the TCA cycle that lead to the production of α-ketoglutarate, the direct precursor of glutamate) are controlled by the RTG genes [183-185].

A number of signaling pathways have been identified that allow the coordination of mitochondrial and nuclear gene expression systems under varying growth conditions [186-189]. However, the origins of mitochondrially-derived peptides and their role in stress signaling has been revealed by a mass spectrometry study that demonstrated the existence of a constant efflux of a large number of peptides from mitochondria in an ATP- and temperature-dependent manner. These cytosol-localized peptides, range in size from 6 to 27 amino acids and originate from the cleavage of proteins localized mainly in the matrix and inner membrane [190]. Another recent study showed that the ABC family transporter effluxes peptides across the mitochondrial membrane and also regulates the mitochondrial unfolded protein response (UPRmt) and the expression of mitochondrial chaperones [191]. This suggests a mechanism by which the degradation and clearance of damaged mitochondrial proteins initiates a mitochondrial stress signal that results in a protective response in the nucleus, which in turn enhances mitochondrial protein homeostasis (proteostasis). Another study has established that peptides of 6–20 amino acids, derived by proteolysis of inner mitochondrial membrane proteins, could be exported through the inner membrane by the ABC transporter, Mdl1 [192]. From this point, it had been assumed that these peptides cross the mitochondrial outer membrane either through porins or the TOM complex into the cytoplasm [192, 193]. These observations suggested that peptide efflux serves as a means of communication between the mitochondria and their cellular environment.

Whether the endogenous Pβ peptide can be exported from the mitochondrial matrix remains to be demonstrated. However, the existence of specialized machinery for the export of matrix peptides of similar size supports the likelihood of translocation of the Pβ peptide from the
mitochondrial matrix. Additionally, the fact that an integral Pβ sequence is not required for PARL-mediated mitochondrial fragmentation suggests that the Pβ peptide might play a role as proposed in mitochondrial-nuclear signaling.

1.4 THESIS RATIONALE

β-cleavage of PARL is an important regulator of the PINK1/Parkin mediated mitophagy pathway. It results in two potentially active moieties β-PARL and Pβ peptide. Despite some evidence that the Pβ peptide is produced by β-cleavage of PARL, the endogenous Pβ peptide is yet to be identified in mammalian cells. Previous studies have demonstrated that Pβ peptide accumulates in the nucleus either by artificially injecting the peptide or overexpressing a synthetic Pβ construct with a GFP tag in mammalian cells. However, the nuclear translocation of the endogenous Pβ peptide remains to be demonstrated. Additionally, the trigger for the nuclear translocation of the Pβ peptide is not yet known. The sequence of the Pβ peptide is highly conserved among mammals that likely highlights its significance. Our previous human molecular genetics study identified a PD-linked mutation at the cleavage site (S77N) that abolishes β-cleavage. While this mutant is defective in eliciting a proper CCCP-induced mitophagy response, this form of the enzyme still cleaves its substrate PINK1. This suggests that the Pβ peptide has biological activity. Therefore, the aim of this study is: 1) to identify the endogenous Pβ peptide in mammalian cells, 2) to assess the mitochondrial-nuclear translocation of the peptide and 3) to determine the trigger for the nuclear accumulation of the Pβ peptide.

In this work, we have identified the endogenous Pβ peptide in the neuroblastoma SH-SY5Y cell line and shown that the endogenous Pβ peptide accumulates in the nucleus after induction of mitochondrial stress. In addition, our work shows that the endogenous Pβ peptide is
unstable and it is degraded by proteasome and lysosome. Furthermore, the enrichment of the endogenous Pβ peptide and its translocation to the nucleus after Dichloroacetic acid (DCA) treatment, opens up new avenues for its possible role in regulation of cell metabolism. Taken together, this work demonstrates the existence of the endogenous Pβ peptide and its enhanced accumulation in the nucleus after reduction of the ATP levels. The nuclear translocation of the Pβ peptide might contribute to mitochondrial-nuclear cross-talk as a part of its role in mitochondrial quality control.
CHAPTER 2

MATERIALS AND METHODS

1. Cell lines and cell culture
HEK 293T, MEFs and SH-SY5Y cell lines were cultured in Dulbecco's Modified Essential Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma). Cells were incubated at 37°C in an atmosphere containing 5% (v/v) CO₂.

2. Plasmids and transfection
The PARLΔPβ-flag construct was generated by deleting the Pβ sequence from the pcDNA vector containing the gene that encodes PARL with a FLAG sequence at the C-terminus. This was done using the inverse PCR method as previously described [194]. Pβ-GFP and Pβ-NLSM-GFP constructs and GFP empty vector were a kind gift from Dr. Lyndal Bayles (Deakin University, Australia). Transient DNA transfections of HEK 293T cells were performed using Xtremegene reagent (Roche, 063 65 787 001). If not otherwise indicated, 1 µg of plasmid encoding PARL-Flag and PARLΔPβ-Flag were used. Cells were harvested 36 h after the transfection. For transfection of plasmids with MEF cell lines, 50,000 cells were seeded per one plate of a 6-well plate. After 24 h, cells were transfected with 2 µg of plasmid using Lipofectamine 2000 reagent (Invitrogen, 11668-027). Disruption of the mitochondrial membrane potential was achieved by incubating the cells with 10 µM CCCP (Sigma, C2759) for 3 h. To inhibit the proteasome, cells were treated with 10 µM MG132 (Sigma) for 16 h, post 24 h transfection with appropriate plasmid. Cells were treated with 0.1 mM DCA (Sigma) and 500 µM hydrogen peroxide (Bioshop) for 24 h and 6 h, respectively, prior to harvesting. Oligomycin
A (Sigma) treatment was done for 24 h using final concentration of 20 µM before harvesting the cells.

3. Western blotting and antibodies

Prior to lysis, the medium from the tissue culture dishes was aspirated and cells were washed once with ice-cold PBS. Cells were then lysed in Lysis Buffer (1% Triton-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 7.5) supplemented with protease inhibitor cocktail (PIC, Bioshop) and 1 µg/ml phenylmethanesulfonyl fluoride (PMSF). Detergent-solubilized membrane proteins were clarified through centrifugation at 800x g for 5 min at 4ºC. Protein samples were dissolved in 1x Laemmli sample buffer and resolved on a precast 4-20% Tris-glycine acrylamide gels (Biorad, 456-1093) and transferred onto PVDF membranes to probe for the Pβ peptide. Membranes were blocked using 5% BSA and incubated with the Pβ peptide specific antibody used in 1:5000 dilution. To probe for other proteins, samples were separated by 10% SDS-PAGE and transferred onto nitrocellulose or PVDF membrane. Membranes were blocked using 5% skim milk powder and incubated with the following antibodies with dilutions as indicated: 1:1000 for polyclonal rabbit ALY (Abcam, 95962), 1:1000 for monoclonal mouse FLAG (M2; Sigma-Aldrich, F1804), 1:1000 for monoclonal mouse GFP (Roche, 11 814 460 001), 1:1000 for monoclonal mouse CVα (Abcam, ab14748), 1:1000 for monoclonal mouse actin (Sigma-Aldrich, A3853). Bound antibodies were detected by enhanced chemiluminescence (ECL). Data shown are representative of 3 independent experiments. Pβ-specific antibody was a kind gift from Dr. Lyndal Bayles (Deakin University, Australia). Rabbit and mouse horseradish peroxidase-conjugated secondary antibodies (Jackson Labs), rabbit and mouse Alexa Fluor secondary antibodies (Invitrogen) were used.
4. Mitochondrial and nuclear fractionation

Cells were harvested after they were 80-90% confluent. Mitochondrial and nuclear fractionations were performed by differential centrifugation method using the mitochondrial fractionation kit (Abcam, ab110170), according to the manufacturer’s instructions. Briefly, cells were harvested and washed once with PBS buffer. Cells were lysed in the fractionation reagent A (Tris, Trisethanolamine, EDTA, Digitonin). Cellular debris and nuclei were removed by centrifugation at 1,000 g for 10 min at 4°C after passing the cell resuspension through a 26 ½ gauge needle to facilitate the cell lysis. The supernatant fraction was centrifuged at 12,000 g for 15 min to obtain the mitochondrial membrane pellet. The cell pellet from the low spin was resuspended in the fractionation reagent B (Tris, digitonin, EDTA) and passed again through the 26 ½ gauge needle for 15 times followed by centrifugation at 1,000 g for 10 min. The pellet obtained is the nuclear fraction. The supernatant from this spin was used to resuspend the mitochondrial membrane pellet and centrifuged again at 12,000 g for 15 min at 4°C to get the final mitochondrial pellet. The mitochondrial pellet was washed twice with the fractionation reagent B. The mitochondrial and nuclear pellets were lysed in Laemmli sample buffer for SDS-PAGE and western blot analyses.

5. Immunoprecipitation

If not indicated differently, all steps were performed at 4°C. The SH-SY5Y cell pellets were washed with PBS and lysed using the IP buffer (1% Triton-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 7.5) supplemented with protease inhibitor cocktail (PIC, Bioshop) and 1 µg/ml phenylmethanesulfonylfluoride (PMSF). Detergent-solubilized membrane proteins were clarified by centrifugation at 800× g for 5 min and subsequently pre-incubated with protein A-coupled magnetic beads (161-4013) for 1 h at 4°C. The supernatant
fraction was incubated with Pb-specific antibody for 2 h. After the addition of protein A coupled magnetic beads, the mixture was incubated overnight. Immunoprecipitates were washed 3 times in IP buffer (without PIC and PMSF). The immunocomplexes were eluted in Laemmli sample buffer and boiled for SDS-PAGE and western blot analyses.

6. Immunofluorescence microscopy

A) Fixation: 2 ml of cell suspensions (in DMEM supplemented with 10% FBS) were plated on coverslips at a density of 75,000 cells/mL and allowed to adhere and grow overnight. After transfections and chemical treatments, the medium was aspirated and replaced with 2 mL of 4% paraformaldehyde (PFA). After 20 min, the PFA was aspirated and subsequently replaced by 2 mL of PBS, pH 7.5.

B) Immunostaining: 0.01% Triton-X 100 (in PBS) was used to first permeabilize cells for 15 min. Then, the cells were incubated with 10% goat serum for 1 hr, prior to addition of primary antibodies. The mouse anti-FLAG (Sigma-Aldrich) and mouse anti-CVα (Abcam) primary antibodies were used at 1:1000 dilutions. The cells were incubated with primary antibodies for 2 h at room temperature, antibody solutions were then aspirated and the coverslips washed with PBS for three times. Cells were then incubated with secondary antibodies conjugated with Alexa fluorophores (Invitrogen) at a dilution of 1:1000 each in 10% goat serum for 2 h at room temperature. Subsequently, 100µL of 1µg/mL 4’,6-diamidino-2-phenylindole (DAPI) was added 5 minutes before the end of the 2 h secondary antibody incubation period. Once again, the antibody solutions were aspirated and the coverslips were washed with PBS 3 times. Coverslips were then mounted on slides using Fluoromount-G (SouthernBiotech, 0100-01) and allowed to dry a minimum of 12 h in the dark prior to imaging.
C) Imaging: Imaging was carried out using a Zeiss AxioVision fluorescence microscope. The 63X magnification objective was used for all experiments and Volocity Imaging Software (Perkin Elmer) was used for all acquisitions. Localization of Parkin was quantified by scoring the subcellular localization of the tagged protein in 100 cells in three separate experiments.
CHAPTER 3

RESULTS

3.1 GENERATION AND OPTIMIZATION OF THE Pβ PEPTIDE SPECIFIC ANTIBODIES

In order to detect Pβ peptide in the mammalian cells, we first generated Pβ peptide-specific antibodies in collaboration with an antibody development company. The Pβ peptide domain is composed of 25 amino acids. Three amino acids at positions S65, T69 and S70 within the Pβ peptide can be phosphorylated and this phosphorylation regulates its production [144, 162]. As a first approach, we have worked with Phospho Solutions, an antibody development company, to generate antibodies against the Pβ peptide. Our strategy was to make antibodies against both the tri-phospho and non-phospho forms of the Pβ peptide. Antibodies were generated by Phospho solutions company for both phospho and non-phospho forms of the Pβ peptide by immunization of rabbits (Figure 10A). To assess the specificity and sensitivity of both the Pβ peptide-specific antibodies, initial test bleeds were analyzed by dot blotting against both purified phospho and non-phospho peptides. Briefly, 2 μL of both phospho and non-phospho peptides were applied directly onto the membrane as a dot in increasing final concentrations on the blot ranging from 10ng/μL to 200ng/μL as shown in Figure 10C. We found that the anti-phospho antibody had a strong signal and specificity to only the phospho peptide in contrast to the non-phospho antibody that reacted with both phospho and non-phospho peptides as shown in Figure 10C. Moreover,
**Figure 10: Generation and optimization of the Pβ specific antibodies.**

**A.** For the preparation of both phospho and non-phospho Pβ peptide specific antibodies (directed against a PARL peptide located near the N terminus), a 25-amino acid-long peptide spanning amino acids 53-77 of PARL was synthesized, purified by high pressure liquid chromatography, conjugated to bovine serum albumin, and used to immunize rabbits according to standard protocols for antisera production followed by affinity-purification of the antisera. (This was done by Phospho solutions, an antibody development company)

**B.** Table showing summary of the parameters used to optimize the sensitivity of both phospho and non-phospho Pβ peptide specific antibodies. Optimized parameters are highlighted in red color.

**C.** Dot blot of the optimized parameters for both phospho and non-phospho Pβ peptide-specific antibodies is shown. Both the antibodies were able to detect as low as 10 ng of the Pβ peptide spotted on the PVDF membrane. Phospho Pβ peptide specific antibody was able to detect only the phospho Pβ peptide whereas the antibody generated against the non-phospho Pβ peptide was able to detect both the phospho and non-phospho Pβ peptides. P and NP represent purified phospho and non-phospho Pβ peptides respectively.

Both antibodies were able to detect 10ng/μL of the purified peptides. To further optimize the use of these antibodies in order to detect the endogenous Pβ peptide in mammalian cells, various parameters were tested, for example, different antibody concentrations, incubation times, different blocking reagents such as milk or BSA, different transfer membranes, nitrocellulose membrane or PVDF membrane as shown in Figure 10B. We found that the optimum working conditions for the phospho Pβ peptide specific antibody were: 1:500 dilution of the antibody in 2% BSA used with PVDF membrane and 2 h incubation at room temperature or overnight at 4°C. For the non-phospho Pβ antibody, the best working conditions were same as the phospho Pβ antibody except for the dilution factor, which was 1:1000. After optimizing the working conditions of the antibodies, further experiments were initiated to detect the Pβ peptide in the mammalian cell lines.
3.2 GENERATION AND EXPRESSION OF PARLΔPβ-FLAG CONSTRUCT IN THE HEK 293T CELL LINE

In order to have a control for our future experiments, we first generated a PARLΔPβ-FLAG construct from which the Pβ peptide sequence had been deleted, as described in the Materials and Methods (Figure 11A). After the construct was generated, we tested its expression in the HEK 293T cell line. The PARLΔPβ-FLAG construct was transiently transfected in HEK 293T cells. We used the PARL-FLAG construct as a positive control. Lysates were prepared and probed for the FLAG tag using a FLAG specific antibody. Two bands were observed in the lane corresponding to cells overexpressing the PARL-FLAG construct, where the upper band corresponds to the full length PARL and the lower band corresponds to PARL lacking the Pβ sequence (Figure 11B). Only the lower band is observed in cells overexpressing the PARLΔPβ-FLAG construct. This result indicates that PARLΔPβ-FLAG construct expresses well in the mammalian cell line HEK 293T. Next, we determined the sub-cellular localization of PARLΔPβ-FLAG construct by using immunofluorescence (IF). HEK 293T cells were transiently transfected with the PARL-FLAG and PARLΔPβ-FLAG constructs separately followed by their analysis by immunofluorescence (Figure 11C). We used CVα as a mitochondrial marker, represented in red. CVα is a component of the complex V of the ETC (electron transport chain) complex located in the mitochondrial inner membrane [27]. The FLAG tag is shown in green. The mitochondrial localization of the PARLΔPβ-FLAG construct is evident from co-localization of the mitochondria with FLAG tag. The PARL-FLAG construct, used as positive control, also exhibits mitochondrial localization. Taken together, these results indicate that the PARLΔPβ-FLAG construct expresses well in the mitochondria validating its use for our future experiments.
Figure 11: Generation and expression of the PARLΔPβ-FLAG construct in the HEK 293T cell line.

A. Schematic of PARL-FLAG and PARLΔ Pβ-FLAG constructs. MTS at the N-terminus represents the mitochondrial targeting sequence and the FLAG tag is at the C-terminus.

B. Western blot analysis of HEK 293T cells overexpressing WT PARL-FLAG and PARLΔPβ-FLAG constructs. CVα, a mitochondrial marker, was used as a loading control.

C. Representative immunofluorescence images of the HEK 293T cells transiently transfected with PARL-FLAG and PARLΔPβ-FLAG constructs. Both PARL-FLAG and PARLΔPβ-FLAG localize in the mitochondria. CVα was used as a mitochondrial marker highlighted in red.

3.3 THE Pβ PEPTIDE IS IDENTIFIED IN THE HEK 293T CELL LINE AFTER OVEREXPRESSION OF PARL

After optimization of the Pβ peptide specific antibodies, we further used these antibodies to detect the Pβ peptide after overexpression of PARL, which has been proposed to produce the Pβ peptide, in the mammalian cell line HEK 293T. However, we were not able to detect the Pβ peptide using the phospho or non-phospho Pβ antibodies in the mammalian cells. Then we obtained another Pβ peptide specific antibody, received as a generous gift from Dr. Lyndal Bayles (Deakin University, Australia), which we tested using the optimized conditions of phospho and non-phospho Pβ peptide specific antibodies. We found that it had strong signal for both phospho and non-phospho peptides as shown in Figure 12. Since this antibody was also able to detect endogenous Pβ in a mammalian neuroblastoma cell line, it was used in all future experiments. Since PARL undergoes β-cleavage at its N-terminus and produces Pβ peptide, we used the PARL-Flag construct for this experiment. The PARLΔPβ-Flag construct was used as a negative control as the Pβ peptide sequence has been removed (Figure 13A).
and PARLΔPβ-Flag constructs were transiently transfected in the HEK 293T cell line. Lysates were prepared and western blotting was used to probe for PARL-FLAG and the Pβ peptide using FLAG-tag specific antibody and the Pβ peptide specific antibody respectively. We observed that PARL undergoes β cleavage as evident from two bands in the lane corresponding to cells overexpressing PARL-FLAG construct as shown in Figure 13B. Moreover, the band corresponding to the Pβ peptide was detected in the cells transiently transfected with PARL-Flag in comparison to PARLΔPβ-Flag overexpressing cells. In addition, we probed for CVα protein as a loading control. Taken together, this data demonstrates that the Pβ antibody was able to detect the Pβ peptide when PARL was overexpressed in the HEK 293T cell line. To further determine the subcellular localization of the Pβ peptide, additional experiments on the Pβ peptide were initiated.

Figure 12: Pβ specific antibody detects both phospho and non-phospho Pβ peptides.

Western blot analysis of purified phospho and non-phospho peptides using the Pβ peptide specific antibody. P and NP are representative of the purified phospho and non-phospho Pβ peptides respectively.
Figure 13: The Pβ peptide is identified in the HEK 293T cell line after the overexpression of the PARL-FLAG construct.

A. Schematic of PARL-FLAG and PARLΔ Pβ-FLAG constructs. MTS at the N terminus represents the mitochondrial targeting sequence and the FLAG tag is at the C terminus.

B. Western blot analysis of HEK 293T cells overexpressing WT PARL-FLAG and PARLΔ Pβ-FLAG constructs. CVα, a mitochondrial marker, was used as a loading control.

C. Western blot analysis indicating that the Pβ peptide is identified in the PARL-Flag expressing cells compared to the cells overexpressing PARLΔ Pβ –Flag. P and NP in the right panel represent purified phospho and non-phospho Pβ peptides respectively.
3.4 THE $\text{P}\beta$ PEPTIDE GOES TO THE NUCLEUS.

Recently, our group has shown that $\beta$ cleavage of PARL increases with induction of mitochondrial stress (unpublished data). Figure 14 shows that $\beta$ cleavage of PARL increases after treatment of HEK cells with various chemicals that perturb mitochondrial health like ATPase inhibitor oligomycin A, complex I inhibitor rotenone and mitochondrial membrane depolarizer CCCP. As a control, HEK cells were also treated with thapsigargin, inhibitor of fusion of autophagosome and lysosomes. Thapsigargin treatment does not lead to enhanced $\beta$ cleavage of PARL. Thapsigargin also results in ER-stress, showing the specificity of mitochondrial stress on $\beta$ cleavage. This result suggests that $\beta$ cleavage of PARL is induced after induction of mitochondrial stress only rather than accumulation of PARL itself.

The $\text{P}\beta$ peptide has a putative nuclear localization signal (NLS) at the N-terminus as reported in a previous study [144], therefore, we further speculated that the $\text{P}\beta$ peptide produced after PARL $\beta$ cleavage might go to the nucleus as a signal to initiate a mitochondrial stress response. Moreover, a previous study has shown that peptides of similar size are liberated from the mitochondria in response to alterations in mitochondrial health [190]. To test this, we determined the subcellular localization of the $\text{P}\beta$ peptide with and without the induction of mitochondrial stress. HEK 293T cells were transiently transfected with the PARL-FLAG construct and treated with several chemicals that influence the mitochondrial health such as the mitochondrial uncoupler, CCCP, and the ROS producer, $\text{H}_2\text{O}_2$. Mitochondrial and nuclear fractionation was carried out by differential centrifugation. Analysis of the mitochondrial and nuclear fractions was done by western blotting using the $\text{P}\beta$ peptide-specific antibody. The $\text{P}\beta$ peptide was enriched in the nuclear fraction of CCCP and $\text{H}_2\text{O}_2$ treated cells compared to the
DMSO treated cells (Figure 15). The cytosolic fraction is not shown here because we could not detect Pβ peptide in the cytosolic fraction in our experiments (data not shown). In addition, we used CVα as a mitochondrial marker and ALY as a nuclear marker. We observed that the mitochondrial fraction had some nuclear contamination but nuclear fraction were clean of mitochondrial contamination. These results indicate that the Pβ peptide accumulates in the nucleus after induction of mitochondrial stress, generated by inner mitochondrial membrane depolarization and oxidative stress.

HEK 293 cells were transiently transfected with FLAG-tagged PARL, and treated with DMSO, ATPase inhibitor oligomycin A, mitochondrial complex I inhibitor Rotenone and mitochondrial membrane depolariser CCCP. As a control, cells were also treated with Thapsigargin, inhibitor of fusion of autophagosomes with lysosomes. The β cleavage levels were analyzed by immunoblotting. CVα, a mitochondrial marker, was used as a loading control.
Figure 15: The Pβ peptide goes to nucleus after induction of the mitochondrial stress in the HEK 293T cells overexpressing PARL-FLAG.

Analysis of mitochondrial and nuclear fractions by western blotting of HEK 293T cells overexpressing WT PARL demonstrates accumulation of the Pβ peptide in the nucleus upon CCCP and H2O2-induced mitochondrial stress. CVα was used as a mitochondrial marker and ALY as a nuclear marker to indicate effective subcellular fractionation. We loaded 100% of mitochondrial and nuclear fractions obtained after subcellular fractionation of the cells. MF and NF represent mitochondrial fraction and nuclear fraction respectively.

3.5 ENDOGENOUS Pβ PEPTIDE IS IDENTIFIED IN THE SH-SY5Y NEURONAL CELL LINE AFTER INHIBITION OF THE PROTEASOME AND LYSOSOME.

Since the Pβ peptide was easily detected using the Pβ peptide specific antibody in the HEK 293T cells after overexpression of PARL, we further carried out experiments to determine if we could detect the endogenous Pβ peptide in the dopamine producing neuronal cell line SH-SY5Y.
However, we were not able to identify the endogenous Pβ peptide even in the disease relevant SH-SY5Y cell line first (data not shown). Therefore we proposed the possibility of the endogenous Pβ peptide being unstable and being degraded after its production. This led us to speculate that the endogenous Pβ peptide might be degraded by either the proteasome or by lysosomes. To test this, we treated SH-SY5Y cells with 10μM of the proteasome inhibitor MG132 or 100nM of the lysosomal degradation inhibitor bafilomycin A, and, with MG132 and bafilomycin A together. Since the Pβ peptide is generated in the mitochondrial matrix, mitochondrial enrichment was done by differential centrifugation. We probed for the endogenous Pβ peptide in the mitochondria-enriched samples by western blotting using the Pβ peptide specific antibody and CVα was used as a mitochondrial marker. We observed that a band corresponding to the Pβ peptide in MG132, bafilomycin A and both MG132 and bafilomycin A treated cells was quite intense as compared to the DMSO as our loading control was comparable in all the treatments (Figure 16A). Additionally, Pβ peptide band observed in the lane corresponding to MG132 and bafilomycin A treatment was less intense compared to only MG132 treatment which suggests that the MG132 alone treatment may lead to aberrantly high intensity of the Pβ peptide band. Figure 16B shows mitochondrial fraction was clean of cytosolic contamination as analyzed by probing with mouse anti-CVα and mouse anti-actin. This data suggests that the endogenous Pβ peptide is degraded by the proteasome and lysosomes. Hence, MG132 or bafilomycin A treatment enhances Pβ peptide stability and leads to its enrichment. To further confirm this result, we performed an IP (immunoprecipitation) experiment in the SH-SY5Y cells. SH-SY5Y cells were treated with or without 10 μM MG132. After harvesting treated cells, the endogenous Pβ peptide was immunoprecipitated using the Pβ
**Figure 16: The endogenous Pβ peptide is identified in the neuronal cell line SH-SY5Y after the inhibition of the proteasome.**

A. Analysis of mitochondrial fractions of the SH-SY5Y cells, treated with the 10 μM MG132, 100 nM bafilomycin and together with 10 μM MG132 and 100 nM bafilomycin, by western blotting using Pβ peptide specific antibody. CVα antibody was used as loading control. We observed that the transfer was not very efficient as evident from protein bands in DMSO lane.

B. Western blot analysis using CVα and actin antibodies. Mitochondrial and cytoplasmic markers indicated that mitochondrial fraction is free of cytosolic contamination. TF, MF and CF represent total fraction, mitochondrial fraction and cytoplasmic fraction respectively. We loaded 100% of mitochondrial fractions obtained after subcellular fractionation of the cells.

C. Western blot analysis of the immunoprecipitated endogenous Pβ peptide after the MG132 treatment of the cells. The endogenous Pβ peptide was immunoprecipitated and probed using the same Pβ peptide specific antibody.

peptide-specific antibody. IP samples were probed for the endogenous Pβ peptide by western blotting using the same Pβ peptide specific antibody. Figure 16C shows the endogenous Pβ peptide is enriched in MG132 treated cells compared to only DMSO treated cells. This result indicates that the endogenous Pβ peptide produced is degraded by the proteasome unless stabilized by treatment of cells with MG132. However, the signal corresponding to the Pβ peptide after MG132 treatment was quite variable in our future experiments as we will see that we were not able to detect the endogenous Pβ peptide in a few experiments with MG132 treatment only unless treated with some other chemical that induces β-cleavage of PARL leading to increase in the production of the endogenous Pβ peptide. After the endogenous Pβ peptide was identified in the SH-SY5Y cell line, further experiments were carried out to determine the subcellular localization of the endogenous Pβ peptide after induction of mitochondrial stress.
3.6 THE ENDOGENOUS Pβ PEPTIDE ACCUMULATES IN THE NUCLEUS AFTER INDUCTION OF MITOCHONDRIAL STRESS IN THE SH-SY5Y CELL LINE.

After the endogenous Pβ peptide was identified in the SH-SY5Y cell line, we further examined the subcellular localization of the endogenous Pβ peptide. SH-SY5Y cells were treated with the mitochondrial uncoupler CCCP and with the ROS producer H$_2$O$_2$. In addition, cells were also treated with 10 μM MG132 for 16 hours. Total, mitochondrial and nuclear fractions were prepared and probed for the endogenous Pβ peptide by western blotting using the Pβ peptide-specific antibody. Figure 17 shows the endogenous Pβ peptide largely ends up in the nuclear fraction under all conditions including DMSO. However, there is increase in the Pβ peptide production after induction of mitochondrial stress by CCCP and H$_2$O$_2$ treatment of cells. Moreover, we observed that the endogenous Pβ peptide is enriched in the nuclear fraction in cells treated with MG132 only. Previous studies have shown that proteasome inhibition by MG132 leads to induction of mitochondrial membrane depolarization leading to mitochondrial and cytosolic oxidative stress [193, 195]. This increase in mitochondrial oxidative stress could be a reason for increased accumulation of the Pβ peptide in the nucleus after MG132 treatment of cells. The quality of the mitochondrial and nuclear fractionation was assessed by staining with rabbit anti-ALY and mouse anti-CVα. Taken together, these results suggest that the endogenous Pβ peptide accumulates in the nucleus after depolarization of mitochondria and generation of oxidative stress.
Figure 17: The endogenous Pβ peptide accumulates in the nucleus after induction of the mitochondrial stress in the SH-SY5Y cell line.

A. Western blot analysis using the anti-serum against the Pβ peptide. SH-SY5Y cells, treated with 10 μM CCCP and 10 μM MG132, were probed for the endogenous Pβ peptide after the mitochondrial and nuclear fractionation. Western blot analysis using CVα and ALY antibodies, mitochondrial and nuclear markers, indicated that mitochondrial fraction has some nuclear contamination. However, we observed clean nuclear fractions. TF, MF and NF are representative...
of the total fraction, mitochondrial fraction and the nuclear fraction respectively. We loaded 100% of mitochondrial and nuclear fractions obtained.

**B.** Western blot analysis using the Pβ peptide-specific antibody. Total, mitochondrial and nuclear fractions were probed for the endogenous Pβ peptide in SH-SY5Y cells, treated with 10 μM H2O2 and 10 μM MG132. Western blot analysis of mitochondrial and nuclear fractions was done using CVα and ALY antibodies. We observed mitochondrial fraction had some nuclear contamination in both DMSO and stressed cells. Minor mitochondrial contamination of nuclear fraction was also observed in both DMSO and treated cells.

### 3.7 THE ENDOGENOUS Pβ PEPTIDE IS ENRICHED IN THE NUCLEUS AFTER INHIBITION OF ATP SYNTHASE ACTIVITY IN THE SH-SY5Y CELL LINE.

Recently, our group has shown that PARL β cleavage is induced after oligomycin A treatment of the cells (unpublished data). Oligomycin A is an ATP synthase inhibitor. ATP synthase activity is required for pumping the protons from the inter-membrane space in the mitochondria to the matrix to produce energy in the form of ATP [27, 28]. Therefore, ATP levels are diminished after oligomycin A treatment of the cells. To further determine the effect of oligomycin A on the subcellular localization of the endogenous Pβ peptide, SH-SY5Y cells were treated with oligomycin A followed by their mitochondrial and nuclear fractionation. In addition, cells were treated with MG132 to stabilize the peptide. Total, mitochondrial and nuclear fractions were probed for the endogenous Pβ peptide by western blotting using the Pβ specific antibody. The endogenous Pβ peptide is enriched in the nuclear fraction upon oligomycin A treatment compared to the DMSO treated cells (Figure 18). This result suggests increased nuclear accumulation of the Pβ peptide after depletion of the mitochondrial ATP levels.
Figure 18: The endogenous Pβ peptide is enriched in the nucleus after inhibition of the ATP synthase activity in the SH-SY5Y cell line.

Western blot analysis using Pβ peptide specific antibody. SH-SY5Y cells were treated with 20 μM oligomycin followed by their sub-cellular fractionation. CVα and ALY antibodies used for western blot analysis indicated some nuclear contamination of mitochondrial fractions. Although, we observed some mitochondrial contamination in the nuclear fractions of DMSO and MG132 treated cells, however, very little mitochondrial contamination of nuclear fraction was observed in Oligomycin and MG132 treated cells. TF, MF and NF are representative of the total fraction, mitochondrial fraction and the nuclear fraction respectively. Mitochondrial and nuclear fraction obtained were 100% loaded on the gel for analyses.

3.8 THE ENDOGENOUS Pβ PEPTIDE ACCUMULATES IN THE NUCLEUS AFTER INHIBITION OF PDK2 IN THE SH-SY5Y CELL LINE.

Recently, our group has revealed that the pyruvate dehydrogenase kinase isoform 2 (PDK2), a mitochondrial matrix enzyme, phosphorylates PARL and abolishes β-cleavage (unpublished
Interestingly, pyruvate dehydrogenase kinase (PDK) is a master regulator of respiration, and is the target of inhibitor dichloroacetic acid (DCA) [168, 169]. In addition, our group reported induction of the β-cleavage of PARL after DCA treatment of cells (unpublished data). DCA is a PDK inhibitor that activates pyruvate dehydrogenase (PDH), increasing glucose oxidation by promoting an influx of pyruvate into the TCA cycle [169, 170]. In addition, a recent study reported induction of partial mitophagy and fragmentation of the mitochondrial network after DCA treatment of SH-SY5Y cells [172]. This is interesting as it suggests that β-cleavage of PARL is coupled to induction of partial mitophagy and causes a shift of metabolism from glycolysis to oxidative phosphorylation in SH-SY5Y cells. To further analyze how treatment of cells with DCA affects endogenous Pβ peptide production, we performed an IP experiment on the endogenous Pβ peptide in the SH-SY5Y cells. SH-SY5Y cells were treated with DCA and MG132 either individually or in combination. After treatment, the Pβ peptide was immunoprecipitated using the Pβ peptide-specific antibody. IP samples were probed for the endogenous Pβ peptide by western blotting using the same Pβ peptide-specific antibody. We found that the endogenous Pβ peptide is enriched in DCA and MG132 treated cells compared to DCA only, MG132 only or DMSO treated cells (Figure 19A). Next, we investigated the subcellular localization of the endogenous Pβ peptide after treatment of SH-SY5Y cells with both DCA and MG132. The endogenous Pβ peptide was probed in the total, mitochondrial and the nuclear fractions by western blotting using Pβ peptide specific antibody. The endogenous Pβ peptide accumulates in the nucleus with DCA and MG132 treatment compared to DMSO treated cells (Figure 19B). These results demonstrate the enhanced nuclear enrichment of the endogenous Pβ peptide after inhibition of PDK isoform 2.
Figure 19: The endogenous Pβ peptide accumulates in the nucleus after inhibition of PDK2 in the SH-SY5Y cell line.

A. Western blot analysis of the SH-SY5Y cells treated with 0.1 mM DCA and 10 μM MG132. Cells were harvested followed by immunoprecipitation of the Pβ peptide using the Pβ peptide specific antibody followed by its identification using the same antibody.

B. Western blot analysis of the SH-SY5Y cells treated with 0.1 mM DCA and 10 μM MG132. Cells were harvested followed by their mitochondrial and nuclear fractionation. Pβ peptide was probed using the Pβ peptide specific antibody. Western blot analysis using CVα and ALY specific antibodies indicated minor mitochondrial contamination of nuclear fractions, however, mitochondrial fractions were contaminated with nuclear components. TF, MF and NF represent total fraction, mitochondrial fraction and nuclear fraction respectively. We loaded 100% of mitochondrial and nuclear fractions obtained after subcellular fractionation of the cells.
3.9 MUTATING POSITIVELY CHARGED RESIDUES IN THE PUTATIVE NUCLEAR LOCALIZATION SIGNAL OF THE Pβ PEPTIDE PREVENTS ITS TRANSLOCATION TO THE NUCLEUS.

Nuclear proteins harbor monopartite and bipartite nuclear localization signals (NLS) both at the N- and C-termini and their function is often unaffected by their location within a fusion protein [144]. Although the Pβ peptide sequence does not contain a canonical monopartite or bipartite nuclear localization signal, it contains three closely spaced doublets of positively charged amino acids (spanning amino acids 54-65) [144]. Clusters of positive charges are characteristic of an NLS [144]. This led us to speculate that the N-terminus sequence of PARL could function as a nuclear localization signal. We transiently transfected HEK 293T cells with the following constructs, received as a generous gift: Pβ-GFP, Pβ-NLSM-GFP (the two conserved doublets of positively charged amino acids RK were mutated to a pair of small residues GS) and GFP empty vector to analyze their expression in mammalian cells (Fig. 20A). Lysates were probed for GFP using a GFP-specific antibody by western blotting. All the constructs expressed well as compared to the negative control (untransfected cells) (Fig. 20B). We further tested whether the Pβ-GFP construct is nuclear localized. HEK 293T cells were transiently transfected with Pβ-GFP and Pβ-NLSM-GFP separately followed by their analysis using immunofluorescence. We used CVα as a mitochondrial marker and DAPI as a nuclear marker. The nuclear localization of the Pβ-NLSM-GFP is indeed largely abrogated in contrast to the Pβ-GFP construct, which is mostly localized to the nucleus (Figure 20). This result suggests that three closely spaced doublets of positively charged amino acids in the Pβ sequence indeed harbors putative NLS and mutating those residues abolishes nuclear localization of the Pβ peptide.
Figure 20: Mutating positively charged residues in the putative NLS of the Pβ peptide prevent its translocation to nucleus.

A. Schematic of Pβ-GFP and PβNLSM-GFP constructs. The nuclear localization signal is mutated in PβNLSM-GFP construct by changing doublets of two positively charged residues RK (highlighted in blue color) to GS.

B. Western blot analysis of HEK 293T cells overexpressing GFP empty vector, Pβ-GFP and PβNLSM-GFP constructs using the GFP-specific antibody. CVα, a mitochondrial marker, was used as a loading control.

C. Representative immunofluorescence images of the HEK 293T cells demonstrates the co-localization of the nucleus and Pβ-GFP construct in contrast to PβNLSM-GFP construct which is localized mostly in the cytosol.

3.10 Pβ PEPTIDE IS NOT INVOLVED IN THE PINK1/ PARKIN MEDIATED MITOPHAGY PATHWAY.

Mutations in PINK1 or Parkin are the most common cause of recessive familial Parkinsonism. Recent studies suggest that PINK1 and Parkin form a mitochondrial quality control pathway that identifies dysfunctional mitochondria, and promotes their degradation by autophagy. In this pathway the mitochondrial kinase PINK1 senses mitochondrial fidelity and recruits Parkin selectively to mitochondria that loses membrane potential. Parkin, an E3 ligase, subsequently ubiquitinates outer mitochondrial membrane proteins and induces autophagic elimination of the impaired organelles [79, 83, 84]. Previously, a study by our group demonstrated that a mutation at position S77N of PARL, observed only in the patients with Parkinson’s disease (PD), impaired PARL’s function by abolishing β-cleavage. This study shows that PARL plays a key role in the PINK1/Parkin mitophagy pathway as the S77N PARL mutant was not able to restore Parkin mitochondrial recruitment in PARL−/− MEFs during induced mitophagy [135]. These findings suggest that the product of β-cleavage, the Pβ peptide, might play a key role in mitophagy by
promoting Parkin recruitment to the depolarized mitochondria. To further understand role of the Pβ peptide in the PINK1/Parkin pathway of the mitophagy, we analyzed Parkin recruitment to depolarized mitochondria in the mouse embryonic fibroblast (MEF) cell line. To test Parkin recruitment, PARL+/+ MEFs and PARL−/− MEFs cell lines were used. A GFP-Parkin construct was used to analyze recruitment of Parkin to mitochondria upon damage in the MEF cell line. PARL+/+ MEFs were transiently transfected with GFP-Parkin construct and cells were treated with and without CCCP. PARL−/− MEFs were transiently transfected with either PARL-FLAG or PARLΔPβ-FLAG constructs and the GFP-Parkin construct followed by their treatment with CCCP to induce membrane depolarization. Co-localization of both mitochondria and Parkin were analyzed by immunofluorescence microscopy (Figure 21A and 21B). To visualize mitochondria, we probed for the mitochondrial chaperonin Hsp60 protein. Figure 20C and 20D shows Parkin recruitment to mitochondria in the PARL−/− MEFs cell line was significantly reduced compared to the PARL+/+ MEFs. However, PARL−/− MEFs transfected with PARL-Flag and PARLΔPβ-Flag constructs exhibited efficient Parkin recruitment to mitochondria in contrast to untransfected PARL−/−MEFs. Taken together, this result suggests that both PARL and PARLΔPβ were able to rescue Parkin recruitment defects observed in the PARL−/− MEFs. Interestingly, our data indicates that PARL-Flag construct lacking the Pβ peptide was able to recruit Parkin to the depolarized mitochondria in PARL−/− MEFs implying that the Pβ peptide might not play a key role in promoting Parkin recruitment to the dysfunctional mitochondria in the PINK1/Parkin mitophagy pathway. Since the PARLΔPβ-Flag construct is able to restore Parkin recruitment in the PARL−/− MEFs, this result suggests that PARLΔPβ might be responsible for promoting Parkin recruitment to the damaged mitochondria by increasing the stabilization of PINK1 on the outer mitochondrial membrane.
Figure 21: Pβ peptide is not involved in the PINK1/ PARKIN mediated mitophagy.

Both PARL+/+ MEFs and PARL−/− MEFs PARL were transiently transfected with GFP tagged Parkin and with PARL-FLAG or PARLΔPβ-FLAG construct.

A. Representative immunofluorescence images of the PARL +/+ MEFs demonstrates the co-localization of the mitochondria and Parkin upon CCCP induced mitophagy.

B. Representative immunofluorescence images of the PARL −/− MEFs demonstrates that the mitochondria and Parkin do not co-localize even upon CCCP induced mitophagy. Parkin remains diffused in the cytosol in the PARL−/− MEFs indicating defects in the Parkin recruitment.

C. Upper Panel: Representative immunofluorescence images of the PARL−/− MEFs transiently transfected with PARL-FLAG. Overexpression of PARL-FLAG in the PARL−/− MEFs resulted in the recruitment of the Parkin to the mitochondria in most of the cells after CCCP treatment as evident from co-localization of the mitochondria and Parkin.

Lower Panel: Representative immunofluorescence images of the PARL−/− MEFs transiently transfected with PARLΔPβ-Flag. Overexpression of the PARLΔPβ-FLAG in the PARL−/− MEFs was able to rescue Parkin recruitment defects observed in these cells. Co-localization of the Parkin and mitochondria was observed in these cells after induced mitophagy.

D. Quantification of the co-localization of the mitochondria and Parkin was analyzed in 100 DMSO treated cells per coverslip. Error bars represent the standard deviation of three independent experiments.

E. Quantification of the co-localization of the mitochondria and Parkin after induced mitophagy was analyzed in 100 cells per coverslip. Error bars represent the standard deviation of three independent experiments. Localization of Parkin was compared with the mitochondrial marker Hsp60 by immunofluorescence microscopy and categorized into mitochondria localized and cytoplasmic localized.
SUMMARY

Together the data presented here provides the first demonstration that the endogenous Pβ peptide could be identified in the neuroblastoma cell line SH-SY5Y after inhibition of the proteasome or lysosomes. Our data suggest that the endogenous Pβ peptide is very unstable and is degraded by the proteasome and lysosomes. Furthermore, we demonstrated that there is increase in the production of the endogenous Pβ peptide and its accumulation in the nucleus, after induction of mitochondrial stress, strongly suggesting that the endogenous Pβ peptide functions in mitochondrial nuclear cross-talk to orchestrate an appropriate response to mitochondrial damage. Finally, our data indicates enhanced nuclear accumulation of the endogenous Pβ peptide after inhibition of PDK 2. Enhanced nuclear accumulation of the endogenous Pβ peptide after DCA treatment further suggests the possibility of a role for the Pβ peptide in regulating shift in cell metabolism.

Previously, our group has demonstrated that PARL senses the overall health of mitochondria and undergoes β cleavage to orchestrate an appropriate mitophagy response. In addition, our group has characterized the PARL S77N mutation, which disrupts PARL β-cleavage. Furthermore, it has been shown that S77N PARL mutant was not able to restore Parkin mitochondrial recruitment in PARL +/- MEFs during induced mitophagy [138]. We further proposed that the product of PARL β-cleavage, Pβ peptide, might influence Parkin recruitment to mitochondria in the PINK1/Parkin mitophagy pathway. Our results indicate that the Pβ peptide might not be involved in this aspect of the mitophagy pathway as PARLΔPβ-FLAG construct was able to rescue Parkin mitochondrial recruitment defects independent of the Pβ peptide.

We believe this work provides new insight into potential mitochondrial-nuclear signaling mediated by a peptide generated from the auto-catalytic processing of an inner mitochondrial membrane protease.
Mitochondria are highly dynamic organelles and their proper function is crucial for the maintenance of cellular homeostasis. Mitochondrial biogenesis and mitophagy are quality control pathways that regulate mitochondrial content and metabolism preserving homeostasis. The tight regulation between these opposing processes is essential for mitochondrial quality control. PARL has been shown to be an important intrinsic player in mitochondrial quality control, a system substantially impaired in Parkinson’s disease (PD) as indicated by reduced removal of damaged mitochondria in affected patients [157]. The main pathological hallmark of PD is a pronounced loss of dopamine-producing neurons in the substantia nigra pars compacta [64]. The neuroblastoma SH-SY5Y cell line has become a popular cell model for PD research because this cell line possesses many characteristics of dopaminergic neurons. Dopamine producing neurons are highly sensitive to mitochondrial damage because these cells have high oxidative metabolism and they rely solely on the ATP produced from mitochondrial oxidative phosphorylation only [64]. Therefore, even mild mitochondrial damage can trigger degeneration of these neuronal-like cells. Hence, the identification of the endogenous Pβ peptide in the dopamine producing neuronal-like SH-SY5Y cell line, compared to other cell lines that we tested such as HEK or MEFs, was not surprising. Importantly, we were able to detect the endogenous Pβ peptide after cells were treated with the proteasome inhibitor MG132 and/or lysosomal degradation inhibitor bafilomycin A. This result suggests that the endogenous Pβ peptide is highly unstable and degraded by the proteasome and/or lysosomes.

We found that the endogenous Pβ peptide translocates in the nucleus in the neuroblastoma SH-SY5Y cell line. Interestingly, a previous study has reported that peptides of a similar size to the Pβ peptide, generated in the mitochondrial matrix are released from
mitochondria to cytosol in an ATP- and temperature- dependent manner [190]. However to date, there is no evidence supporting the accumulation of a mitochondrial-derived peptide to the nucleus in mammals. Therefore, this is the first time that a peptide generated in the mitochondrial matrix has been shown to translocate to the nucleus. Previous studies have reported generation of peptides in the mitochondrial matrix after the degradation of unfolded proteins in the mitochondrial matrix, also termed the mtUPR [191, 192]. However, this is the first time where the peptide generated from autocatalytic processing of a mitochondrial inner membrane protease has been demonstrated to accumulate in the nucleus. Our findings are consistent with a recent study demonstrating the nuclear accumulation of the overexpressed GFP tagged Pβ peptide [43]. However, the reason that prompted increase in the Pβ peptide production and its enhanced nuclear accumulation remained elusive.

Our work presented here determined the trigger for increase in the endogenous Pβ peptide production and its enhanced nuclear enrichment. It was found that the Pβ peptide production and its accumulation in the nucleus increases after induction of mitochondrial stress. Mitochondrial stress induced by mitochondrial depolarization or oxidative stress resulted in the overall reduction of mitochondrial ATP levels. Our findings are consistent with a recent work done by our group which demonstrates that β-cleavage of PARL is induced after generation of mitochondrial stress. Therefore, our results reinforce the importance of the PARL’s β-cleavage in inducing a mitophagy response by promoting Parkin recruitment. Mitochondrial and nuclear fractionation result of untreated cells indicate the basal level of the endogenous Pβ peptide production and its translocation to the nucleus. Interestingly, we observed increased nuclear accumulation of the endogenous Pβ peptide even after inhibition of the proteasome only, without direct induction of the mitochondrial stress. However, previous studies have reported that proteasome inhibition causes damage to mitochondria, leading to increase in ROS production
and resulting in enhanced cytosolic oxidative stress [193,195]. These findings indicate that there is increase in the nuclear enrichment of the endogenous Pβ peptide only after cells are treated with chemicals that perturb mitochondrial health.

In addition to role of the Pβ peptide in responding to low levels of ATP by its enhanced enrichment in the nucleus, this study reveals that the Pβ peptide also responds to a shift in oxidative metabolism from glycolysis to mitochondrial oxidative phosphorylation. It was found that the endogenous Pβ peptide is enriched in dichloroacetate (DCA) treated SH-SY5Y cells after immunoprecipitation of peptide using the Pβ peptide specific antibody. Furthermore, mitochondrial and nuclear fractionation of the DCA treated cells revealed increased production of the endogenous Pβ peptide and its enhanced nuclear accumulation. Studies have shown that DCA is inhibitor of pyruvate dehydrogenase kinase (PDK), a master regulator of the cell metabolism [168, 169]. PDK inhibition by DCA leads to activation of the pyruvate dehydrogenase (PDH) that shifts pyruvate metabolism from glycolysis to oxidative phosphorylation [168, 170]. In addition, a recent study done by our group has shown that β-cleavage of PARL is induced after DCA treatment of the cells (unpublished work). Our results are consistent with the recent work demonstrating that induction of β-cleavage of PARL after DCA treatment of the cells result in enhanced enrichment of the Pβ peptide to the nucleus. Interestingly, a recent study reported that DCA treatment of SH-SY5Y cells affected mitochondrial network as these neuronal-like cells exhibited reorganization of the mitochondrial network leading to shorter and more fragmented mitochondrial filaments, and led to induction of partial mitophagy [172]. Therefore, mitochondrial damage, evident from the fragmented mitochondrial network, caused by DCA treatment leads to induction of partial mitophagy and causes a shift in cell metabolism in the SH-SY5Y cells. This suggests that DCA affects the mitochondrial morphology and induce partial mitophagy causing enhanced nuclear accumulation.
of the Pβ peptide which further leads to changes in cellular energy metabolism. Therefore, the increase in the nuclear enrichment of the Pβ peptide after DCA treatment could be due to induction of partial mitophagy, which is indication of mitochondrial damage.

Function of the Pβ peptide: Mitochondrial or Nuclear?

In order to determine whether the Pβ peptide plays a role in the PINK1/Parkin mitophagy pathway, Parkin recruitment to the damaged mitochondria was assessed by immunofluorescence. Using MEFs derived from PARL knockout mice, we found that the PARLΔPβ-FLAG construct was able to rescue Parkin mitochondrial recruitment defects observed in the PARL-/- MEFs, independent of the Pβ peptide. This observation seems to indicate a mechanism whereby β-PARL might be promoting Parkin recruitment to the damaged mitochondria during induced mitophagy. One explanation for the observed phenomenon is that β-PARL might be required to support PINK1 stabilization on the outer mitochondrial membrane. The stabilization of PINK1 on the outer mitochondrial membrane further phosphorylates ubiquitin and activates Parkin for its translocation to the depolarized mitochondria during induced mitophagy [130, 131]. This result indicates that the Pβ peptide might not be playing a role in promoting the Parkin recruitment to the depolarized mitochondria during the induced mitophagy.

As mitochondrial damage results in dysfunctional mitochondria that are efficiently removed by the PINK1/Parkin mitophagy pathway, this leads to a depletion of the mitochondrial pool in the cell. This loss of mitochondria is remunerated by the process of mitochondrial biogenesis in order to ensure that a healthy mitochondrial population is maintained in the cell. Our work demonstrates that the endogenous Pβ peptide goes to the nucleus after induction of mitochondrial stress. Additionally, we show that the Pβ peptide might not be playing role in promoting Parkin recruitment during induced PINK1/Parkin mitophagy. Therefore, it is possible
that the Pβ peptide might influence mitochondrial biogenesis which is a key aspect of mitochondrial quality control. During stress, such as accumulation of ROS and the depolarization of mitochondrial membrane potential, mitochondrial ATP levels drop significantly. Therefore, there is a possibility that the Pβ peptide, once accumulates in the nucleus, might up-regulate the transcription of genes encoding enzymes of the tricarboxylic acid (TCA) cycle and/or subunits of the electron transport chain (ETC) to restore cellular ATP levels. Future studies on this project should focus on elucidating role of the Pβ peptide in mitochondrial biogenesis by analyzing upregulation of transcription of the genes encoding TCA cycle enzymes and subunits of the ETC.

Our work also shows enhanced nuclear enrichment of the endogenous Pβ peptide after a metabolic switch from glycolysis to oxidative phosphorylation. The nuclear accumulation of the Pβ peptide after DCA treatment of cells suggests that the Pβ peptide might play a key role in the metabolism shift by activating signaling pathways required to compensate for the enhanced bioenergetic state of the cell. In addition, DCA treatment also induces partial mitophagy which indicates that the Pβ peptide might also play role in enhancing mitochondrial biogenesis to maintain the healthy mitochondrial pool in the cell. Pβ peptide might activate signaling pathways by up-regulating transcription of the genes encoding enzymes of the TCA cycle, components of the ETC, or mitochondrial biogenesis genes such as PGC1α, SIRT1.

Together, these findings suggest a model whereby upon mitochondrial damage there is significant reduction in mitochondrial ATP levels which causes dephosphorylation of the PARL N-terminus. This results in induction of β-cleavage of the PARL and generation of the Pβ peptide. The Pβ peptide generated in the mitochondrial matrix accumulates in the nucleus (Figure 22B). In the nucleus, Pβ peptide might upregulate the expression of genes responsible for
mitochondrial biogenesis. However, in a healthy mitochondrion, phosphorylation of the PARL N-terminus is promoted by the normal mitochondrial ATP levels which impedes the β-cleavage of PARL. Therefore, there is a basal level of mitochondrial biogenesis when generation of the Pβ peptide is inhibited (Figure 22A). This strongly indicates that PARL is not simply a protease, but that it also has other important regulatory functions such as the proposed mitochondrial–nuclear communication mediated by the Pβ peptide. In support of this, a recent study demonstrated that the Pβ peptide robustly increased the mRNA expression of PARL and genes involved in mitochondrial biogenesis (PGC1β and NRF1) and mitochondrial fusion (MFN-1 and -2) and OPA1 protein expression [43]. These findings indicate that the Pβ peptide might play a crucial role in regulating the transcription factor(s) to alter the expression of mitochondrial biogenesis genes. However, these studies were done using the GFP-tagged Pβ peptide. As the GFP tag is a huge tag for a small peptide, future work on the Pβ peptide should validate these findings by using an untagged version of the Pβ peptide or by using the PARL-FLAG, PARLΔPβ-FLAG constructs.

We believe that our work will add a new dimension to the mitochondrial-nuclear signaling pathways as this is the first time that a protease residing in the inner mitochondrial membrane has been shown to respond to mitochondrial damage by undergoing autocatalytic processing and releasing the Pβ peptide. More importantly, our work raises the exciting possibility that transcription of mitochondrial biogenesis genes might be influenced by the Pβ peptide, possibly addressing debate on the role of the Pβ peptide in mitochondrial quality control.
Figure 22: Proposed model of the Pβ peptide in mitochondrial-nuclear signaling

A. In a healthy mitochondrion, normal mitochondrial ATP levels facilitate the phosphorylation of the PARL N-terminus. The phosphorylation of the PARL inhibits the self-regulated proteolysis (β-cleavage) that releases the PARL N-terminal domain termed the Pβ peptide. There is a basal level of mitochondrial biogenesis in the absence of the Pβ peptide.

B. During stress, such as accumulation of ROS and the depolarization of mitochondrial membrane potential, mitochondrial ATP levels drop significantly as a result of an inhibition of the electron transport chain. Reduced ATP levels result in the dephosphorylation of the PARL N-terminus, which leads to β-cleavage. β-cleavage generates the Pβ peptide, which translocates to the nucleus and activates expression of several genes involved in cell metabolism and mitochondrial biogenesis by upregulating transcription of PGC1α, master regulator of mitochondrial biogenesis.
FUTURE DIRECTIONS

The work presented here reveals a new aspect of mitochondrial-nuclear cross talk mediated by the Pβ peptide generated in the mitochondrial matrix. This study reports new data about what additional role PARL might have in mitochondrial quality control in addition to its role as an inner mitochondrial membrane protease. Our work has identified the endogenous Pβ peptide in the neuronal-like SH-SY5Y cell line and demonstrated that it goes to the nucleus with induction of mitochondrial stress. However, it is still unclear how Pβ peptide functions and mediates mitochondrial-nuclear communication.

Therefore, further studies on the Pβ peptide are definitely needed to address the following major questions: (1) does the Pβ peptide induce up-regulation of mitochondrial biogenesis genes, (2) does the Pβ peptide bind to DNA in the nucleus, (3) how does the Pβ peptide translocate from the mitochondria to the nucleus.

1. Does the Pβ peptide induce up-regulation of mitochondrial biogenesis genes? In this study, we have shown that the endogenous Pβ peptide production and its accumulation in the nucleus increases after induction of mitochondrial stress. Additionally, our experiments suggest that the endogenous Pβ peptide might not play a role in Parkin recruitment to damaged mitochondria in the induced PINK1/Parkin mitophagy pathway. Therefore, it is important to determine whether the endogenous Pβ peptide plays a role in up-regulating the transcription of genes required for mitochondrial biogenesis. A previous study has reported a robust increase in the mRNA expression of PARL and genes involved in mitochondrial biogenesis (PGC1β and NRF1) and mitochondrial fusion (MFN-1 and MFN-2; and OPA1) after treating the mammalian cells with synthetic Pβ peptide [43]. Importantly, SIRT1 protein expression was increased by the Pβ
peptide in conjunction with elevated mitochondrial biogenesis which suggested a potential Pβ-SIRT1 regulation of mitochondrial mass [43]. As mitochondrial stress results in dysfunctional mitochondria that are efficiently removed by the process of mitophagy, there is a requirement for increased mitochondrial biogenesis to compensate for loss of the total healthy mitochondrial pool in the cell. Studies have shown that PGC-1α, a cold-inducible coactivator of nuclear receptors, stimulates mitochondrial biogenesis and respiration through regulation of the NRFs. PGC-1α stimulates powerful induction of NRF-1 and NRF-2 gene expression [98, 99]. NRF1 encodes a protein that functions as a co-activator of the transcription factor which activates expression of some key metabolic genes regulating cellular growth and nuclear genes required for respiration, mitochondrial DNA transcription and replication [98, 99]. Both NRF-1 and NRF-2 have been shown to activate the transcription of a large number of genes involved in respiratory chain function such as β-ATP synthetase, COX IV, and cytochrome c [98, 99]. Therefore, future studies should focus on analyzing the mRNA transcripts of genes involved in mitochondrial biogenesis using quantitative real-time PCR (qPCR). Quantitative real-time PCR (qPCR) has become the standard for precise measurement of mRNA transcripts in cell culture experiments as it allows for measurements of mRNA transcript copy numbers. For this experiment, we will first do an unbiased screen for the candidate genes that might be up-regulated by the Pβ peptide. We will do a RNA microarray using PARL/- MEFs. PARL-FLAG and PARLΔPβ-FLAG constructs will be transfected separately to see which mRNA transcripts are induced. We will use vector only as a control with induction of mitochondrial stress as well. This experiment will be performed both with and without induction of mitochondrial stress. We expect an increase in the number of mRNA transcripts for genes involved in mitochondrial biogenesis, such as PGC-1α, NRF1, 2 and SIRT1, in cells transfected with PARL-FLAG compared to PARLΔPβ-FLAG construct after induction of mitochondrial stress. In addition, promising lead candidates obtained
from above unbiased screen will be analyzed further to determine what signaling pathways Pβ peptide might be involved in.

2. Does the Pβ peptide bind to transcription factors in the nucleus? In order to study the link between mitochondrial stress and mitochondrial-nuclear cross talk mediated by the Pβ peptide, it would be important to determine how the Pβ peptide functions. Small peptides can have potent biological activity, such as hormonal responses and apoptosis [196]. For instance, humanin is a recently discovered peptide of 24 amino acids that binds the apoptosis-inducing Bax protein and prevents Bax from associating with mitochondria, therefore preventing apoptosis [197]. It seems likely that Pβ peptide might function through a specific interaction(s) with a nuclear factor(s).

Our work here shows that the endogenous Pβ peptide production and its accumulation in the nucleus increases after generation of mitochondrial stress, which could contribute to regulation of nuclear activities, e.g. by interacting with a transcriptional co-activator or transcription factor(s). Therefore, it will be interesting to determine binding partners of the Pβ peptide within the nucleus to elucidate its function. We will first do an unbiased search to screen for the transcription factors that Pβ peptide might be interacting with. For this experiment, we will immobilize the Pβ peptide with appropriate linker on beads and perform a pull down of nuclear extract and use liquid chromatography coupled mass spectrometry (LC-MS)/ MS to see what binds to the Pβ peptide. We will further use Bio-Layer Interferometry (BLI), a label-free technology for measuring biomolecular interactions, to confirm interaction of the Pβ peptide with the hits obtained from the unbiased screen. BLI is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer [198]. Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real-time. The binding between a ligand immobilized on the biosensor tip
surface and an analyte in solution produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift, $\Delta \lambda$, which is a direct measure of the change in thickness of the biological layer [198]. For this experiment, purified Pβ peptide will be immobilized on the surface of a biosensor tip followed by interaction to transcription factor hits obtained from the unbiased screen. After the attachment, the thickness of the layer on the surface will be measured in terms of the wavelength shift ($\Delta \lambda$). We would expect an increase in the thickness of the layer on the surface, detected by a positive shift in the wavelength, indicating the association of the Pβ peptide with the transcription factor.

3. How does the Pβ peptide translocate from the mitochondria to the nucleus? Previous studies have implicated the mitochondria-localized ATP-binding cassette (ABC) transporter in signalling the stress of unfolded/misfolded proteins (mitochondrial unfolded protein response, also called UPRmt) in the mitochondrial matrix to the nucleus. These studies have established a correlation between mitochondrial unfolded protein stress and mitochondrial transporter dependent efflux of peptides derived from proteolysis of matrix proteins [186-188]. In order to elucidate the mechanism of mitochondrial-nuclear signaling mediated by the Pβ peptide, it will be important to investigate if the mitochondrial localized ABC transporter is implicated in signaling the mitochondrial stress to the nucleus. Previous studies on yeast have reported ATP-binding cassette (ABC) adenosine triphosphatase protein Mdl1 as an intracellular peptide transporter localized in the inner membrane of mitochondria [192]. It has also been shown that Mdl1 is required for export of peptides generated by proteolysis of inner-membrane proteins by the m-AAA protease in the mitochondrial matrix [192]. Thus, pathways of peptide efflux from mitochondria exist that may allow communication between mitochondria and their cellular environment. Another study done in C.elegans reports that the ABC transporter protein HAF-1, localized in the inner mitochondrial membrane, functions in pumping peptides from the matrix to
the inter membrane space and plays a role in mitochondrial peptide efflux similar to Mdl1 [192, 200]. Even though the source of the Pβ peptide generated in the mitochondrial matrix in mammalian system is not same as peptides generated in the yeast, they are still produced in the mitochondrial matrix. Therefore, based on the studies done in yeast, we propose that the Pβ peptide might also be translocated by ABC transporter proteins localized in the inner mitochondrial membrane in mammalian cells. Since ABC10 is the closest mammalian ortholog to yeast Mdl1 [199, 200], future studies should focus on studying ABCB10 as a potential transporter protein required for the Pβ peptide translocation from the mitochondrial matrix. For this experiment, mutations will be introduced in the mammalian transporter protein ABC10 gene to inactivate the gene or clustered regularly interspaced short palindromic repeats (CRISPRs /Cas) system enabled targeted gene inactivation approach will be used to inactivate the ABC10 gene in mammalian cells. Then, the subcellular localization of either endogenous or overexpressed Pβ peptide should be examined by mitochondrial and nuclear fractionation of the mammalian cells expressing the mutant ABC10 gene. Upon induction of mitochondrial stress, we expect enrichment of the Pβ peptide in the mitochondrial fraction compared to the nuclear fraction in the cells expressing mutant ABC10 gene.

We believe that our work shown here will open up new avenues of mitochondrial – nuclear cross talk and add new insight to the signaling network of bioactive peptides. It is possible that the Pβ peptide might play a key role in mitochondrial quality control as it might regulate a stress response pathway by orchestrating an appropriate response to rebalance the healthy mitochondrial population in the cell.
REFERENCES


86. Lemasters, J. J. et al. (2005) Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. Rejuvenation Res. 8, 3–5.


