Characterization of the Mitochondrial Peptide Pβ

By

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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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ABSTRACT

Mitophagy, the degradation of damaged mitochondria, and mitochondrial biogenesis, the formation of new mitochondria, are two critical mitochondrial quality control pathways that maintain a healthy mitochondrial network and thus a healthy cell. We propose that the mitochondrial protease presenilins-associated rhomboid-like (PARL) coordinates these two processes. PARL undergoes regulated cleavage, termed β cleavage, in response to mitochondrial stress to produce N-terminally cleaved β PARL and a 25 amino acid peptide, Pβ. We have recently demonstrated that β PARL promotes mitochondrial fragmentation and has reduced proteolytic activity for a key component of PINK1/Parkin-mediated mitophagy, PINK1, which may instigate mitophagy. In contrast, Pβ’s putative role and mechanism of action in mitochondrial biogenesis remains poorly defined. In this thesis, I determined that the mitochondrial Pβ peptide translocates to the nucleus where it associates with chromatin. Together, these results further support PARL as a key coordinator of mitophagy and mitochondrial biogenesis.
ACKNOWLEDGEMENTS

I entered research only thinking about studying mitochondria. It doesn’t escape me how lucky I am to have gotten to study the organelle that I love in such a supportive and academically rigorous environment. The largest stroke of luck was meeting my supervisor, Dr. Angus McQuibban, who took a chance on me, both as an undergraduate and graduate student. He has been equal parts optimistic and analytical, and I’ve learned a lot from him, both as a researcher and as a person. I am incredibly grateful for his patience, time, guidance, honesty, and support.

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<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔΨ&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>Aβ42</td>
<td>Amyloid β 42</td>
</tr>
<tr>
<td>ABCB8/10</td>
<td>ATP-binding cassette (ABC) B8/B10</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate (AMP) kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>Cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>Ccp1</td>
<td>Cytochrome c peroxidase 1</td>
</tr>
<tr>
<td>CICD</td>
<td>Caspase-independent cell death</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRP1</td>
<td>Dynamin-related protein 1</td>
</tr>
<tr>
<td>Dyn2</td>
<td>Dynamin 2</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FADH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Flavin adenine dinucleotide (hydroquinone form)</td>
</tr>
<tr>
<td>Fis1</td>
<td>Mitochondrial fission 1 protein</td>
</tr>
<tr>
<td>FOXO3</td>
<td>Forkhead box O3</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HtrA2</td>
<td>High-temperature requirement protein A2</td>
</tr>
</tbody>
</table>
IMM  Inner mitochondrial membrane
IMS  Intermembrane space
IP   Immunoprecipitation
MDL1 Multi-drug resistance-like 1
MiD49/51 Mitochondrial dynamics protein of 49/51 kDa
Mff  Mitochondrial fission factor
Mgm1 Mitochondrial genome maintenance 1
MFN1/2 Mitofusin 1/2
MPP  Mitochondrial processing peptidase
MPP+ 1-methyl-4-phenylpyridinium
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTS  Mitochondrial targeting sequence
NADH Nicotinamide adenine dinucleotide (reduced)
NLS  Nuclear localization sequence
NRF1/2 Nuclear respiratory factor 1/2
OIA  Oligomycin A
OMM  Outer mitochondrial membrane
OPA1 Dominant optic atrophy 1
OXPHOS Oxidative phosphorylation
Pβ   PARL-β
PARL  Presenilins-associated rhomboid-like
PD   Parkinson’s disease
PDK2 Pyruvate dehydrogenase kinase 2
PDP1/2 Pyruvate dehydrogenase phosphatase 1/2
PGAM5 Phosphoglycerate mutase 5
PGC-1α/β Proliferator-activated receptor γ (PPARγ) coactivator-1 (PGC-1) α/β
PINK1 Phosphatase and tensin homolog (PTEN)-induced putative kinase 1
PKA  Protein kinase A
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC</td>
<td>PGC-1-related coactivator</td>
</tr>
<tr>
<td>Rbd1</td>
<td>Rhomboid-1</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Silent information regulator 2 (SIR2) protein 1</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of the inner membrane</td>
</tr>
<tr>
<td>TFAM</td>
<td>Transcription factor A, mitochondrial</td>
</tr>
<tr>
<td>TFEB</td>
<td>Transcription factor EB</td>
</tr>
<tr>
<td>TFB1M/TFB2M</td>
<td>Transcription factor B1/2, mitochondrial</td>
</tr>
<tr>
<td>TMH</td>
<td>Transmembrane helix</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the outer membrane</td>
</tr>
<tr>
<td>UBL</td>
<td>Ubiquitin-like</td>
</tr>
<tr>
<td>ULK1</td>
<td>Unc-51-like autophagy activating kinase 1</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
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</table>
CHAPTER 1
INTRODUCTION

1.1 Mitochondrial Biology

The innocuously named mitochondria (coined by German anatomist Carl Benda in 1898; from the Greek mitos, “thread” and chondrion, “granule”) are central to complex eukaryotic life as we know it\(^1\). Proper understanding of this vital organelle requires understanding of its origin: mitochondria are derived from a bacterium of α proteobacterial ancestry that was engulfed by a host cell of archaeal origin\(^2\)–\(^4\). This ancient endosymbiotic event occurred roughly 1.6 billion years ago and gave rise to all known eukaryotes\(^5\)–\(^8\). The endosymbiotic origin of mitochondria is evidenced most obviously by their unique retention of genetic information and double membrane composition.

1.1.1 The Mitochondrial Genome

As semi-autonomous organelles, mitochondria possess their own genomes\(^9\)–\(^11\). Owing to their bacterial origin, mitochondrial DNA (mtDNA) in almost all multicellular organisms is circular\(^12\). Human mtDNA is a 16 569 bp circular DNA molecule that tightly sequences 37 genes: two rRNAs, 22 tRNAs, and 13 polypeptides coding for core hydrophobic subunits of enzyme complexes in the oxidative phosphorylation (OXPHOS) system\(^13\). OXPHOS is made up of the electron transport chain (ETC) (also known as the
respiratory chain; composed of Complexes I-IV) and ATP synthase (also known as Complex V)\textsuperscript{11,13}. The vast majority of mitochondrial proteins, including the machinery required to transcribe and translate mtDNA, is encoded by the nuclear genome and imported into mitochondria\textsuperscript{9–11}.

1.1.2 Mitochondrial Organization

The double membrane structure of the mitochondrion was first visualized by Palade and Sjöstrand independently through several high-resolution electron micrographs, which were published in 1952 and 1953\textsuperscript{14–17}. These images revealed two membranes dividing mitochondria into four distinct compartments: the outer mitochondrial

Figure 1-1: Overview of mitochondrial structure and morphology: A Transmission electron micrograph of a mitochondrion. Mitochondria are composed of an outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM) which forms cristae, and mitochondrial matrix. Adapted with permission\textsuperscript{4}. B Representative confocal immunofluorescence microscopy images of mitochondrial morphology in mouse embryonic fibroblasts (MEFs), visualized by TOM20 immunostaining. Nuclei were stained with DAPI. When treated with vehicle control, DMSO, elongated mitochondrial morphology is maintained. When treated with 100ng/mL rotenone for 1 hour, mitochondrial morphology becomes fragmented. Reprinted with permission\textsuperscript{50}.

1.1.2 Mitochondrial Organization

The double membrane structure of the mitochondrion was first visualized by Palade and Sjöstrand independently through several high-resolution electron micrographs, which were published in 1952 and 1953\textsuperscript{14–17}. These images revealed two membranes dividing mitochondria into four distinct compartments: the outer mitochondrial
membrane (OMM); the intermembrane space (IMS), which is contained between the two membranes; the inner mitochondrial membrane (IMM), which is folded in on itself to form many ridges termed cristae; and the mitochondrial matrix, which is enclosed by the IMM (Fig. 1-1A)\textsuperscript{14–17}.

However, these electron micrographs did not immediately expose the degree of specialization of the two mitochondrial membranes. In the human liver, an estimated 6% of total mitochondrial protein is located in the OMM\textsuperscript{18}. Of this 6%, many proteins embedded in the OMM are porins (transport proteins that form large aqueous channels) and voltage–dependent anion channels (VDAC)\textsuperscript{18,19}. As a consequence, ions and small uncharged molecules less than 5 kilodaltons (kDa) freely pass through the OMM\textsuperscript{18,19} The majority of mitochondrial proteins larger than 5 kDa contain an N-terminal mitochondrial targeting sequence (MTS) that is comprised of an amphipathic α helix with a +3 to +6 net charge\textsuperscript{20}. The MTS is recognized by the translocase of the outer membrane (TOM), which is the general transporter protein complex in the OMM\textsuperscript{21}. Due to the porous nature of the OMM, the IMS is chemically similar to the cytosol at a pH of approximately 7\textsuperscript{19}.

At first glance, the most prominent feature of the IMM is its convolutedness as it forms many cristae which project into the mitochondrial matrix\textsuperscript{15,19}. These convolutions are so abundant that in the human liver, the IMM constitutes approximately one third of total cell membrane\textsuperscript{18}.

The other defining feature of the IMM is its extreme impermeability\textsuperscript{19,20}. Unlike the OMM, ions and other molecules require specific membrane-spanning transport complexes to enter or exit the IMM\textsuperscript{18,21}. In the human liver, these transport complexes contribute to the 21% of total mitochondrial proteins found in the IMM\textsuperscript{18,21}. In particular,
the high proportion of cardiolipin (up to 20% of the IMM), a phospholipid that is characterized by four fatty acid chains rather than the conventional two, is a key contributor to the IMM’s impermeability. As a result, an electrochemical mitochondrial membrane potential (ΔΨₘ) of approximately 180 mV is generated across the IMM by many copies of the IMM-embedded ETC chain. Consequently, the pH of the mitochondrial matrix is approximately 7.9. In addition to being the driving force of ATP production for aerobically respiring cells, maintenance of ΔΨₘ also determines the import of many proteins into or across the IMM, including the OXPHOS machinery itself. A leading hypothesis is that ΔΨₘ electrophoretically positions the positively charged MTS to initiate import across or into the IMM through the translocase of the inner mitochondrial membrane (TIM).

**1.2 Mitochondrial Function**

Often referred to as “the Powerhouse of the Cell”, mitochondria are indeed critical to energy production, as well as many other key cellular processes including metabolism of fatty acids and amino acids, calcium signalling, heat production, various signalling pathways, and cell death. For the sake of brevity, only two of the mitochondrion’s many important functions, energy production and cell death, are highlighted below.

**1.2.1 Energy Production**

As the generators of more than 90% of our cellular energy, the most prominent responsibility of mitochondria is to produce energy in the currency of ATP by aerobic respiration. Ideally, pyruvate, the byproduct of glycolysis, is metabolized through the tricarboxylic acid (TCA) cycle (also known as the Krebs cycle or the citric acid cycle) in
the mitochondrial matrix\textsuperscript{24}. Although not as preferred, fatty acids and amino acids may also feed into the TCA cycle\textsuperscript{24}. Electron-rich reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (hydroquinone form) (FADH\textsubscript{2}) that are produced by the TCA cycle and glycolysis transfer electrons to the ETC\textsuperscript{18}. Energy released by electrons passed down the ETC by a series of redox reactions is used to pump H\textsuperscript{+} ions across the IMM to the IMS to generate and maintain the electrochemical gradient, $\Delta \Psi_m$\textsuperscript{26,27}. H\textsuperscript{+} ions are then channeled back into the matrix by ATP synthase, which uses the electromotive force to drive phosphorylation of adenosine diphosphate (ADP) to ATP\textsuperscript{19}.

1.2.2 Cell Death

Mitochondria are less commonly referred to as the judge and executioner of the cell\textsuperscript{28,29}. In mammals, the best characterized mitochondria-centric cell death pathway is intrinsic apoptosis, which is occasionally termed mitochondrial apoptosis\textsuperscript{29,30}. Intrinsic apoptosis is a controlled cell death pathway that activates in response to non-receptor-mediated stimuli such as toxins, viral infections, and free radicals\textsuperscript{31}. As the convergence point for many apoptosis-inducing cues, mitochondria regulate apoptosis through mitochondrial outer membrane permeabilization (MOMP), which is mediated by oligomerization and pore formation by the proapoptotic proteins Bax and Bak\textsuperscript{32,33}. MOMP is considered the point of no return as it commits the cell to death\textsuperscript{30}. This event releases IMS proteins that promote apoptosis\textsuperscript{29}. The most important of these is cytochrome c, which activates the caspase protease cascade in the cytosol, which ultimately leads to cell death\textsuperscript{32}. In the absence of caspase activity, MOMP still results in cell death through an ill-defined mechanism termed caspase-independent cell death (CICD)\textsuperscript{34}. 

5
1.3 Mitochondrial Dysfunction in Parkinson’s Disease

The importance of mitochondrial function in the eukaryotic cell is emphasized by the commonality of mitochondrial dysfunction in various diseases\(^3\). Briefly, a few lines of evidence implicating mitochondrial dysfunction in Parkinson’s disease (PD) etiology are emphasized below.

PD is the most common neurodegenerative movement disorder; it affects a growing estimate of 10 million people worldwide\(^{35,36}\). PD is characterized by progressive deterioration of dopaminergic neurons in the *substantia nigra*, which contributes to cardinal PD motor impairments\(^{36}\). Neurons are particularly sensitive to mitochondrial dysfunction due to their high energetic demand, their need for high calcium buffering capacity due to action potential-driven calcium influxes, and because their predominant mode of energy production is by OXPHOS\(^{37}\). Dopaminergic neurons in the *substantia nigra* are especially sensitive to mitochondrial stress, although the exact reasons remain to be determined\(^{38}\).

The first causative association of mitochondrial dysfunction in PD pathogenesis occurred when accidental infusions of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced selective dopaminergic neurodegeneration and resultant rapid onset of PD-like symptoms\(^{39,40}\). Although little evidence suggests that MPTP itself is toxic, researchers later found that in glial cells, MPTP could be oxidized to produce 1-methyl-4-phenylpyridinium (MPP\(^+\)), which selectively inhibits Complex I of the ETC\(^{39,40}\). Other Complex I inhibitors such as rotenone, pyridaben, trichloroethylene, and fenpyroximate also cause dopaminergic neurodegeneration in various animal models\(^{41}\).
Consistent with this, Complex I activity is impaired in the substantia nigra of PD patients, likely due in part to oxidative stress\textsuperscript{41}. Notably, dopamine is a relatively unstable neurotransmitter that can oxidize to generate dopamine quinone and reactive oxygen species (ROS) which may in turn impair Complexes I and III of the ETC\textsuperscript{42}. Aside from obvious perturbations in the ETC and consequent energy production, Complex I impairment also increases superoxide formation\textsuperscript{42}. In line with this, postmortem brain samples from PD patients have shown evidence of oxidative damage\textsuperscript{43}.

Mutations in numerous genes that cause mitochondrial dysfunction also cause familial forms of PD. PD-linked mutations in Parkin, PTEN-induced kinase 1 (PINK1), α-synuclein, DJ-1, UCHL-1, LRRK2, NURR1, VPS35, and HtrA2 are all directly or indirectly linked to abnormal mitochondrial function\textsuperscript{41}. Some of these mutations are suggested to affect core mitochondrial functions such as mitochondrial import or the ETC; still others disrupt critical mitochondrial quality control pathways that protect the mitochondrial network, and thus the cell, from damage\textsuperscript{41}.

1.4 Mitochondrial Quality Control

The severe consequences of impaired mitochondrial function have driven the evolution of several quality control mechanisms to maintain mitochondrial homeostasis. These quality control systems vary dramatically in scale. For example, the mitochondrion’s own proteolytic system degrades misfolded and oxidatively denatured proteins within the mitochondrion\textsuperscript{44}. Conversely, oxidized, damaged proteins may also be enriched in mitochondrial-derived vesicles (MDVs) that bud off mitochondria and are ultimately degraded by the lysosome\textsuperscript{45}. Lastly, whole, severely damaged mitochondria
may be degraded by a specialized autophagic pathway termed mitophagy. Highlighted below are a few mitochondrial quality control pathways that affect the entire mitochondrion.

### 1.4.1 Mitochondrial Dynamics

Between 1914 and 1915, Lewis and Lewis first described a key characteristic of mitochondria: their dynamic network. Of note, mitochondrial fission and fusion are constant, regulated processes that are critical components of multiple mitochondrial and cellular functions, including other mitochondrial quality control pathways.

Mitochondrial fusion is suggested to be a first line of defense by diluting mitochondrial damage across the mitochondrial network. Conversely, mitochondrial fission resulting in fragmentation is associated with dissipation of \( \Delta \Psi_m \); it separates severely damaged mitochondria to be subsequently degraded (Fig. 1B). Fission facilitates and, according to multiple studies, is required for mitophagy.

In mammals, mitochondria fusion is mediated by dynamin-like GTPases: membrane-bound mitofusin 1 (MFN1) and MFN2 for the OMM; and various isoforms of optic atrophy 1 (OPA1) for the IMM. In response to mitochondrial depolarization, long isoforms of OPA1 are cleaved by the inducible protease OMA1 to inhibit fusion and thus encourage mitochondrial fragmentation. Additionally, initiation of mitophagy results in MFN1/2 ubiquitination and proteasomal degradation, which further fragments damaged mitochondria for eventual degradation.

Mammalian mitochondrial fission is also driven by a pair of cytosolic dynamin-like GTPases: Dynamin-related protein 1 (Drp1) and Dynamin 2 (Dyn2). Sites of mitochondrial fission are initially marked by contact with the endoplasmic reticulum,
followed by recruitment of Drp1 by mitochondrial OMM receptor proteins: fission protein 1 (Fis1); mitochondrial fission factor (Mff); mitochondrial dynamics protein of 49 kDa (MiD49); and MiD51. Drp1 assembles on the OMM into a helical ring-like structure that constricts the mitochondrion to a diameter of approximately 100 nm. Dyn2 subsequently completes mitochondrial constriction until fission occurs. In addition to fusion machinery degradation, mitophagy initiation also results in displacement of protein kinase A (PKA), which inhibits Drp1 by phosphorylation, from mitochondria. Drp1 and fission machinery is also implicated in other processes, including apoptosis.

1.4.2 PINK1/Parkin-mediated Mitophagy

First reported and named in 2005 by Lemasters, mitophagy, which results in whole degradation of mitochondria, is considered to be the last line of defense in mitochondrial quality control. Mitophagy is a mitochondria-specific subcategory of macroautophagy, which is characterized by the engulfment of cargo by a double-membraned vesicle, the autophagosome, which then fuses with the lysosome for degradation. Since its fairly recent discovery, mitophagy has burgeoned as a research field: multiple mitophagy mechanisms have been identified and their pathophysiological roles in development and disease have been heavily scrutinized. The best studied mitophagy pathway is PINK1/Parkin-mediated mitophagy, which is triggered by mitochondrial depolarization. There are some indications that PINK1/Parkin-mediated mitophagy also occurs under basal conditions, however a recent 2018 study in PINK1 knockout mice suggests that this pathway is unessential to basal mitophagy. Much of this attention has stemmed from PINK1 and Parkin’s implication in PD; mutations in the E3 ubiquitin ligase Parkin and the
mitochondrial Serine/threonine kinase PINK1 are the most common causes of autosomal recessive PD\textsuperscript{66–68}.

Suggestions that PINK1 and Parkin participate in the same mitochondrial maintenance pathway were first raised by genetic studies in \textit{Drosophila melanogaster}\textsuperscript{68–72}. Initial studies demonstrated that Parkin over-expression could partially rescue PINK1 deletion mutants but not vice versa, suggesting that PINK1 acts upstream of Parkin\textsuperscript{69–71}.

\textbf{Figure 1-2: Representation of PINK1/Parkin-mediated mitophagy:} A PINK1/Parkin-mediated mitophagy is inhibited in healthy polarized mitochondria by PINK1 import into mitochondria for processing by MPP and PARL proteases, which releases cleaved PINK1 to the cytosol where it is rapidly degraded. B In damaged depolarized mitochondria, PINK1 is instead stabilized on the OMM where it phosphorylates OMM proteins, ubiquitin and Parkin, which is recruited to the OMM. Parkin in turn ubiquitinates OMM proteins, which recruit the autophagic machinery necessary to target the damaged mitochondrion for degradation.
The pathway was shown to be conserved and further elucidated in the mammalian system\textsuperscript{73–75}. In healthy mitochondria, endogenous PINK1 protein levels are constitutively low due to rapid degradation (Fig.1-2A)\textsuperscript{48}. PINK1 is imported and anchored to the IMM by the TOM and TIM transporters by its MTS, which is subsequently cleaved off by the mitochondrial processing peptidase (MPP) in the matrix\textsuperscript{72,76}. The serine protease, presenilins-associated rhomboid-like (PARL) then cleaves PINK1 between A103 and F104 in the IMM\textsuperscript{77–80}. PARL-cleaved PINK1 is untethered from the IMM and relocates to the cytosol, where it is rapidly degraded by the proteasome according to the N-end rule\textsuperscript{77–81}. Recently, one study has also proposed that the PINK1 cleavage product binds to Parkin in the cytosol to inhibit Parkin mitochondrial localization and resultant mitophagy\textsuperscript{82}. Under normal conditions, Parkin exists in a compact native autoinhibited conformation in the cytosol that is mediated by tight intramolecular association between its ubiquitin-like (UBL) domain and C-terminal region\textsuperscript{83}. The importance of this regulation is highlighted by pathogenic Parkin mutations, K27N, R33Q, R42P, and A46P in the UBL domain, that disrupt this autoinhibition\textsuperscript{83}.

Mitochondrial damage leading to mitochondrial depolarization inhibits PINK1 import to the IMM (Fig1-2B)\textsuperscript{23,76,77}. Instead, PINK1 is stabilized on the OMM of the damaged mitochondrion with its kinase domain facing the cytosol by TOM, although mechanistic details are still under active investigation\textsuperscript{76,84,85}. Multiple studies have shown that PINK1 kinase activity is required to recruit and activate Parkin\textsuperscript{84–86}. Stabilized PINK1 phosphorylates Parkin’s linker region between the In-Between Ring and RING2 domains at T175 and T217, which activates Parkin E3 ligase activity and recruits Parkin to
mitochondria. PINK1 also phosphorylates both Parkin’s UBL domain and ubiquitin at S65. PINK1 phosphorylation of S65 in Parkin’s UBL domain is proposed to prime Parkin for further activation by S65-phosphorylated ubiquitin (p-Ub). Rigorous biophysical and structural studies collectively suggest that p-Ub binds to phosphorylated Parkin with high affinity to allosterically induce conformational changes that promote its E2 recruitment and further stimulate Parkin E3 ligase activity. PINK1 is suggested to phosphorylate monoubiquitin and/or polyubiquitin chains covalently conjugated to OMM proteins at basal levels to further recruit Parkin, which has a high affinity for p-Ub. Recruited Parkin further ubiquitinates OMM proteins that are phosphorylated by PINK1, thus forming a positive feedback loop.

PINK1 has also been suggested to directly phosphorylate a number of other OMM proteins. Of note, one study reported Miro1, a component of the primary motor/adaptor complex that links mitochondria to the microtubule cytoskeleton, as a direct PINK1 substrate. However, two other studies have been unable to replicate this result. Nevertheless, these studies agree that PINK1 and Parkin mediate Miro1 proteasomal degradation to inhibit mitochondrial motility. Another study identified the OMM fusion protein MFN2 as a PINK1 substrate at T111 and S442. Chen and Dorn demonstrated that PINK1-dependent MFN2 phosphorylation facilitates Parkin recruitment and resultant mitophagy. Others report MFN1/2 as targets for PINK1/Parkin-mediated proteasomal degradation to promote mitochondrial fragmentation, which is a necessary primer for mitophagy.

Once recruited, Parkin conjugates OMM proteins with K48- and K63-linked ubiquitin chains. Sarraf et al. identified 36 Parkin OMM substrates with high
confidence, suggesting that chain-linkage types and density rather than specific substrates target the damaged mitochondrion for mitophagic degradation\textsuperscript{103}. This theory is supported by the observation that ectopic PINK1 expression on peroxisomes was sufficient to recruit Parkin and trigger pexophagy (peroxisome-specific autophagy)\textsuperscript{85}. To date, six ubiquitin-binding autophagy receptor proteins associated with mitophagy have been identified: NBR1, NDP53, OPTN, p62/SQSTM1, TAX1BP1, and TOLLIP\textsuperscript{104}. Studies suggest that NDP52 and OPTN are essential and TAX1BP1 is important for PINK1/Parkin-mediated mitophagy\textsuperscript{105,106}. These receptors recruit autophagy machinery necessary for autophagosome formation and eventual lysosomal degradation\textsuperscript{107}.

### 1.4.3 Mitochondrial Biogenesis

Multiple studies have demonstrated that mitophagy has the capacity to clear most or all mitochondria in the cell\textsuperscript{73,108,109}. While mitophagy protects the cell from excessive mitochondrial damage, persistent mitophagy resulting in mitochondrial depletion has an equal repercussion: cell death\textsuperscript{109}. Therefore it is critical for the cell to replace damaged mitochondria by activation of the mitochondrial biogenesis program. Due to the mitochondrion’s endosymbiotic origin, biogenesis of these organelles presents unique challenges: firstly, mitochondria cannot be made \textit{de novo}; secondly, mitochondrial genes reside in both mitochondrial and nuclear genomes. As a result, mitochondrial biogenesis is a complex, highly regulated coordination of several distinct processes including mtDNA expression, synthesis and import of nuclear-encoded proteins, coordinated assembly of mitochondrial complexes, expansion of OMM and IMM, and mtDNA replication\textsuperscript{110–112}.

Mitochondrial biogenesis is regulated mainly at the level of transcription (\textbf{Fig1-3}). The core controllers that modulate mitochondrial biogenesis are the proliferator-activated
receptor γ (PPARγ) coactivator-1 (PGC-1) family of transcriptional coactivators and the transcription factors, nuclear respiratory factor 1 (NRF1) and NRF2\textsuperscript{113,114}. The PGC-1 family is composed of PGC-1α, PGC1-β, and PGC-1-related coactivator (PRC)\textsuperscript{115}. While the PGC-1 family regulates overlapping mitochondrial gene expression programs, they significantly differ in their physiological expression and modes of regulation\textsuperscript{115–117}. PGC-1α, the founding member of the family, is the best studied and suggested to be the most regulated (Fig1-3)\textsuperscript{116,118}; however, this observation may in part due to the emphasis of study in adipocyte and muscle cell differentiation\textsuperscript{119}. Both PGC-1α/β are ubiquitously expressed, but at especially high levels in tissues with high metabolic demands, such as heart, skeletal muscle, kidney, and brain tissue\textsuperscript{120–122}. In contrast, PRC, the least characterized member of the family, appears to be restricted to regulating mitochondrial biogenesis in proliferating cells\textsuperscript{123}. PGC-1α, and to a lesser extent PGC-1β, are considered master regulators of mitochondrial biogenesis as they increase expression of various key transcription factors, including NRF1/2, and act as their transcriptional coactivators to stimulate activity (Fig1-3)\textsuperscript{124–126}. While PGC-1α/β share many overlapping mitochondrial gene targets, they have been shown through multiple studies to be activated independently and affect mitochondrial biogenesis in an additive and independent manner\textsuperscript{127,128}.

Upon activation, the transcription factors NRF1/2 stimulate gene expression of multiple key mitochondrial structures, including OXPHOS, mitochondrial transcription, translation, protein import, and assembly machinery\textsuperscript{113,119,129,130}. Critically, NRF1/2 coordinate nuclear and mitochondrial gene expression to facilitate OXPHOS assembly. As their name suggests, the nuclear respiratory factors NRF1/2 bind to the promoters and
activate expression of the nuclear genes that encode subunits of the five respiratory OXPHOS complexes and cytochrome c in a partially redundant manner\textsuperscript{131–133}. NRF1/2 also regulate mitochondrial gene expression by directly activating genes encoding mitochondrial transcription factor A (TFAM), mitochondrial transcription factor B1 (TFB1M), and TFB2M, which are crucial regulators of mtDNA transcription and replication, and mitochondrial ribosome assembly (Fig1-3)\textsuperscript{129,131}. As the majority of mitochondrial proteins are nuclear-encoded, the synthesis of mitochondrial import machinery is a crucial component of mitochondrial biogenesis. Importantly, NRF1/2 also activate expression of TOMM20, a key receptor subunit of the main mitochondrial translocase TOM that is involved with initial precursor protein recognition\textsuperscript{130,131}.

Mitochondrial biogenesis is another mitochondrial quality control pathway whose dysfunction is implicated in the pathogenesis of multiple neurodegenerative diseases. PGC-1α and its downstream effectors are down-regulated in brain tissue of PD, Alzheimer’s disease (AD), and Huntington’s disease (HD) patients\textsuperscript{114,134}. Decrease in PGC-1α in PD is partially owed to repressive PGC-1α promoter methylation\textsuperscript{134}. Additionally, one study demonstrated that polymorphisms in \textit{NRF1} and \textit{TFAM} genes significantly correlated with HD age of onset\textsuperscript{135}.  

\textsuperscript{129,131}
Figure 1-3: Representation of Mitochondrial Biogenesis: Mitochondrial biogenesis is controlled by many transcriptional regulators. One of these key master regulators, PGC-1α, is activated by AMPK phosphorylation and SIRT1 deacetylation. In conjunction with various transcription factors, including NRF1/2, PGC-1α/β upregulate nuclear mitochondrial gene expression. The mitochondrial transcription factor Tfam, as well as TFB1/2M, is also expressed and activate mtDNA transcription with PGC-1α. Coordinated nuclear and mitochondrial protein expression, as well as protein import, complex assembly, membrane expansion and mtDNA replication are necessary processes in mitochondrial biogenesis.
1.4.4 Coordination of Mitophagy and Mitochondrial Biogenesis

The mitochondrial content of the cell is controlled by the balance between the opposing processes of mitophagy and mitochondrial biogenesis. However, as mitophagy and mitochondrial biogenesis have only gained attention within recent decades, the additional regulatory layer of coordination between the two processes is still not fully understood. While certain known coordinating mechanisms favor one pathway and repress the other, the pathways highlighted below share the common characteristic of activating both mitophagy and mitochondrial biogenesis to allow for efficient mitochondrial turnover.

1.4.4.1 Parkin

The E3 ubiquitin ligase Parkin has been extensively studied in the context of mitophagy; however, its participation in mitochondrial biogenesis was also determined in the same time period. Work from Kuroda et al. suggests that Parkin promotes mitochondrial biogenesis by interacting with and enhancing TFAM mtDNA transcriptional activity. Moreover, a recent study from Shin et al. re-identified the Parkin interacting substrate, PARIS (ZNF746), which is repressed by Parkin ubiquitination and degraded by the ubiquitin-proteasome system. PARIS inhibits mitochondrial biogenesis by binding to PGC-1α’s promoter region to repress PGC-1α and its target genes (Fig1-3). The authors emphasized the importance of PARIS in mitochondrial homeostasis and PD by demonstrating that mice over-expressing PARIS displayed progressive loss of dopaminergic neurons, which could be rescued by Parkin or PGC-1α over-expression. Parkin inactivation in sporadic PD patients is suggested to contribute to
mitochondrial dysfunction by disrupting PINK1/Parkin-mediated mitophagy and by PARIS accumulation resulting in PGC-1α repression\textsuperscript{110,144}.

1.4.4.2 PGC-1α

Likewise, although PGC-1α is often described as a master regulator of mitochondrial biogenesis, new evidence suggests that it also acts as an autophagy regulator. In one HD mouse model study, researchers found that PGC-1α overexpression ameliorated neurodegeneration and huntingtin aggregation by upregulation of mitochondrial biogenesis and by activation of transcription factor EB (TFEB), a master regulator of the autophagy-lysosome pathway\textsuperscript{145}. Additionally, another study found that acute exercise-induced mitophagy in skeletal muscle was impaired in PGC-1α -/- mice\textsuperscript{146}. In particular, PGC-1α appeared to mediate expression of autophagy factors LC3B and p62 independent of TFEB or Forkhead box O3 (FOXO3), a PGC-1α-associated transcriptional regulator of autophagy\textsuperscript{146}. This suggests that in addition to regulating mitochondrial biogenesis, PGC-1α regulates autophagy through known and yet-to-be elucidated mechanisms.

1.4.4.3 AMPK

AMP-activated kinase (AMPK) is activated in response to high cellular AMP levels, which may indicate nutrient deprivation and environmental stress\textsuperscript{110,147}. Once activated, AMPK stimulates mitochondrial biogenesis by activating PGC-1α through direct phosphorylation and by promoting PGC-1α deacetylation by Silent Information Regulator 2 (SIR2) protein 1 (SIRT1) (Fig1-3)\textsuperscript{148,149}. Recently, AMPK was also shown to mediate mitophagy by directly phosphorylating the DRP1 receptor MFF to promote mitochondrial fragmentation\textsuperscript{50}. Additionally, the autophagy-initiating kinase, Unc-51-like autophagy activating kinase 1 (ULK1) is also a direct AMPK activation target\textsuperscript{150,151}. Egan et al.
showed mitophagy defects in ULK1- and AMPK-deficient primary murine hepatocytes, although the mechanism of ULK1-mediated mitophagy is not yet fully understood\textsuperscript{150–152}.

1.5 The Mitochondrial Rhomboid Protease, PARL

The mitochondrial rhomboid protease PARL is commonly described as a key protease of PINK1 that prevents aberrant mitophagy of healthy mitochondria\textsuperscript{77–80}. Work from our group and others has revealed that PARL plays a critical but not entirely understood role in multiple mitochondrial and cellular functions.

1.5.1 Identification of the Rhomboid Superfamily

The first rhomboid protease, Rhomboid-1, was identified in 1984 in a genetic screen where researchers identified a mutation in \textit{Drosophila} embryos that resulted in an abnormal rhombus-like head skeleton\textsuperscript{153}. Rhomboid-1 and its homologues are predominantly characterized in the activation of epidermal growth factors (EGFs)\textsuperscript{154}. It was later shown that Rhomboid-1 activated the EGF-like protein Spitz by cleaving it in its transmembrane domain\textsuperscript{155–157}. Thus, Rhomboid-1 and its six \textit{Drosophila} homologues became the founding members of the well-conserved rhomboid superfamily of intramembrane proteases\textsuperscript{157–159}. Rhomboid homologues have since been found in nearly every sequenced genome across virtually all life forms and constitute the most widespread family of intramembrane proteases\textsuperscript{157–159}. The rhomboid superfamily is composed of proteolytically active RHO secretory pathway rhomboids and PARL mitochondrial rhomboids, and proteolytically inactive iRhoms and Derlin proteins\textsuperscript{159}. 
1.5.2 Rhomboid Structure

Rhomboids are the best mechanistically characterized intramembrane proteases but are still not well understood\textsuperscript{160}. Much of our structural and mechanistic understanding is gleaned from seven crystal structures of GlpG, the rhomboid homologue in \textit{Escherichia coli}, which were solved just over a decade ago\textsuperscript{161–163}. All active prokaryotic and eukaryotic rhomboids share a catalytic domain comprised of six transmembrane helices (TMH)\textsuperscript{164}. These crystal structures reveal that the bacterial rhomboid is almost entirely immersed in the detergent micelle with its universally conserved catalytic serine on TMH-4 (S277 in human PARL) submerged approximately 10 Å from the presumed membrane surface\textsuperscript{161–164}. Molecular dynamics studies indicate that although proteolysis occurs in the membrane, water molecules necessary for catalysis are still able to access the active site, which is composed of a catalytic dyad, serine on TMH-4 and histidine on TMH-6 (H335 in human PARL)\textsuperscript{165–167}.

Most eukaryotic members of the PARL and RHO subfamilies possess seven TMHs, having gained an additional TMH at the N-terminus (PARL subfamily) or at the C-terminus (RHO family)\textsuperscript{158}. One group has noted the difficulty in expressing PARL’s 6 TMH catalytic domain due to issues of topology and misfolding, which suggests that the additional N-terminal TMH-A may facilitate PARL import and folding\textsuperscript{164}. Homology modeling of PARL’s 6 TMH core indicates that disruption of PARL’s ‘1+6’ structure may displace D319, which is implicated in PARL’s catalytic activity\textsuperscript{164}. To date, the structural and functional contribution of the 7\textsuperscript{th} TMH, TMH-A in PARL, remains an open question.
1.5.3 Identification of the Mitochondrial Rhomboid

The first mitochondrial rhomboid protease, Rhomboid-1 (Rbd1), was identified in 2002 in *Saccharomyces cerevisiae* as a protease of cytochrome c peroxidase 1 (Ccp1). While Ccp1 deletion had minimal consequences, Δ*rbd1* yeast cells were unable to grow on glycerol media and displayed prominent mitochondrial abnormalities, including mitochondrial fragmentation and aggregation, loss of mtDNA nucleoids, and respiratory defects. This drastic phenotype was one of the first pieces of evidence that Rbd1 and its homologues play a critical role in mitochondrial biology. Less than a year after its initial discovery, Rbd1’s second substrate, mitochondrial genome maintenance 1 (Mgm1), was identified. Mgm1, like its mammalian homologue, the IMM fusion GTPase OPA1, is a key participant in mitochondrial fusion and mtDNA maintenance. Rbd1-dependent Mgm1 cleavage and production of short Mgm1 (s-Mgm1) is required to carry out these functions. Part of Rbd1’s effect on mitochondrial biology is through Mgm1 cleavage as expression of s-Mgm1 partially rescues Δ*rbd1* mitochondrial defects. Importantly, the Δ*rbd1* phenotype could be rescued by expression of its human mitochondrial homologue PARL; this was the first demonstration that mitochondrial rhomboid function is conserved from yeast to humans.

Mitochondrial Rbd1 homologues in other model organisms, including *Drosophila*, became more heavily scrutinized in light of Rbd1’s regulation of mitochondrial biology. The few *Drosophila* deficient in the Rbd1 homologue Rhomboid-7 that survived pupation displayed neurological defects, male sterility, and did not survive past three days into adulthood. Notably, rhomboid-7-deficient *Drosophila* testis and skeletal muscle displayed mitochondrial abnormalities suggestive of defective mitochondrial fusion. *Rhomboïd-7* silencing in *Drosophila* S2 cells also resulted in severe mitochondrial
fragmentation, similar to opa1-like (homologue of Mgm1 and OPA1) silencing\textsuperscript{172}. However, evidence of conserved Rhomboid-7 cleavage of Opa1-like is conflicting\textsuperscript{173,174}. Surprisingly, Rhomboid-7-overexpressing \textit{Drosophila} displayed similar defects to Rhomboid-7-deficient \textit{Drosophila}: increased lethality, neurological defects, and severe mitochondrial malfunction\textsuperscript{173}. These converging phenotypes suggest the necessity of regulating Rhomboid-7 proteolytic activity.

Studies of Rhomboid-7 in \textit{Drosophila} also led to the first line of evidence connecting the mitochondrial rhomboid to PD\textsuperscript{174}. Whitworth \textit{et al.} identified three PD-linked genes as genetic interactors of \textit{rhomboid-7}: \textit{pink1}, \textit{parkin}, and the serine protease high temperature requirement A2 (\textit{htrA2}; also known as \textit{omi})\textsuperscript{174}. Further work suggests that Pink1 and HtrA2 are Rhomboid-7 proteolytic substrates and that this cleavage is required for substrate function\textsuperscript{174}.

<table>
<thead>
<tr>
<th>Species</th>
<th>Rhomboid</th>
<th>Putative Substrates</th>
<th>Function</th>
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<td>Rbd1</td>
<td>Ccp1, Mgm1</td>
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<td>\textit{Drosophila melanogaster}</td>
<td>Rhomboid-7</td>
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<td>Mammals</td>
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<td>Mitochondrial Fusion, Mitophagy, Apoptosis</td>
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\textbf{Table 1-1: List of mitochondrial rhomboids and their putative substrates}
1.5.4 The Mammalian Mitochondrial Rhomboid, PARL

The only human mitochondrial rhomboid protease, presenilins-associated rhomboid-like (PARL), was identified in 2001 in a yeast two-hybrid screen as a putative interactor of presenilins, which are implicated in AD\(^{175}\). Unlike what PARL’s name suggests, this interaction was ruled as artefactual as PARL is located in the IMM, separated by an additional OMM, whereas the presenilins are located in the plasma membrane; this false positive may be attributed to the yeast two-hybrid system’s poor suitability for membrane proteins\(^{169,171}\). To correct this misnomer and retain PARL’s historical name, it has recently been suggested that the meaning of the PARL acronym be changed to “PINK1/PGAM5-associated rhomboid-like”\(^{176}\).

1.5.4.1 PARL α/β/γ Cleavage

As a eukaryotic mitochondrial rhomboid, mammalian PARL is an IMM protease with seven TMHs: the evolutionarily conserved 6 TMHs (TMH1-6) containing the catalytic core, and the additional TMH-A that is appended to PARL’s N-terminus. TMH-A is suspected to exert some regulatory control on PARL. As a nuclear-encoded protein, PARL import into the IMM results in removal of its N-terminal MTS by MPP in the matrix; this event is referred to as α cleavage (Fig 1-4A)\(^{164,177,178}\).

PARL undergoes a sequential cleavage event at its N-terminus in the matrix between amino acids S77 and A78, which is termed β cleavage (Fig 1-4A)\(^{78,177}\). β cleavage is dependent on PARL catalytic activity as mutation of PARL’s catalytic serine, S277G, abolishes β cleavage\(^{78,177}\). However, as PARL cleaves in the IMM and the site of β cleavage is in the mitochondrial matrix, the protease responsible for β cleavage remains an active topic of debate. The protease responsible for β cleavage may be PARL itself.
through some unknown mechanism or an as-of-yet unknown protease whose recruitment or activity requires PARL proteolytic activity.

Unlike α cleavage, which is a constitutive event, β cleavage is triggered by mitochondrial stress. Our group has demonstrated that PARL β cleavage is exacerbated by the mitochondrial stressors, Oligomycin A (OIA), Rotenone, and carbonyl cyanide m-chlorophenyl hydrazone (CCCP), but not by the endoplasmic reticulum (ER) stressor, Thapsigargin. Mechanistically, β cleavage is additively inhibited by phosphorylation of S65, T69, and S70, N-terminal to the site of β cleavage. Pyruvate dehydrogenase kinase 2 (PDK2), a key regulator of energy production and metabolism, was recently identified as the kinase responsible for PARL phosphorylation and β cleavage inhibition. Shi and McQuibban hypothesize that PDK2 promotes β cleavage by reducing PARL phosphorylation rates in response to reduction of mitochondrial energy metabolism. The matrix phosphatase responsible for reversing PARL phosphorylation and promoting β cleavage remains an open question.

Also unlike α cleavage, whose site is highly conserved amongst animal orthologues of PARL, the β cleavage site is strictly conserved in mammals (Fig 1-4B). This suggests that β cleavage may mediate some mammalian-specific regulation on PARL in response to mitochondrial stress. The importance of PARL β cleavage in mitochondrial and cellular health is emphasized by our group’s discovery of the PD-associated missense mutation S77N at PARL’s β cleavage site, which abolishes this cleavage event (Fig 1-4C).

β cleavage results in two moieties: N-terminally cleaved PARL, termed β PARL, and a 25 amino acid peptide, termed PARL β (Pβ) (Fig 1-4A). Our group and others
have found that exogenous expression of β PARL results in a fragmented mitochondrial morphology similar to WT PARL over-expression phenotypes\textsuperscript{78,178}. Fragmented mitochondrial morphology is not recapitulated when PARL catalytic activity (S277G) or β cleavage (S77N) is disrupted\textsuperscript{78,178}. Additionally, our group has shown that β cleavage alters PARL cleavage efficiency of its substrate PINK1\textsuperscript{179}. The effect of PARL β cleavage on PINK1 of PINK1/Parkin-mediated mitophagy is further expanded below.

Speculations that the second product of PARL β cleavage, the small peptide Pβ, may carry some as-of-yet unknown role in mitochondrial and cellular biology have stemmed from its strong conservation in vertebrates and especially in mammals\textsuperscript{78,177}. Interestingly, Sík \textit{et al.} identified a non-canonical nuclear localization sequence (NLS) in Pβ composed of three closely spaced doublets of positively charged amino acids; the first and second are conserved in vertebrates whereas the third pair is mammalian-specific\textsuperscript{177}. Indeed, two studies exogenously expressing Pβ-GFP fusion constructs found that Pβ appeared to partially localize to the nucleus\textsuperscript{177,180}. Sík \textit{et al.} determined that mutation of Pβ-GFP’s putative NLS abrogated nuclear localization\textsuperscript{177}. However, the results of these localization studies are obscured by the usage of Pβ-GFP as GFP itself can localize to the nucleus to some extent\textsuperscript{181}.

One group has suggested that PARL’s N-terminus undergoes a third sequential cleavage, this time in the loop region separating TMH-A from the core catalytic domain, termed Y cleavage (\textbf{Fig 1-4A})\textsuperscript{164,177}. Homology modeling of Y PARL suggests that removal of TMH-A results in structural changes that disrupted PARL proteolytic activity\textsuperscript{164}. In line with this, exogenous expression of Y PARL results in elongated mitochondrial
morphology, similar to catalytically dead PARL. However, our group has had difficulties recapitulating this data.

Figure 1-4: PARL β cleavage is a disease-relevant regulatory event: A A representation of PARL structure and cleavage events. PARL contains 7 transmembrane helices (TMH) organized in a “1+6” manner, where the latter 6 make up the conserved rhomboid catalytic core and the N-terminal TMH is a mitochondrial rhomboid-specific addition. PARL undergoes 3 cleavage events: constitutive α cleavage; mitochondrial-stress regulated β cleavage, which is additively inhibited by phosphorylation; and ϒ cleavage, which is mechanistically paired to β cleavage. B PARL β cleavage site amino acid sequence alignment by CLUSTALW. S77, the β cleavage site, is highlighted in red. Alignment demonstrates a high degree of sequence similarity in mammals. S77N mutation was identified in Parkinson’s disease (PD) patients. C Western blot of FLAG-tagged PARL constructs in HEK293Ts. S77N abolishes PARL β cleavage.
1.5.4.2 The Role of PARL in Mitochondrial Morphology

As previously noted, WT PARL overexpression in cultured cells induces mitochondrial fragmentation, a phenotype that is dependent on both PARL catalytic activity and β cleavage\textsuperscript{78,178}. These observations suggest the importance of mammalian PARL, especially β PARL, the product of β cleavage, in regulation of mitochondrial dynamics and morphology. However, studies of mitochondrial morphology in Parl \(-/-\) mice are conflicting. Two studies, one in Parl \(-/-\) mouse embryonic fibroblasts (MEFs) and one in PARL-depleted mouse skeletal muscle and cultured human myotubes, found no major disruption of mitochondrial morphology\textsuperscript{180,182}. The second study observed changes in mitochondrial cristae structure\textsuperscript{180}. PARL proteolytic activity for the IMM fusion protein OPA1 is suggested to be conserved as yeast two-hybrid screens and co-immunoprecipitation experiments indicate interaction\textsuperscript{182}. However, other studies suggest that PARL is dispensable for OPA1 processing as OPA1 is a substrate for many other proteases\textsuperscript{183,184}.

1.5.4.3 The Role of PARL in Apoptosis

The implication of PARL in intrinsic apoptosis is also conflicting. One study argued that PARL is an anti-apoptotic protein by demonstrating that Parl \(-/-\) mice had a drastically reduced life span owing to progressive multisystemic atrophy that was sustained by increased apoptosis\textsuperscript{182}. Increased apoptosis was attributed to higher susceptibility to cytochrome c release in Parl \(-/-\) MEFs and primary myoblasts in response to several intrinsic apoptotic inducers, including staurosporine (STS)\textsuperscript{182}. In contrast, a 2017 study identified PARL as a pro-apoptotic protein. Saita et al. showed that PARL-deficient human cells were more resistant to apoptotic inducers, including STS\textsuperscript{185}. They demonstrated that PARL cleaved the pro-apoptotic protein Smac (also known as DIABLO) which
subsequently inhibited apoptosis inhibitors to promote caspase activity and resultant apoptosis\textsuperscript{185}.

PARL may also influence apoptosis through two other substrates: the mitochondrial kinase phosphoglycerate mutase 5 (PGAM5), which is implicated in multiple cell death pathways including apoptosis and necroptosis\textsuperscript{186–188}; and HtrA2, a controversial PARL substrate implicated in apoptosis\textsuperscript{185,189}. However, these connections remain circumstantial at best. To date, there are no studies observing PARL β cleavage in cell death pathways.

1.5.4.4 The Role of PARL in Mitophagy and Parkinson’s disease

According to current mitophagy models, PARL’s role in mitophagy extends only to prevent aberrant mitophagy of healthy polarized mitochondria because it cleaves imported PINK1 to target it to the cytosol for degradation. While other proteases have been documented to proteolyze PINK1, PARL likely acts as the favoured PINK1 protease as PARL ablation or expression of catalytically inactive S277G PARL results in impaired PINK1 processing\textsuperscript{78,176,190}. One study also noted mitochondrial PINK1 retention and premature Parkin recruitment in PARL-deficient human cells\textsuperscript{190}.

As the PD-linked S77N mutation disrupts PARL β cleavage, we hypothesized that β cleavage and resultant β PARL expression may play a role in mitophagy. Indeed, although S77N PARL retains catalytic activity, exogenous expression of S77N PARL showed impaired mitochondrial fragmentation and Parkin recruitment in response to mitochondrial depolarization by treatment with the ionophore, CCCP, in comparison to WT PARL-expressing cells\textsuperscript{78}. Recent work from Shi and McQuibban demonstrated that β PARL was less efficient at cleaving PINK1 in comparison to S77N PARL\textsuperscript{179}. WT PARL, which exists as both full length and β PARL when over-expressed, showed intermediate
PINK1 cleavage efficiency. Furthermore, PARL β cleavage was responsive to a number of stresses that may occur prior to depolarization, including ATP depletion by the ATP synthase inhibitor, OIA. Of note, the PARL kinase, PDK2, is highly sensitive to ATP depletion, owing to its role in metabolism. Thus, we hypothesize that PARL β cleavage initiates PINK1/mediated mitophagy prior to ΔΨm depletion through β PARL’s reduced proteolytic efficiency for PINK1, leading to PINK1 OMM stabilization. Interestingly, over-expression of β PARL but not S77N PARL induced mitochondrial fragmentation, a necessary prerequisite of mitophagy. Assuming inefficient PINK1 processing results in stabilization on the OMM, PINK1 may drive PARL-mediated mitochondrial fragmentation by phosphorylating and targeting MFN1/2 for degradation.

1.5.4.5 The Role of PARL in Mitochondrial Biogenesis

One study conducted in 2010 by Civitarese et al. has connected PARL to mitochondrial biogenesis. The authors showed that Parl knockdown in mouse skeletal muscle cells resulted in decreased mitochondrial mass and concomitant decreased protein expression in PGC-1α, OPA1, and MFN1/2. Surprisingly, transfection of untagged Pβ in human myotubes increased mRNA expression of the mitochondrial biogenesis genes, PGC-1β and NRF1; mitochondrial fusion genes, MFN1/2; and PARL itself. In addition, Pβ-transfected myotubes also displayed increased protein expression of the OPA1 and SIRT1, a PGC-1α activator. Pβ treatment also appeared to increase mitochondrial mass and oxygen consumption, in line with increased mitochondrial biogenesis. However, the mechanism by which the 25 amino acid peptide Pβ may activate mitochondrial biogenesis remains unresolved.
1.6 Thesis Rationale

Mounting evidence suggests that PARL plays a crucial but as-of-yet unclear role in mitochondrial and cellular biology. Specifically, recent reports indicate that PARL β cleavage coordinates two critical mitochondrial quality control processes, mitophagy and mitochondrial biogenesis, through its cleavage products, β PARL and Pβ, respectively. However, Pβ’s putative role in mitochondrial biogenesis remains poorly characterized. This study sought to delineate the mechanism of Pβ regulation of mitochondrial biogenesis by identifying its expression and subcellular localization in cells and its potential interactions. In this thesis, I demonstrated that Pβ localizes to the nucleus, where it associates with chromatin. My work suggests that Pβ may act at the transcriptional level to upregulate mitochondrial biogenesis and thus enforce mitochondrial quality control.
Chapter 2
MATERIAL AND METHODS

2.1 Reagents

2.1.1 Cell Culture and Transfection
HEK293 cell lines were cultured in Dulbecco’s Modified Essential Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma) at 37°C in 5% (v/v) CO₂. SH-SY5Y cell lines were cultured in DMEM/F12 with L-Glutamine (Sigma), 15mM HEPES (Gibco) supplemented with 10% FBS at 37°C in 5% (v/v) CO₂. Both adherent and floating cell populations were maintained. HEK293s were transiently transfected using XtremeGENE9 (Roche) according to manufacturer’s instructions.

2.1.2 Chemical Reagents and Antibodies
Oligomycin A treatment was conducted for 24 hours to induce mitochondrial stress. 5μM MG132 treatment was conducted for 16 hours in SH-SY5Y cells to stabilize Pβ signal by inhibition of the proteasome.

The following antibodies were used: rabbit anti-Pβ (a generous gift from Dr. Lyndal Bayles, Deakin University), mouse M2 anti-FLAG (Sigma, F1804), rabbit-PARL (Abcam, ab45231), mouse anti-ATP5α (Abcam, ab95962), mouse anti-actin (Sigma, A3853), mouse anti-tubulin (Sigma, T9026), rabbit anti-Lamin B1 (Abcam, ab16048), rabbit anti-ALY (a generous gift from Dr. Alex Palazzo, University of Toronto) rabbit anti-H3 (Sigma,
SAB4200651), and rabbit and mouse horseradish peroxidase-conjugated secondary antibodies (Jackson Labs).

2.1.3 Plasmids
C-terminally 3X FLAG-tagged WT PARL was generated as previously described\textsuperscript{78}. β PARL was generated by Pβ deletion from WT PARL by inverse PCR, as previously described\textsuperscript{179}.

2.2 Subcellular Fractionations
2.2.1 Abcam Subcellular Fractionation
Subcellular fractionations were conducted by differential centrifugation using a method adapted from manufacturer’s instructions for the Mitochondrial Isolation Kit for Cultured Cells (Abcam, ab110170). Cells were trypsinized, harvested, and washed twice in chilled PBS buffer. On ice, collected cells were lysed in 10:1 volumes of reagent A (Tris, trisethanolamine, EDTA, digitonin) for 5 minutes, homogenized by 26½ gauge needle 30 times, and incubated for an additional 5 minutes. A total fraction was collected and remaining sample was centrifuged at 1000xg for 10 minutes at 4°C to pellet the nuclear fraction. The supernatant fraction was subsequently centrifuged at 16,000xg for 15 minutes to separate the pelleted mitochondrial fraction and the cytosolic fraction. The mitochondrial pellet was washed once in reagent A. The pelleted nuclear fraction was resuspended in reagent B (Tris, EDTA, digitonin) and homogenized with a 26½ gauge needle 30 times. The nuclear fraction was centrifuged at 1000xg for 10 min and washed twice with reagent B. Fractions were protein precipitated if necessary and resuspended in 2X Lammli sample buffer.
2.2.2 CSK Subcellular Fractionation

Subcellular fractionations were conducted using a protocol adapted from Mirzoeva and Petrini\textsuperscript{191}. Cells were trypsinized, harvested, and washed twice with chilled PBS. Cells were lysed in cytoskeleton (CSK) buffer (10mM PIPES pH 6.8, 300mM sucrose, 100mM NaCl, 3mM MgCl\textsubscript{2}, 1mM EGTA, 0.5% Triton X-100, and protease inhibitors) for 7 minutes on ice. A total fraction was obtained prior to centrifugation at 5000xg for 5 minutes at 4°C to pellet the nuclear fraction. The nuclear pellet was washed twice in CSK buffer. The supernatant was centrifuged at 16 000xg for 15 minutes to obtain the pelleted mitochondrial fraction and the cytoplasmic fraction. The mitochondrial pellet was washed once with CSK buffer. Fractions were protein precipitated if necessary and resuspended in 2X Lammli sample buffer.

2.3 Chromatin Fractionations

2.3.1 DNase-based Chromatin Fractionation

Chromatin fractionations were conducted using a protocol adapted from Mirzoeva and Petrini\textsuperscript{191}. Cells were trypsinized, harvested, and washed twice with chilled PBS. Cells were lysed in CSK buffer for 7 minutes on ice prior to centrifugation at 5000xg for 5 minutes at 4°C to obtain the pelleted nuclear fraction and the triton-soluble supernatant. The triton-soluble fraction was centrifuged at 16 000xg for 15 minutes to obtain the pelleted mitochondrial fraction and the cytoplasmic fraction. The mitochondrial pellet was washed once with CSK buffer. The nuclear pellet was washed once with CSK buffer and incubated in CSK buffer and 1:5 volumes of DNase I (New England Biolabs) at 37°C for 1 hour. The fraction was then centrifuged at 5000xg for 5 minutes at 4°C. The supernatant was taken as the DNase-soluble fraction and the pellet was taken as the DNase-insoluble
nuclear pellet. Fractions were protein precipitated if necessary and resuspended in 2X Lammlli sample buffer.

2.3.2 Acid-based Chromatin Fractionation

Chromatin fractionations were adapted from a previously described protocol by Huang et al.\textsuperscript{192}. Cells were trypsinized, harvested, and washed twice with chilled PBS. Cells were lysed in 5 volumes of lysis buffer (10mM HEPES pH 7.4, 10mM KCl, 0.05% NP-40, and protease and phosphatase inhibitors) for 20 minutes on ice prior to centrifugation at 2000xg for 10 minutes at 4°C to obtain a pelleted nuclear fraction and a cytosolic supernatant. The pelleted nuclear fraction was subsequently washed once in lysis buffer prior to incubation in low salt buffer (10mM Tris-HCl pH 7.4, 0.2mM MgCl\textsubscript{2}, 1% Triton-X 100 and protease and phosphatase inhibitors) for 15 minutes on ice. The lysate was centrifuged at 5000xg for 10 minutes at 4°C to obtain the pellet and the supernatant containing nucleoplasmic proteins. The pelleted fraction was incubated in 5 volumes of HCl 0.2N for 20 minutes on ice and centrifuged at 15000xg to obtain an insoluble nuclear pellet fraction and the supernatant containing chromatin-associated proteins. The collected supernatant was neutralized with the same volume of 1M Tris HCl pH 8. Fractions were protein precipitated if necessary and resuspended in 2X Lammlli sample buffer.

2.3 Immunoprecipitation

Cells were trypsinized, harvested, and washed twice with chilled PBS. Collected cells were subsequently lysed with IP lysis buffer (150mM NaCl, 10mM Tris-HCl, 1mM EDTA, 1% TX-100, 0.5% NP-40, and protease inhibitors) at 4°C for 25 minutes and centrifuged at 1500xg for 10 minutes. To immunoprecipitate PARL, 1:100 rabbit
polyclonal anti-PARL was incubated with supernatant at 4°C overnight, followed by incubation with protein A magnetic beads (Bio-Rad, ab214286) at 4°C for 2 hours, according to manufacturer's instructions.

2.4 Western Blotting

2.4.1 Protein Precipitation

Protein was precipitated as previously described by Wessel and Flügge\textsuperscript{193}. Briefly, sample volume was raised to 600μL with ddH\textsubscript{2}O and 600μL methanol and 100μL chloroform were added. The resulting sample was mixed thoroughly followed by centrifugation at 10 000xg for 5 minutes at room temperature (RT). The upper layer was extracted and 600μL methanol was added. The two phases were carefully mixed by low vortexing or by tilting the tube lengthwise. Mixed samples were centrifuged at 16 000xg for 5 minutes at RT. The supernatant was removed and the protein pellets were dried at 42°C. Dried pellets were subsequently resuspended in 2X Laemmli buffer.

2.4.2 SDS-PAGE and Western Blotting

Cell lysates were resuspended in 2X Laemmlı buffer followed by 15 minute incubation at 95°C or 65°C for PARL to avoid aggregation. Samples were electrophoresed on 15% polyacrylamide gels or 4-20% Mini-PROTEAN\texttextsuperscript® TGX™ Precast Protein Gels (Biorad, ab 456-1094) for endogenous Pβ. Gels were transferred for 45min for Pβ detection or 1 hour for PARL detection at 110V onto methanol-activated PVDF membranes. Membranes were blocked in 5% bovine serum albumin (BSA) in TBST for 1 hour, incubated in 1° antibody in 1% BSA overnight, and 2° antibody in 1% BSA for 2 hours at room temperature. Chemiluminescence was detected via Clarity Western ECL Substrate (Biorad, 170-5061) and Versadoc imager (Biorad).
Chapter 3
RESULTS

3.1 Over-expressed Pβ localizes to the nucleus

Pβ contains a putative non-canonical vertebrate-specific nuclear localization sequence (NLS), which has lead two groups to demonstrate partial nuclear localization of the small peptide through fluorescence microscopy experiments over-expressing GFP fusion constructs\textsuperscript{177,180}. However, these results are obscured by the usage of Pβ-GFP as GFP itself can localize to the nucleus to some extent\textsuperscript{181}. Furthermore, as these constructs are expressed in the cytoplasm, there is currently no published evidence that the mitochondrial peptide is exported from mitochondria. To address these issues, I determined untagged Pβ subcellular localization. As PARL β cleavage is an N-terminal event, C-terminally 3XFLAG-tagged PARL was transiently expressed in HEK293s. PARL-FLAG localizes to the IMM and produces untagged Pβ in the mitochondrial matrix upon β cleavage. Because mitochondrial stress instigates PARL β cleavage, HEK293s were treated with 2.5μM Oligomycin A (OIA), an ATP synthase inhibitor and potent mitochondrial stressor, or DMSO as control for 24 hours. I was unable to detect free Pβ localization by immunofluorescence microscopy due to the simultaneous recognition of full-length PARL in mitochondria (data not shown). Therefore, I conducted two separate
subcellular fractionations which isolated total, cytoplasmic, mitochondrial and nuclear fractions to rigorously determine free Pβ subcellular localization. Fractionations were verified by mitochondrial ATP5α, a subunit of ATP synthase that faces the mitochondrial matrix, and the nuclear lamina protein, Lamin B1. Pβ, as detected by Pβ-specific antibody gifted by Dr. Lyndal Bayles (Deakin University), was largely enriched in the nuclear fraction in both fractionations, corroborating previous studies suggesting nuclear Pβ localization (Fig. 3-1A,B)\textsuperscript{177,180}. Little to no Pβ was reliably detectable in the mitochondrial fraction in subsequent repetitions, which suggests efficient Pβ clearance by efflux and/or degradation from the mitochondrial matrix.
Figure 3-1: Overexpressed Pβ localizes to the nucleus in HEK293s: HEK293 cells were transiently transfected with PARL-FLAG and treated with 2.5μM Oligomycin (OIA) or DMSO for 24 hours. HEK293s were fractionated according to manufacturer’s instructions using the Mitochondrial Isolation Kit for Cultured Cells (Abcam ab110170) (A) or using a fractionation protocol as previously described (B). Both fractionations isolated Tot (total), Cyto (cytoplasmic), Mito (mitochondrial) and Nuc (nuclear) fractions. C To determine the effect of increasing mitochondrial stress on Pβ production, HEK293s transiently expressing PARL-FLAG were treated with higher concentrations of OIA, 10μM and 20μM. β PARL-FLAG was also transiently transfected as a control for antibody specificity. Cells were fractionated using Abcam protocol as in Fig 1A. PARL β cleavage was monitored by ratio of full length PARL (upper band) to β PARL (lower band).
Surprisingly, Pβ was detected at comparable amounts in both 2.5μM OlA- and DMSO-treated fractions, contrary to previous reports of cleavage dependency on mitochondrial damage\textsuperscript{179}. To determine whether the lack of change could be due to insufficient mitochondrial stress, PARL-transfected HEK293s were treated with higher concentrations of OlA (10μM and 20μM) for 24 hours and subcellular fractionations were conducted. PARL β cleavage was monitored by the ratio of full length PARL to β PARL in response to mitochondrial stress. Notably, nuclear Pβ expression and mitochondrial PARL β cleavage were prominent and comparable in DMSO- and 10μM OlA-treated HEK293s (Fig. 3-1C). Preliminary results suggest that while PARL β cleavage correspondingly increased in cells treated with 20μM OlA, the highest concentration tested, the expression of the cleavage byproduct Pβ paradoxically decreased. Pβ decrease in 20μM OlA-treated HEK293s may be due to increased Pβ degradation in response to increased mitochondrial and thus cellular stress, although it is unclear in which subcellular compartment this degradation may occur.

As a control for antibody specificity, I also transiently transfected HEK293s with β PARL, an N-terminally truncated construct where the Pβ sequence has been deleted in-frame. In these cells, only a lower molecular weight band corresponding to β PARL was present in the mitochondrial fraction (Fig. 3-1C). No bands corresponding to Pβ were detectable by western blot, verifying that the doublet visualized by Pβ antibody is Pβ-specific (Fig. 3-1C).

These results corroborate and extend previous observations of nuclear Pβ-GFP localization by establishing that overexpressed, untagged Pβ efficiently translocates from the mitochondrial matrix to the nucleus in HEK293s.
3.2 Endogenous Pβ localizes to the nucleus

As Pβ localization studies heavily rely on overexpression systems, I next aimed to determine the subcellular localization of endogenous Pβ, made possible by the possession of a Pβ-specific antibody. Experiments observing endogenous Pβ were conducted in the human neuroblastoma cell line, SH-SY5Y, which was a gracious gift from Dr. Anurag Tandon (University of Toronto). SH-SY5Ys are a common cell line to study neurodegenerative diseases, especially PD, in part due to their catecholaminergic properties\textsuperscript{194}. These properties include tyrosine hydroxylase expression and dopamine and norepinephrine/noradrenaline production, which are intact in undifferentiated SH-SY5Ys\textsuperscript{194,195}. Most importantly, we chose this cell line as endogenous PARL and PARL β cleavage is detectable in SH-SY5Ys\textsuperscript{179}.

**Figure 3-2:** Previous work from our lab demonstrates that PARL β cleavage is sensitive to OIA treatment in SH-SY5Ys\textsuperscript{177}: SH-SY5Ys were treated with increasing concentrations of OIA for 24 hours. PARL was immunoprecipitated to increase signal. PARL β cleavage was monitored by ratio of full length PARL (upper band) to β PARL (lower band). ATP5α is the loading control. Reprinted with permission\textsuperscript{177}.
Our lab has previously shown that PARL does not undergo β cleavage in SH-SY5Ys in the absence of mitochondrial stress (Fig. 3-2)\textsuperscript{179}. Previous unpublished results also suggest that endogenous Pβ is difficult to detect by western blot without the addition of MG132, a potent proteasome inhibitor, which inhibits Pβ degradation. To induce β cleavage and ensure Pβ visualization, I treated SH-SY5Ys with 2.5μM OlA, which was previously shown to be sufficient to initiate PARL β cleavage (Fig.3-2), or DMSO for 24 hours. SH-SY5Ys were also treated with 5μM MG132 for 16 hours to aid Pβ visualization. Cells were subsequently fractionated using two independent protocols. In agreement with Pβ overexpression studies, both published and presented above, free endogenous Pβ was largely enriched in the nuclear fraction in both fractionations (Fig. 3-3A,B). Surprisingly, Pβ was comparably expressed in DMSO- and OlA-treated cells. This result is congruous with Pβ overexpression fractionations in HEK193s, but contrary to previous reports that PARL β cleavage and thus Pβ expression are tightly controlled by induction with mitochondrial stress in SH-SY5Ys\textsuperscript{179}. Nevertheless, my findings provide evidence that Pβ translocates from mitochondria to the nucleus in SH-SY5Ys.
Figure 3-3: Endogenous Pβ localizes to the nucleus in SH-SY5Ys: SH-SY5Y cells were treated with either 2.5μM OIA or DMSO for 24 hours. Cells were also treated with 5μM MG132 for 16 hours. SH-SY5Ys were fractionated using an Abcam mitochondrial isolation kit for cultured cells (A) or as described previously by Mirzoeva and Petrini (B). Both fractionations isolated Tot (total), Cyto (cytoplasmic), Mito (mitochondrial) and Nuc (nuclear) fractions. ATP5α (α subunit of ATP Synthase) is the mitochondrial marker. Lamin B1 (component of nuclear matrix) is the nuclear marker.
3.3 Endogenous Pβ associates with chromatin in SH-SY5Ys

Civitarese et. al. have shown that transfection of naive synthetic Pβ peptide in mouse cardiomyocytes resulted in transcriptional upregulation of mitochondrial biogenesis genes, PGC1β and NRF1; mitochondrial fusion genes, MFN1, MFN2, and OPA1; and PARL itself\textsuperscript{180}. Combined with multiple reports of Pβ nuclear localization, we and others have hypothesized that Pβ peptide may act as transcriptional upregulator to activate mitochondrial biogenesis in response to mitochondrial damage. To assess whether Pβ’s suggested role in nuclear mitochondrial gene expression could occur directly at the level of DNA, I determined whether Pβ associates with chromatin.

Initially, I aimed to test Pβ chromatin association in HEK293s transiently transfected with WT PARL and treated with 5\(\mu\)M OlA or DMSO for 24 hours. HEK293s were subsequently fractionated to obtain nuclear fractions. Chromatin-associated proteins were eluted from nuclear fractions by DNase I incubation, as previously documented\textsuperscript{191}. The chromatin-associated fraction was verified by the selective enrichment of histone octamer subunit H3 and the disenrichment of nuclear structural protein and insoluble nuclear pellet marker Lamin B1, mitochondrial marker ATP5α, and cytosolic marker Tubulin. In HEK293s, overexpressed Pβ largely localized to the insoluble nuclear pellet fraction, suggesting that overexpressed Pβ does not associate with chromatin (Fig. 3-4). As overexpression studies may be prone to artefactual mislocalization, I next aimed to determine whether endogenous Pβ associates with chromatin in SH-SY5Ys.
SH-SY5Ys were treated with 5μM OA or DMSO control for 24 hours and 5μM MG132 for 16 hours to aid Pβ visualization. I then fractionated cells to determine chromatin association by DNase I incubation, as previously described. Contrary to data obtained from overexpression studies in HEK293s, endogenous Pβ was solely detected in the chromatin-associated fraction (Fig. 3-5A). In line with previous fractionations, Pβ chromatin association was comparable in DMSO- and 5μM OA-treated SH-SY5Ys. To further validate this result, I conducted a second independent fractionation that isolates chromatin-associated proteins by acid extraction. SH-SY5Ys were treated with DMSO.
or 5μM OIA for 24 hours and MG132 for 16 hours. This fractionation also isolated nucleoplasmic proteins whose fraction was validated by the enrichment of Aly, a nuclear export factor\textsuperscript{196}, and disenrichment of other markers: cytosolic Tubulin, mitochondrial ATP5α, chromatin-associated H3, and the nuclear structural protein, Lamin B1. In agreement with the previous chromatin fractionation, Pβ was highly enriched in the chromatin-associated fraction, regardless of presence of mitochondrial stress (Fig. 3-5B). Taken together, these results advance studies establishing Pβ nuclear localization by demonstrating that endogenous Pβ peptide associates with chromatin in the nucleus and may act at the level of transcription to regulate mitochondrial biogenesis.
Figure 3-5: Endogenous Pβ associates with chromatin in SH-SY5Ys: A SH-SY5Y cells were treated with 5μM OIA or DMSO for 24 hours. Cells were also treated with 5μM MG132 for 16 hours. SH-SY5Ys were fractionated as previously described by Mirzoeva and Petrini with the additional step of DNase I incubation. The fractionation resulted in a Tot (total) fraction; a TS (triton soluble) fraction containing cytosolic and mitochondrial proteins; a DS (DNase I soluble) fraction containing chromatin-associated proteins; and an NP (nuclear pellet) fraction containing nuclear DNase I insoluble proteins. B A second fractionation isolating chromatin-associated proteins was conducted on SH-SY5Ys treated with 5μM OIA or DMSO for 24 hours and MG132 for 16 hours, as previously described\textsuperscript{191}. The fractionation resulted in a Cyto (cytoplasmic) fraction; a Nuc Plas (nucleoplasmic) fraction; a Chr (chromatin-associated) fraction; and a Nuc Pellet (nuclear pellet fraction). ATP5α is the mitochondrial marker. H3 is the chromatin-associated marker. Lamin B1 is the nuclear pellet marker. Tubulin (major component of cytoskeleton) is the cytosolic marker. Aly (mRNA export factor) is the nucleoplasmic marker.
3.4 MG132 does not affect Pβ expression or localization

Our lab has previously shown that endogenous PARL undergoes β cleavage and subsequent Pβ expression upon mitochondrial stress in SH-SY5Ys (Fig. 3-2)\textsuperscript{179}. However, as shown in sections 3.2 and 3.3, results in this thesis found no difference in Pβ production in the presence or absence of OlA, a mitochondrial stressor, at concentrations that were previously shown to be sufficient and necessary for PARL β cleavage. One hypothesis is that this incongruity may be due to incubation with MG132, a common proteasome inhibitor, to increase Pβ stability and detection. It is possible that treating cells with 5μM MG132 for 16 hours may indirectly cause mitochondrial stress and subsequent β cleavage and Pβ production. This is supported by previous studies reporting observations of mitochondrial stress with prolonged treatment of MG132\textsuperscript{197–200}. Indeed, Maharjan et. al. have shown that an 8 hour treatment with 10μM MG132 in Chinese hamster ovary cells resulted in loss of $\Delta \Psi_m$ and increased mitochondrial ROS\textsuperscript{200}.

To determine whether MG132 treatment affected Pβ production, I treated SH-SY5Ys with 5μM OlA or DMSO for 24 hours and 5μM MG132 or DMSO for 16 hours. To test whether PARL β cleavage would increase with additional mitochondrial stress, I also treated SH-SY5Ys with 20μM OlA for 24 hours and 5μM MG132 for 16 hours. Chromatin fractionations were subsequently conducted on treated cells. MG132 treatment did not affect Pβ detection, as previously suggested, nor did it affect Pβ production as I had hypothesized (Fig. 3-6). Thus, future studies of endogenous Pβ in SH-SY5Ys should omit MG132 treatment. In agreement with section 3.3, detected Pβ was heavily enriched in the chromatin-associated fraction regardless of the presence or absence of mitochondrial stress when compared to the chromatin-associated fraction control, H3 (Fig. 3-6).
Additionally, there was no noticeable difference in Pβ detection with the added mitochondrial stress in the form of 20μM OIA in comparison to 5μM OIA or DMSO control.

Figure 3-6: MG132 does not affect Pβ detection or localization in SH-SY5Ys: SH-SY5Ys were treated with 5μM OIA, 20μM OIA, or DMSO for 24 hours. Cells were also treated with 5μM MG132 or DMSO for 16 hours. SH-SY5Ys were fractionated as previously described by Mirzoeva and Petrini to obtain a TS (triton soluble) fraction containing cytoplasmic and mitochondrial proteins and DS (DNase soluble) fraction containing chromatin-associated proteins. ATP5α is the mitochondrial marker. H3 is the chromatin-associated marker. Actin (major component of cytoskeleton) is the cytosolic marker.
3.5 Endogenous PARL β cleavage occurs in absence of mitochondrial stress in SH-SY5Ys

Previously, our lab has shown that PARL β cleavage is virtually undetectable in SH-SY5Ys without mitochondrial stress whereas 2.5μM OIA for 24 hours is sufficient to induce cleavage (Fig. 3-2)\(^{179}\). However, my results presented thus far have consistently shown that Pβ, a product of PARL β cleavage, is easily detected in the absence of stress. Furthermore, Pβ detection is comparable in DMSO, 2.5μM, 5μM, 10μM, and 20μM OIA-treated conditions. These concentrations are more than sufficient to cause mitochondrial stress as 1μM OIA treatment in undifferentiated SH-SY5Ys inhibits mitochondrial respiration\(^{201}\). These results suggest that Pβ production in SH-SY5Ys is insensitive to mitochondrial stress. As SH-SY5Ys are particularly susceptible to cell line changes, I hypothesized that PARL β cleavage may be altered in current SH-SY5Ys in comparison to published data from our group\(^{180}\).

To compare PARL β cleavage in SH-SY5Ys, I treated cells with 5μM OIA or control DMSO for 24 hours. As previously described\(^{179}\), endogenous PARL was then immunoprecipitated from cells using an antibody specific to the C-terminus of PARL, which is capable of detecting full length and N-terminally-cleaved forms of PARL. PARL immunoprecipitation (IP) is necessary to detect full-length PARL and β PARL and to decrease relative background. A parallel IP was conducted without PARL antibody to control for possible nonspecific interactions. As expected, no bands were detected in this negative control (Fig 3-7). Contrary to previously published results but congruous with fractionations conducted in this thesis, β PARL was reliably detected in DMSO-treated SH-SY5Ys, indicating that PARL β cleavage occurs constitutively in the absence of
mitochondrial stress (Fig. 3-7). Additionally, β cleavage was comparable in DMSO and 5μM OIA conditions, indicating that PARL β cleavage is not responsive to mitochondrial stress in SH-SY5Ys (Fig. 3-7).

**Figure 3-7: Endogenous PARL β cleavage occurs in the absence of mitochondrial stress in SH-SY5Ys:** SH-SY5Ys were treated with 5μM OIA or DMSO for 24 hours. PARL was immunoprecipitated (IP) to increase signal. PARL β cleavage was monitored by ratio of full length PARL (upper band) to β PARL (lower band). ATP5α is the loading control.
Chapter 4
CONCLUSIONS & FUTURE DIRECTIONS

4.1 Brief Summary of Results

Previous reports have proposed that the PARL β cleavage product Pβ localizes to the nucleus and instigates mitochondrial biogenesis\textsuperscript{177,180}. In this thesis I have supported and extended these findings by demonstrating that free untagged Pβ, which originates in the mitochondrial matrix, translocates to the nucleus. Importantly, I have established that endogenous nuclear Pβ localization is maintained in SH-SY5Ys, a common PD model cell line. A previous study demonstrated that Pβ may upregulate transcription of mitochondrial biogenesis genes\textsuperscript{180}. To this end, I have provided evidence that endogenous Pβ interacts with chromatin in the nucleus, which suggests that Pβ affects nuclear mitochondrial gene expression transcriptionally, although it is unclear whether this interaction is direct or indirect. Additionally, I have shown that MG132, a proteasome inhibitor, is not required to stabilize endogenous Pβ in SH-SY5Ys as previously suggested. Finally, I demonstrated that PARL undergoes β cleavage in current SH-SY5Ys without the addition of mitochondrial stress, contrary to previous reports\textsuperscript{179}. Together, these data support the hypothesis that the small mitochondrial peptide Pβ acts as a transcriptional regulator by demonstrating that endogenous Pβ stably translocates to the nucleus where it associates with chromatin.
Figure 4-1: Model of PARL coordination of mitophagy and mitochondrial biogenesis: In response to mitochondrial stress, PARL is dephosphorylated and β cleavage occurs, resulting in β PARL and Pβ. β PARL’s reduced proteolytic activity for PINK1 results in PINK1 accumulation on damaged mitochondria and initiation of mitophagy prior to global depolarization. Conversely, Pβ relocalizes from the mitochondrial matrix to the nucleus where it associates with chromatin to instigate mitochondrial biogenesis by upregulation of mitochondrial genes, including PGC-1β, NRF1, and MFN1/2.
4.2 Model: PARL as a Coordinator of Mitophagy and Mitochondrial Biogenesis

The coordinated regulation of mitophagy and mitochondrial biogenesis, two processes that control overall mitochondrial mass in the cell, is critical to maintain both mitochondrial and cellular health. Our group proposes that through regulated cleavage of the IMM intramembrane protease, PARL synchronistically activates mitochondrial biogenesis and mitophagy in response to mitochondrial stress (Fig.4-1).

In healthy, polarized mitochondria, PARL cleaves PINK1 in the IMM, which results in subsequent PINK1 cytoplasmic relocalization and rapid proteasomal degradation. The current understanding is that PARL is not involved with the initiation of PINK1/Parkin-mediated mitophagy as PINK1 import to the IMM is disrupted by mitochondrial depolarization. However, we have previously proposed that PARL may initiate PINK1/Parkin-mediated mitophagy prior to mitochondrial depolarization. Our group has shown that PARL β cleavage is sensitive to various mitochondrial stresses, including ATP depletion and ROS production. β PARL, the N-terminally cleaved product of β cleavage, has reduced catalytic efficiency for PINK1 and instigates mitochondrial fragmentation, a necessary primer for mitophagy. In our model, increased β PARL production in response to mitochondrial stress leads to less efficient PINK1 cleavage. Inefficient PINK1 cleavage results in PINK1 OMM accumulation and consequent mitophagic initiation prior to global mitochondrial depolarization (Fig.4-1).

The other product of PARL β cleavage, the 25 amino acid peptide Pβ, has also been proposed to play a role in mitochondrial biogenesis. Treatment of human myotubes with Pβ resulted in upregulation of mitochondrial biogenesis and mitochondrial
fusion genes and increased mitochondrial mass\textsuperscript{180}. Pβ has been proposed to function in the nucleus, as suggested by two studies determining Pβ-GFP localization\textsuperscript{177,180}. My thesis supports these studies by demonstrating that untagged, endogenous Pβ stably translocates from the mitochondrial matrix to the nucleus. More excitingly, data indicates that Pβ associates with chromatin, which suggests that Pβ’s mechanism of regulating mitochondrial biogenesis occurs at the transcriptional level (Fig.4-1).

Thus, we propose that in response to mitochondrial stress, PARL β cleavage simultaneously activates mitophagy via β PARL and mitochondrial biogenesis via Pβ to ensure mitochondrial turnover to maintain a healthy mitochondrial network.

4.3 Perspectives

4.3.1 Pβ Translocates to the Nucleus

Two studies have shown that Pβ-GFP localizes to the nucleus\textsuperscript{177,180}. These studies are partially confounded by the use of GFP fusion constructs, as GFP itself has been documented to localize to the nucleus\textsuperscript{181}, and the usage of constructs expressed in the cytosol, as it is unclear whether Pβ is capable of exiting mitochondria. These issues were circumvented by expressing mitochondrial-localized PARL constructs that produce untagged Pβ upon β cleavage. Fractionation data indicates that Pβ, which is physiologically produced in the mitochondrial matrix, is enriched in the nuclear fraction, thus corroborating previously published studies. More excitingly, further experiments showed that endogenously-expressed Pβ also localizes to the nucleus, indicating that this process is physiologically conserved. Previous studies from our group suggests that endogenous Pβ is rapidly degraded by the proteasome. In contrast, my data
demonstrates that MG132, a proteasome inhibitor, is dispensable for endogenous Pβ detection. This suggests that Pβ is not significantly degraded by the proteasome and instead largely accumulates in the nucleus; however it does not negate the possibility that Pβ is a substrate for other cytoplasmic peptidases.

4.3.2 Pβ Associates with Chromatin

One report in 2010 by Civitarese et al implicates Pβ in mitochondrial biogenesis, although its putative mechanism of action has not yet been elucidated\textsuperscript{180}. Data in this thesis indicates that Pβ peptide associates with chromatin, which suggests that Pβ transcriptionally regulates mitochondrial biogenesis. Historically, small peptides have been observed to strongly associate with DNA in spite of chromatin deproteinization with NaCl, SDS, chloroform/isoamyl alcohol and phenol\textsuperscript{202,203}. Interestingly, Pβ chromatin association also appears to be fairly strong as lysis protocols previously documented to solubize various DNA-bound complexes were unable to extract Pβ from chromatin\textsuperscript{204–206}. These results immediately raise multiple questions, including what the effect of this interaction is on immediate gene transcription. One study demonstrated that global peptide removal from DNA by alkaline extraction resulted in increased \textit{in vitro} transcription in comparison to deproteinized DNA preparations\textsuperscript{202}. This study suggests that the majority of chromatin-bound peptides act in an inhibitory fashion. However, the majority chromatin-bound peptides in this study are suggested to be approximately 1kDa\textsuperscript{202}, smaller than the 25 amino acid Pβ. If Pβ acts as a transcriptional repressor, it may increase expression of mitochondrial biogenesis and fusion genes by inhibiting mitochondrial biogenesis repressors. However, it is also possible that Pβ directly binds
chromatin to act as an activator or interacts with an undetermined protein complex to influence gene expression.

4.3.3 Experiments in SH-SY5Ys Produce Variable Results

Our group has previously demonstrated that PARL β cleavage is tightly controlled in SH-SY5Ys; it is undetectable by immunoblotting unless induced by various mitochondrial stresses\textsuperscript{179}. Titration of the ATP synthase inhibitor, OIA, demonstrated that concentrations as low as 2.5μM were sufficient to trigger PARL β cleavage\textsuperscript{179}. In contrast, under the same conditions and with the same reagents, I detected equivalent amounts of PARL β cleavage in SH-SY5Ys treated with or without 5μM OIA. A notable difference between the two experiments is the source of SH-SY5Ys. The tumour-derived SH-SY5Ys are notoriously susceptible to cell line changes due to factors including passage number, confluence, differentiation, and differences in subculturing. A prominent consideration with SH-SY5Ys is the presence of two morphologically distinct phenotypes that were inherited from the parental SK-N-SH cell line: neuroblast-like cells and epithelial-like cells\textsuperscript{207–209}. Another consideration is the existence of both adherent and floating cell populations\textsuperscript{209}. Both of these characteristic ratios of cell populations may be drastically altered with differing subculturing methods or due to drift over time. Of note, both subculturing methods used by our group previously and in this thesis maintained both floating and adherent cell populations. Nevertheless, if difficulties in reproducing results in SH-SY5Ys are due to tendency of the cell line to change over time, alternative cell models should be evaluated to ensure project continuity.

One cell model that should not be pursued is the overexpression system in HEK293s. Data in this thesis indicates that overexpressed Pβ does not associate with
chromatin upon nuclear translocation, as endogenous Pβ in SH-SY5Ys does, but instead accumulates in the insoluble nuclear fraction in HEK293s. This difference may be due to disparity in the two cell lines’ expression profiles or in expression levels of Pβ. If it is the former, this may suggest that Pβ chromatin association is cell line-specific and/or dependent on other cell-specific actors to mediate its association. If it is the latter, this may suggest that Pβ chromatin association is saturable.

4.3.4 Pβ: a Peptide with Purpose?

As a potential regulator of mitochondrial biogenesis, Pβ joins the small but recently growing group of peptides implicated in mitochondrial quality control and homeostasis. One of the founding members of this group is humanin, a 21 or 24 amino acid peptide (depending on the location of its translation) expressed from an open reading frame (ORF) in the mitochondrial 16S rRNA, has been demonstrated to be anti-apoptotic, anti-inflammatory, and neuroprotective by inhibiting various protein-binding partners. Given their roles in maintaining mitochondrial homeostasis, humanin and other mitochondrial peptides are targets for therapeutic development against various diseases and conditions including neurodegeneration and diabetes.

Pβ is not the first peptide produced by proteolysis that has been proposed to regulate transcription. Amyloid-β42 (Aβ42), a 44 amino acid product of amyloid precursor protein (APP) proteolysis and a hypothesized central player in Alzheimer’s disease (AD) pathogenesis, has been demonstrated to localize to the nucleus and is suggested to directly associate with gene promoters to repress their expression. While Pβ is not pathogenic, it will be interesting to determine whether any parallels in transcriptional regulation may be identified in the future.
4.4 Future Directions

Work presented in this thesis further characterizes Pβ, a product of PARL β cleavage, by providing a foundation to elucidate its putative mechanism of action in affecting mitochondrial biogenesis, a pathway that can replace damaged mitochondria degraded by mitophagy. However, the work presented in this thesis proposes more questions than it has answered. Presented below are some of the many questions that remain.

4.4.1 What is the Phosphatase Responsible for β Cleavage?

The mitochondrial matrix kinase, PDK2, was identified as the kinase responsible for the phosphorylation of PARL’s N-terminus to inhibit PARL β cleavage. However, the phosphatase responsible for lifting these repressive post-translational modifications remains unknown. PARL’s putative phosphatase is a key regulator of PARL β cleavage and Pβ production, and by extension, a key regulator in mitochondrial quality control and homeostasis. The most obvious candidate is Pyruvate Dehydrogenase Phosphatase (PDP), which reverses PDK phosphorylation of Pyruvate Dehydrogenase\(^{220}\). To date, two isoforms of PDP have been identified: PDP1, which is most highly expressed in the heart, brain, and testis; and PDP2, which is most highly expressed in the heart, liver, and kidney\(^{220–222}\). It is possible that like PDK2, only one isoform is responsible for PARL dephosphorylation. Another possibility is that another unidentified phosphatase located in the matrix or IMM dephosphorylates PARL. To identify the PARL phosphatase, matrix/IMM phosphatases may be systematically overexpressed and cells may be monitored for increased PARL β cleavage by western blot. Phosphatases whose altered expression affects PARL β cleavage may be validated for PARL interaction by
coimmunoprecipitation (co-IP), similar to Shi and McQuibban’s approach to identify PARL’s kinase, PDK2\(^{179}\).

**4.4.2 What Allows Pβ Export from Mitochondria?**

As Pβ’s effect on mitochondrial biogenesis likely necessitates its nuclear localization, mitochondrial Pβ retention or export may serve as an additional layer of regulation on Pβ activity. As Pβ possesses six highly conserved positively charged residues, it is an extremely poor candidate for unfacilitated diffusion across the highly impermeable IMM and almost certainly requires a dedicated transporter. Two identified putative peptide exporters in the IMM are ATP-binding cassette (ABC) B8 (ABCB8) and ABCB10, which belong to a highly heterogeneous subfamily of ABC half transporters involved in intracellular trafficking and compartmentalization of peptides\(^{223}\). ABCB10 and its orthologues are the better studied of the pair. Studies have shown that the ABCB10 orthologues, multi-drug resistance-like (MDL1) from *Saccharomyces cerevisiae* and HAF-1 from *Caenorhabditis elegans*, are conserved in their function as mitochondrial peptide exporters\(^{224–227}\). Peptides exported by the yeast MDL1 are estimated to be approximately 0.6-2.1 kDa, the upper limit of which is comparable to Pβ’s expected molecular weight\(^{224}\).

To determine whether ABCB8 and/or ABCB10 facilitates Pβ efflux from mitochondria, either transporter may be knocked down by siRNA or knocked out by CRISPR/Cas9 gene editing. Resultant Pβ localization may be observed by subcellular fractionation and western blot.

Data from this thesis and others show that Pβ is strongly detected in the nuclear fraction in HEK293s under all conditions except when treated with the highest concentrations of OIA, which depletes mitochondrial ATP. Under this condition, reduced Pβ production was observed despite increased PARL β cleavage. We hypothesize that
the paradoxical reduction in Pβ detection at high levels of mitochondrial stress reduces Pβ-mediated mitochondrial biogenesis in response to significant mitochondrial damage, in part because mitochondrial biogenesis is a highly energy-consuming process. If ATP-dependent ABCB8/10 are Pβ exporters, nuclear Pβ reduction may be a result of impaired efflux from the mitochondrial matrix due to mitochondrial ATP depletion followed by Pβ degradation by proteases in the matrix.

Mitochondrial matrix peptide export is largely focused on traversing the IMM. Peptides are hypothesized to exit mitochondria by diffusion through OMM porins or TOM, although these mechanisms require clarification\textsuperscript{223,228–230}. As a mitochondrial peptide, Pβ may be exported across the OMM in a similar fashion.

4.4.3 Does Pβ Undergo any Post-Cleavage Modifications?

A consistent observation when visualizing Pβ by western blot is the recognition of two specific bands. Importantly, these bands ran at a similar molecular weight to purified phosphorylated and unphosphorylated Pβ (Fig.4-2). This led us to speculate that Pβ exists in two populations in the nucleus – phosphorylated and unphosphorylated. When

![Figure 4-2: Pβ doublet and phosphorylated/unphosphorylated Pβ run at similar molecular weights. A Western blot of PARL β cleavage and Pβ production in HEK293s expressing WT PARL or β PARL. A Pβ doublet is detectable in cells expressing WT PARL, which undergoes β cleavage, but not in cells expressing β PARL, which does not undergo β cleavage. B Western blot of purified synthetic phosphorylated and unphosphorylated Pβ, which run at a similar molecular weight as the Pβ doublet detected in cell lysates.](image)
attached to PARL, Pβ’s phosphorylation sites, S65, T69, S70, additively inhibit PARL β cleavage\textsuperscript{178}. Thus, we hypothesize that putative Pβ phosphorylation likely occurs post-cleavage. To determine whether the upper Pβ-specific band is due to phosphorylation, cell lysates may be dephosphorylated by incubation with non-specific phosphatases such as calf intestinal alkaline phosphatase (CIAP) or protein phosphatase-1 (PP1) prior to SDS-PAGE and monitored for decrease of the upper Pβ-specific band by western blot. Alternatively, PARL constructs where phosphorylation sites have been mutated to non-phosphorylatable alanine may be generated and monitored by western blot to determine whether Pβ produced by these constructs is visualized as a doublet or a singlet. As a more unbiased approach, Pβ may be immunoprecipitated from cell lysates prepared using strong detergents and post-translational modifications may be identified by mass spectrometry\textsuperscript{231,232}.

If a subpopulation of Pβ is phosphorylated, an immediate concern, aside from the identity of the putative kinase, is whether phosphorylation alters Pβ activity. To this end, future experiments should include conditions where Pβ phosphorylation is inhibited. As overexpression data in HEK293s differs from endogenous data in SH-SY5Ys, it would be necessary to generate cell lines wherein PARL phosphorylation sites are mutated to alanine rather than rely on overexpression systems. These missense mutations may be generated via the CRISPR-Cas9 gene-editing system and homologous repair incorporating templates containing our mutations of interest.

**4.4.4 Does Pβ Interact with Other Proteins?**

One method to determine Pβ mechanism and function is to identify its potential protein interactors. As Pβ associates with chromatin, an immediate question is whether this interaction is mediated by other proteins. While certain peptides are proposed to bind
directly to DNA to inhibit transcription, we cannot negate the possibility that Pβ’s putative role in transcriptional regulation may be mediated by association with transcription factors, modulators, scaffolds, or other proteins. Ideally, Pβ interactors would be identified by Pβ immunoprecipitation followed by mass spectrometry analysis (IP-MS). However, I have been unable to successfully immunoprecipitate Pβ from chromatin-associated fractions (data not shown). It is possible that the epitope that Pβ antibody recognizes is sterically unavailable when Pβ is bound to its interactor. This likelihood is increased due to Pβ’s comparatively small size. Alternatively, Pβ interactors may be identified by affinity chromatography. Briefly, synthesized Pβ may be immobilized on resin, followed by pull down assays with whole cell lysates. Eluted proteins may be identified by MS and validated for physiological Pβ interaction by co-IP. To determine what residues or post-translational modifications mediate these interactions, parallel experiments may be conducted with Pβ synthesized with mutated key residues or additional modifications.

Another method to identify Pβ interactors involves size exclusion chromatography (SEC) in cells where Pβ is expressed or abolished. Proteins in SEC fractions where Pβ is expected to be eluted may be identified by MS and compared to identify putative Pβ interactors, which would then be validated by co-IP. A benefit of this approach in comparison to affinity chromatography is the likelier identification of physiological interactors.

In addition to identifying putative facilitators of Pβ chromatin association, both suggested methods should recognize interactors that may be involved in Pβ transport and stability in the mitochondria, cytosol and nucleus. Depending on the sensitivity of
these assays, Pβ modulators, such as its putative kinase or phosphatase, may also be identified.

4.4.5 What Genes Associate with Pβ?

Another immediate goal that arises from observations of Pβ chromatin association is to identity Pβ-binding sequences. While human myotubes transfected with Pβ showed increased expression of select mitochondrial biogenesis and fusion genes (PGC-1β, NRF1, MFN1/2), it is unclear whether upregulation of these genes is due to direct or indirect activation. To identify Pβ-associated genes, chromatin immunoprecipitation (ChIP) experiments may be conducted to identify Pβ-associated DNA sequences via high-throughput sequencing (ChIP-seq). This data will help to determine Pβ DNA-binding specificity and characterize possible Pβ consensus sequences or motifs. Importantly, the category of associated genes will shed light on Pβ’s control of mitochondrial biogenesis. Furthermore, this data will determine whether Pβ regulates multiple mitochondrial biogenesis genes or mediates their upregulation indirectly by associating with master regulator genes like PGC-1β.

Of note, I was unable to immunoprecipitate endogenous Pβ from SH-SY5Ys treated with 0.75% or 1% formaldehyde using a protocol adapted from Donner et al. using Pβ-specific antibody. A possible solution is to express a tagged version of Pβ, such as Pβ-GFP, which has been demonstrated to localize to the nucleus. Using this system, Pβ may be immunoprecipitated by its epitope tag. Importantly, tagged Pβ must be validated for nuclear localization and chromatin association as tags may interfere with these activities. For instance, Pβ tagged with 3XFLAG at the N- or C-terminus is exclusively cytosolic (data not shown) and thus is a poor construct to characterize Pβ.
Chromatin fractionations and ChIP experiments are not reliable methods to establish direct Pβ DNA binding, despite novel proposed bioinformatics methods to distinguish direct and indirect interactions from ChIP-seq data\textsuperscript{218}. To determine whether Pβ can directly associate with DNA, an \textit{in vitro} electrophoretic mobility shift assay (EMSA; also known as gel shift assay) may be conducted with synthesized Pβ and labelled DNA probes whose sequences are identified by ChIP-Seq as putative Pβ-associated sites. If Pβ directly associates with these sequences, a band shift should be apparent when both Pβ and the probe are present compared to the probe alone.

If Pβ-DNA direct interaction is verified by EMSA, this technique will be a powerful tool to identify the core amino acid and nucleotide residues responsible for Pβ chromatin association. As both Pβ peptide and the DNA probe would be synthesized, both may be systematically mutated and tested by EMSA for changes in band shifts. Additional post-translational modifications, such as phosphorylation, may also be manipulated.

\textbf{4.4.6 What is the Effect of Pβ on Mitochondrial Biogenesis?}

Although Civitarese \textit{et al.} have identified a handful of mitochondrial genes whose increased expression is dependent on Pβ, the full scope of Pβ-mediated gene expression remains to be elucidated. To better clarify Pβ’s role in mitochondrial biogenesis, we propose to employ RNA-sequencing (RNA-Seq), which is a less biased approach to compare transcriptome profiles of Pβ-expressing and Pβ-deficient cells. As overexpressed Pβ does not appear to associate with chromatin, I intend to pursue this endeavor in cells expressing endogenous levels of Pβ. For comparison, cell lines with abolished Pβ expression must be established. Using the CRISPR/Cas9 gene-editing system combined with homologous repair using designed templates, PARL β cleavage, and thus Pβ production, may be abolished by introducing the PD-linked substitution S77N.
(generating full length PARL) or by deleting Pβ sequence in-frame (generating β-PARL). Alternatively, mutations of Pβ’s putative NLS (R54T, K55S, R58T, K59S\cite{177}) may be introduced to prevent nuclear Pβ localization while simultaneously maintaining PARL β cleavage and protease activity. The transcriptome profiles of these cell lines in comparison to WT cells will also help delineate transcriptional changes due to Pβ production versus PARL activity. Upregulated mitochondrial gene transcripts should be validated for consequent increases in mitochondrial protein expression by western blot. In turn, Pβ-dependent increases in mitochondrial biogenesis should be verified by various mitochondrial biogenesis assays as described by Civitarese et al\cite{180}.

### 4.5 Conclusions

The importance of PARL β cleavage in mitochondrial and cellular health is emphasized by the PD-linked mutation, S77N, which abolishes this N-terminal cleavage event and expression of its products. One of these products, the 25 amino acid peptide Pβ, is suggested to localize to the nucleus to regulate mitochondrial biogenesis. In this thesis, I have demonstrated that endogenous Pβ is exported from mitochondria and accumulates in the nucleus. Additionally, data suggests that Pβ associates with chromatin, offering a potential mechanism of mitochondrial biogenesis regulation. These exciting advances in characterizing Pβ peptide are accompanied by new, pressing questions whose imminent answers will shed light on coordination of mitochondrial quality control pathways to maintain mitochondrial health, and ultimately cellular and human health.
REFERENCES


122. Lin, J., Puigserver, P., Donovan, J., Tarr, P. & Spiegelman, B. M. Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel


APPENDIX

Pβ Deletion Outline Using CRISPR/Cas9 System

A

<table>
<thead>
<tr>
<th>Guide</th>
<th>score</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guide #1</td>
<td>80</td>
<td>GTCCCTGGTCTGATCTTCCGAGG</td>
</tr>
<tr>
<td>Guide #2</td>
<td>78</td>
<td>AACCTGAAGATCAGACCCAGGG</td>
</tr>
<tr>
<td>Guide #3</td>
<td>76</td>
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<tr>
<td>Guide #4</td>
<td>73</td>
<td>TATGCTTCACTTGTCCCTTGG</td>
</tr>
</tbody>
</table>

Appendix 1-1: Pβ-targeted gRNAs knock down overexpressed PARL-FLAG in HEK293s: A Top CRISPR gRNA design hits for second exon of PARL, which includes Pβ nucleotide sequence, as generated by the CRISPR Design Tool (http://crispr.mit.edu/). Scores are calculated by faithfulness of on-target activity minus off-target hit scores. B PARL second exon is highlighted. Pβ nucleotide sequence is underlined. The estimated Cas9 cleavage site if Guide #1 or #2 is used is coloured corresponding to the gRNA in A. C pX458 plasmids containing Cas9-GFP and Pβ-targeted gRNAs corresponding to Guides #1 (gRNA1.1 and 1.2) and #2 (gRNA 2.1 and 2.2) were cotransfected with PARL-FLAG in HEK293s. Pβ-targeted gRNAs decrease PARL-FLAG expression in comparison to cells cotransfected with pX458 alone (empty).
A

β PARL ssODN:
gacgctactgttgttctcatctgttatatatgtgccccatataggtttaactctttattcaacaaaa
atgcggagcttttgattcctcctgttggagaaacagtcttttatccctcccctataaagg
agtctcataaaac

B

S77N PARL ssODN:
tttatgagactcctttataggataggagaaagagactgggcatcctgggtctgatcttcgaggttcaaccttc
tgggtgccttttc

Appendix 1-2: Pβ deletion homologous repair templates: A A homologous repair template to selectively delete Pβ sequence and generate β PARL was designed by sequencing a single-stranded oligodeoxynucleotides (ssODN) combining the 70 nucleotides immediately upstream and downstream of Pβ sequence. B A homologous repair template to introduce S77N mutation which abolishes β cleavage and generates full-length PARL was produced from a 140 nucleotide sequence encompassing S77, the expected Cas9 cleavage site, gRNA PAM sequence. Two point mutations were introduced: C→T (resulting in S77N mutation) and C→G (to mutate gRNA PAM sequence to inhibit Cas9 cleavage after homologous repair).
Appendix 1-3: Genomic DNA primer test was designed to validate β PARL clones:
A PARL second exon is CAPITALIZED. Pβ nucleotide sequence is highlighted. Primers were designed to flank Pβ upstream and downstream and are colour-coded and mapped to PARL second exon and immediately surrounding intron region. B Primer pairs amplified fragments from WT SH-SY5Y genomic DNA at sizes corresponding to expected sizes. If Pβ nucleotide sequence is deleted in β PARL clones, bands are expected to run roughly 75 bp faster.