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Review

# The mitochondrial rhomboid protease: Its rise from obscurity to the pinnacle disease-relevant genes☆

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ABSTRACT

The Rhomboid proteases belong to a highly conserved family of proteins that are present in all branches of life. In *Drosophila*, the secretory pathway-localized rhomboid proteases are crucial for epidermal growth factor (EGF) signaling. The identification of a mitochondrial-localized rhomboid protease shed light on other functions of rhomboid proteases including the maintenance of mitochondrial morphology and the regulation of apoptosis. More recent work has revealed other functions of the mitochondrial rhomboid protease in mitochondrial and cellular biology, failure of which have been implicated in human diseases. In this review, we will summarize the current knowledge and disease relevance of the mitochondrial-localized rhomboid protease. This article is part of a Special Issue entitled: Intramembrane Proteases.



**Contents**



*Abbreviations:* TMD, transmembrane domain; IMS, intermembrane space; PD, Parkinson's disease; T2D, type 2 diabetes

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## 1. Introduction

Proteolysis within the membrane bilayer is conceptually challenging, but several groups of proteases have evolved elegant and sophisticated mechanisms to conduct such a feat. In this review, we will discuss in detail the discovery and ongoing characterization of mitochondrial-localized rhomboid proteases. This family has emerged as a critical mediator of mitochondrial biology and is also deeply rooted in human disease etiologies.

### 1.1. Intramembrane proteolysis

Intramembrane proteolysis is a key regulatory mechanism conserved throughout evolution [1–4]. To date, intramembrane proteases can be classified into three major classes: (i) the site-2 metalloproteases (S2P), (ii) the signal peptide peptidase (SPP) and presenilin (PS) aspartyl proteases and (iii) the Rhomboid family of serine proteases. One of the major challenges of intramembrane proteolysis is the requirement for water in the hydrophobic lipid bilayer to allow for the hydrolysis of a peptide bond. Until recently, it was inconceivable that proteolysis can occur within the membrane. Intense research in the field has elucidated the structure, function and regulatory mechanisms of these intramembrane proteases. The S2P, SPP and PS proteases are highlighted in other reviews of this special issue. We will focus our review on the Rhomboid family of serine proteases, and more specifically, the mitochondrial sub-family of Rhomboids.

### 1.2. Identification of the Rhomboid superfamily of enzymes

Rhomboids are the newest class of intramembrane proteases and are a relatively new superfamily of proteins. The first rhomboid was identified in a genetic screen performed in *Drosophila*, where fly embryos of the mutant had a mis-shapened rhombus-like head skeleton. This mutant phenotype led to the naming of the gene as "rhomboid" [5]. It was later discovered that Rhomboid was also required for the establishment of the dorsal–ventral axis during oogenesis. It was proposed that the spatial localization of Rhomboid was important in selectively activating the epidermal growth factor (EGF) [6]. The identification of six other rhomboids in *Drosophila* and the finding that Rhomboid-1 activates the EGF-like protein, Spitz, by cleaving it in its transmembrane domain (TMD) defined a new family of intramembrane proteases [7–9]. It is now known that rhomboid proteases are indispensible regulators of EGF signaling in *Drosophila*. The Golgi-localized rhomboid proteases, Rhomboid-1, -2, -3 and -4 can activate EGF signaling *in vivo* by cleaving the EGF-like proteins Spitz, Keren and Gurken [10]. However, Rhomboids are also present in organisms that lack EGF signaling, and hence, must have additional conserved functions [11].

### 1.3. Identification of the mitochondrial rhomboid protease

The identification of a mitochondrial-localized rhomboid protease and its substrates shed light on another function of Rhomboids that is distinct from EGF signaling. The mitochondrial rhomboid proteases regulate mitochondrial morphology and function [12–17]. First identified in yeast as a protein required for the cleavage and maturation of cytochrome c peroxidase, Ccp1, the role of the mitochondrial rhomboid proteases in mitochondrial biology was not highlighted until the identification of Mgm1 as the other substrate of the yeast mitochondrial rhomboid protease, Rbd1/Pcp1 [12–14,18]. The mitochondrial rhomboid proteases are now known to be crucial regulators of mitochondrial dynamics and function. Impaired function of the mammalian mitochondrial rhomboid protease is associated with impaired mitochondrial function and quality control that are proposed to contribute to type 2 diabetes and Parkinson's disease (discussed below).

### 1.4. Structure and catalysis of mitochondrial-localized rhomboid proteases

The specificity of rhomboid proteases for their substrates is better characterized for the non-mitochondrial rhomboid proteases than it is for the mitochondrial rhomboid proteases, and is discussed in great detail in other reviews of this special issue. A recent study examining substrate specificity of the mitochondrial rhomboid proteases indicates that the yeast and human mitochondrial rhomboid proteases are not selective in the sequence that they cleave. Although hydrophobicity is required, the sequence can be highly variable. Replacing the entire rhomboid cleavage region (RCR) of Mgm1 with two different hydrophobic sequences not physiologically cleaved by Rbd1 did not alter the efficiency of Rbd1-dependent cleavage of Mgm1. However, this cleavage was dependent on a 13 amino acid stretch of negatively charged residues C-terminal to the RCR. Mutating these residues resulted in impaired Mgm1 cleavage by both the yeast and mammalian mitochondrial rhomboid proteases, demonstrating possible conservation of substrate recognition [21].

Although the Rhomboid family was discovered to be proteases only a decade ago, intense research has enabled us to learn more about their localization and biological functions *in vivo*. It is now clear that the Rhomboid superfamily includes the secretory pathway-localized rhomboid proteases, the inactive rhomboids (iRhoms), the mitochondrial-localized rhomboid proteases and more recently, the Derlin proteins [11,22]. In this review, we will focus on the mitochondrial-localized rhomboid proteases, summarizing the current findings on their substrates, regulatory mechanisms and disease-relevance.

## 2. The mitochondrial rhomboid proteases

### 2.1. The yeast mitochondrial rhomboid protease, Rbd1

The existence of a mitochondrial-localized rhomboid protease was first discovered in yeast in 2002. Rbd1/Pcp1 was found to be required for the cleavage and maturation of cytochrome c peroxidase, Ccp1 [18]. Shortly after, in 2003, three independent studies identified another substrate of Rbd1, the dynamin-like GTPase, Mgm1 [12–14]. The defect in the proteolytic processing of Mgm1 and Ccp1 in the Δ*rbd1* strain could by rescued by a plasmid-borne copy of WT Rbd1 but not a catalytically inactive mutant. More importantly, this defect could also be rescued by human PARL (presenilins-associated rhomboid-like), the human homolog of Rbd1 that also localized to mitochondria, although its function was still unknown at the time. This was the first indication that the localization and function of the mitochondrial rhomboid proteases are conserved from yeast to mammals [12]. Despite these important findings in yeast, Ccp1 and Mgm1 remain the only known substrates of the yeast mitochondrial rhomboid protease (Table 1). Although Ccp1 was the first identified substrate of Rbd1, Mgm1 has since become its key substrate in yeast and most studies have focused on understanding the regulation of Mgm1 processing and its biological function in mitochondrial membrane dynamics.

**Table 1**
List of mitochondrial rhomboids and their known substrates.

| Species | Rhomboid | Substrate | Function | Refs. |
|---|---|---|---|---|
| *Saccharomyces cerevisiae* | Rbd1/Pcp1 | Ccp1 | | [12,18] |
| | | Mgm1 | Mitochondrial fusion | [12–14] |
| *Drosophila melanogaster* | Rhomboid-7 | Opa1-like | Mitochondrial fusion, apoptosis | [16] |
| | | Pink1 | Mitophagy | [28] |
| | | Omi | Apoptosis | [28] |
| Mammals | PARL | PINK1 | Mitophagy | [43,53–55] |
| | | OMI | Apoptosis | [96] |
| | | PGAM5 | Apoptosis | [17] |

Mgm1 exists as two isoforms — the inner membrane (IM)-tethered form, long-Mgm1 (l-Mgm1), and the Rbd1-cleaved, intermembrane space (IMS)-soluble form, short-Mgm1 (s-Mgm1). A proper balance of l-Mgm1 and s-Mgm1 is crucial for proper mitochondrial fusion [23,24]. The phenotype of cells lacking Rbd1 mirrors that of cells lacking Mgm1. They lose their mitochondrial DNA (mtDNA), resulting in a petite strain with fragmented mitochondria characteristic of mitochondrial fusion mutants [12–14]. However, an *in vivo* mitochondrial fusion assay demonstrated that mitochondria in cells lacking Rbd1 were still able to fuse, indicating that Rbd1, unlike Mgm1, is not an obligate member of the mitochondrial fusion machinery despite being an important regulator of mitochondrial fusion [13]. Recent studies have shown that mitochondria in cells lacking Psd1, a protein that promotes mitochondrial fusion, can still undergo complete fusion even though they appear fragmented or aggregated [25,26]. Since the mitochondrial fusion machinery is still present in Δ*rbd1* mitochondria, it is possible that fusion can still occur, but perhaps at a reduced rate, similar to that of Δ*psd1* mitochondria [26]. Nevertheless, the abnormal mitochondrial morphology in Δ*rbd1* cells and the role of Rbd1 in the cleavage of Mgm1 strongly implicates the mitochondrial rhomboid protease as a critical regulator of mitochondrial membrane fusion.

### 2.2. The Drosophila mitochondrial rhomboid protease, Rhomboid-7

The identification of a mitochondrial rhomboid protease in yeast led to further studies that discovered functional conservation in other organisms including *Drosophila melanogaster*. Over-expression of the *Drosophila* mitochondrial rhomboid protease, Rhomboid-7, resulted in increased processing of *Drosophila* optic atrophy 1-like (Opa1-like), the fly ortholog of yeast Mgm1 [16]. Rhomboid-7 is also required for mitochondrial fusion during fly spermatogenesis and muscle maturation. *Rhomboid-7* mutant flies have a reduced lifespan, difficulties walking and are unable to fly [15]. In contrast, the over-expression of Rhomboid-7 resulted in severe mitochondrial aggregation in the larval brain, reduced ATP levels and dysfunctional neuromuscular junctions [16]. Similar to their yeast counterparts, mutations in *rhomboid-7* result in phenotypes similar to that of flies with mutations in *opa1-like* [15]. Loss-of-function mutations in *rhomboid-7* also result in severe light-induced neurodegeneration of photoreceptors, a phenotype characteristic of the human disease dominant optic atrophy, a result of mutations in the human gene OPA1 [15,27]. In addition, the over-expression of Rhomboid-7 resulted in increased apoptosis, a process that is known to be regulated by mammalian OPA1 [16]. The results from these studies strongly support the genetic analyses that rhomboid proteases are highly conserved, and that their function and substrates are likely conserved throughout evolution [11].

The first evidence that the mitochondrial-localized rhomboid protease is implicated in Parkinson's disease (PD) came from studies in *Drosophila* that identified PTEN-induced putative kinase 1 (*pink1*), high temperature requirement A2 (*htrA2*, a serine protease, also known as *omi*) and *parkin* (an E3 ubiquitin ligase) as genetic interactors of *rhomboid-7* [28]. *Pink1*, *parkin* and *omi* are PD-linked genes; loss-of-function mutations in *PINK1* and *PARKIN* are linked to autosomal recessive PD, whereas mutations in *OMI* are associated with sporadic PD [29–31]. Rigorous *Drosophila* genetics had previously identified that *pink1* and *parkin* function in the same pathway, with *pink1* acting upstream of *parkin* [32]. Using a similar approach, Whitworth et al. determined that *rhomboid-7* interacts genetically with *pink1*, *parkin* and *omi*. Rhomboid-7 also interacts physically with Pink1 and Omi, acting as an upstream protease that is required for their cleavage and function (Table 1) [28]. Genetic epistasis analyses from this study also demonstrated that Omi might function downstream of Pink1, a result that was contradicted by that of a later study suggesting that *omi* is not a component of the *pink1*/*parkin* pathway [28,33]. Nevertheless, a growing body of evidence strongly supports the role of the mitochondrial rhomboid protease in the proteolytic processing of PINK1, implicating it as a key player in the pathogenesis of PD.

The first study indicating that Rhomboid-7 is required for proper mitochondrial morphology and apoptosis was published in 2006. Since then, only a handful of articles have further described the role of Rhomboid-7 in flies, possibly due to reduced interest in Rhomboid-7-mediated apoptosis in flies. Although *Drosophila* genetics is an incredibly powerful tool for studying genetic interactions, evidence indicates that the mechanism of apoptotic activation in *Drosophila* is not conserved in mammals. The release of cytochrome c from the mitochondrial IMS to the cytosol is a key step in the activation of apoptosis in mammalian cells (discussed in more detail in Section 4.2.1). However, cytochrome c appears to be dispensable for apoptosis in *Drosophila* cells [34]. Moreover, in mammalian cells, upon apoptotic stimulation, Omi translocates from mitochondria to the cytosol where it participates in the activation of pro-apoptotic proteins, whereas in *Drosophila*, Omi remains near mitochondria [35,36]. These differences, among others, could have dampened the interest in studying Rhomboid-7-mediated apoptosis in *Drosophila*, especially in the context of human diseases, tilting the balance of these studies to mammalian models.

### 2.3. The mammalian mitochondrial rhomboid protease, PARL

The mammalian mitochondrial rhomboid protease, presenilins-associated rhomboid-like (PARL) was originally identified in a yeast two-hybrid screen as a putative metalloprotease that interacts with the presenilins implicated in Alzheimer's disease [37]. It was later discovered that PARL is not a functional interacting partner of the presenilins, but rather is mitochondrial-localized, where it is required for cristae maintenance and the regulation of cytochrome c release during apoptosis [12,38,39]. The original screen, performed in 2001, preceded the first description of a mitochondrial-localized rhomboid protease in yeast in 2002. The original identification of PARL as an interactor of the presenilins could be a caveat of the classical yeast two-hybrid system, which is poorly suited to membrane proteins [40]. To overcome this, a membrane yeast two-hybrid system has since been introduced [41]. Nevertheless, the name PARL is still used today to refer to the mammalian mitochondrial rhomboid protease.

In mammals, PARL regulates mitochondrial cristae remodeling and cytochrome c release during apoptosis. Changes in mitochondrial cristae structure that supported cytochrome c release during apoptosis was observed earlier in *Parl*$^{-/-}$ cells than in WT cells, indicating that PARL plays an anti-apoptotic role in mammalian cells. *Parl*$^{-/-}$ mice have a reduced lifespan and increased muscle wasting due to increased apoptosis [39]. Recently, PARL was also shown to regulate mitochondrial adaptation to heat shock by protecting cells from apoptosis. Cells lacking PARL were more sensitive to thermal stress, releasing cytochrome c more quickly than WT cells [39,42]. In addition to regulating apoptosis, PARL also regulates mitochondrial morphology, although its role in this process is not well characterized. Over-expressing PARL in mammalian cells results in mitochondrial fragmentation, indicating that PARL regulates mitochondrial morphology [38,43]. However, the mitochondrial morphology of *Parl*$^{-/-}$ cells is similar to that of WT cells, suggesting that PARL is not involved in the maintenance of proper mitochondrial morphology [39,44]. Although the exact role of PARL in mitochondrial dynamics remains to be determined, recent research has uncovered another role of PARL in mitochondrial quality control that is becoming the focus of intense research (discussed further in Section 4.2.2). Since PARL is a protease, a better understanding of PARL functions can be achieved by identifying and characterizing more of its substrates. It is our belief that the newfound interest in PARL with respect to its role in PD will facilitate the identification of more PARL substrates.

The relationship between PARL and OPA1 was first studied in mice where it was observed that the shorter, IMS-soluble form of OPA1 was reduced in *Parl*$^{-/-}$ mouse mitochondria, suggesting that OPA1 is a substrate of PARL, similar to their yeast and *Drosophila* orthologs [39]. However, this was challenged by results from other studies indicating

that PARL was not required for the processing of OPA1 [44–47]. These conflicting data generated debate within the field as to whether PARL was truly required for the proteolytic processing of OPA1. In addition, other proteases, namely the m-AAA proteases paraplegin and AFG3L2 (albeit also with conflicting evidence), the ATP-independent protease OMA1, and the metalloprotease YME1 have also been implicated in OPA1 processing [44–50]. It is now widely accepted that OPA1 is unlikely to be a physiological substrate of PARL, and strong evidence points towards OMA1 and YME1 as more biologically relevant proteases that cleave OPA1 [45–49,51].

Similar to the *Drosophila* orthologs, mammalian PINK1 and HtrA2/Omi are also processed by PARL (Table 1) [43,52–57]. Although it was originally reported that PINK1 was not a substrate of PARL, it was later shown by the same group, as well as other groups, that PINK1 is indeed a substrate of PARL [43,52–56]. This discrepancy between observations was attributed to the lack of a good antibody for PINK1 and the fact that PINK1 is also processed by other unknown proteases in the absence of PARL [54,54]. Indeed, very recent work has shown that PINK1 is proteolytically processed in a manner dependent on the mitochondrial processing peptidase (MPP), ClpXP and AFG3L2 [45,50,56].

PARL cleaves PINK1 at A103 and PARL-dependent cleavage of PINK1 is required for its proper localization [43,54]. Upon import into mitochondria, the mitochondrial targeting sequence (MTS) of PINK1 is removed by MPP, resulting in the formation of an ~60-kD fragment [53,56]. Subsequent cleavage by PARL at A103 results in the formation of an ~52-kD fragment that is released back into the cytoplasm by some unknown mechanism [43,53–55]. In the absence of catalytically active PARL, PINK1 is primarily mitochondrial-localized [43,53,55]. PINK1 cleavage and cytoplasmic localization can only be detected in the presence of WT PARL and not the catalytically inactive S277G/A PARL mutant. More importantly, PARL-dependent cleavage of PINK1 is required for its downstream function of Parkin recruitment to damaged mitochondria to initiate mitophagy, a mitochondrial quality control mechanism (Section 4.2.2, Fig. 1) [43,58]. Cells expressing the S277G catalytically inactive PARL mutant had impaired PINK1-dependent Parkin recruitment to damaged mitochondria upon the induction of mitophagy. It has been proposed that dysfunctional mitophagy could contribute to the pathogenesis of PD [59]. The result that PARL enzymatic activity is required for Parkin recruitment during mitophagy suggests that PARL might be a key player in the prevention of PD. In support of this, a mutation in the N-terminus of *PARL* was identified in PD patients, implicating PARL as a PD-linked gene. This mutation resulted in an S77N amino acid substitution that resulted in impaired PARL function. PARL S77N was unable to induce PARL-dependent mitochondrial fragmentation and Parkin recruitment upon mitochondrial damage in MEFs lacking PARL [43]. This was the first indication that PARL is a PD-linked gene. The role of PARL in disease will be discussed in more detail in Section 4 (PARL and human diseases).

Another recently identified substrate of PARL is phosphoglycerate mutase 5 (PGAM5), a mitochondrial Ser/Thr protein phosphatase, implicated in apoptosis (Table 1). Upon the loss of mitochondrial membrane potential, PARL cleaves PGAM5 in its TM domain at S24. Prolonged exposure to the decoupler, CCCP, resulted in increased cleavage of PGAM5 with a concomitant accumulation of full-length PINK1. This result suggests that mitochondrial damage could be a signal for PARL to cleave another substrate, resulting in a different downstream effect that may signal a change in mitochondrial health. Indeed, PINK1 and PGAM5 are not competitive substrates of PARL. Reducing the expression of PINK1 by siRNA did not increase PARL-dependent cleavage of PGAM5. Together, these data suggest that the function of PARL changes when mitochondrial health deteriorates, highlighting the importance of the mitochondrial rhomboid protease in regulating the responses to mitochondrial damage [17].

The discovery that mitochondrial dynamics plays a crucial role in the cellular response to mitochondrial damage (either by apoptosis or mitophagy) suggests that PARL might be the master regulator of mitochondria-mediated stress responses (reviewed in [60–62]). PARL has been shown to regulate mitochondrial morphology, although the

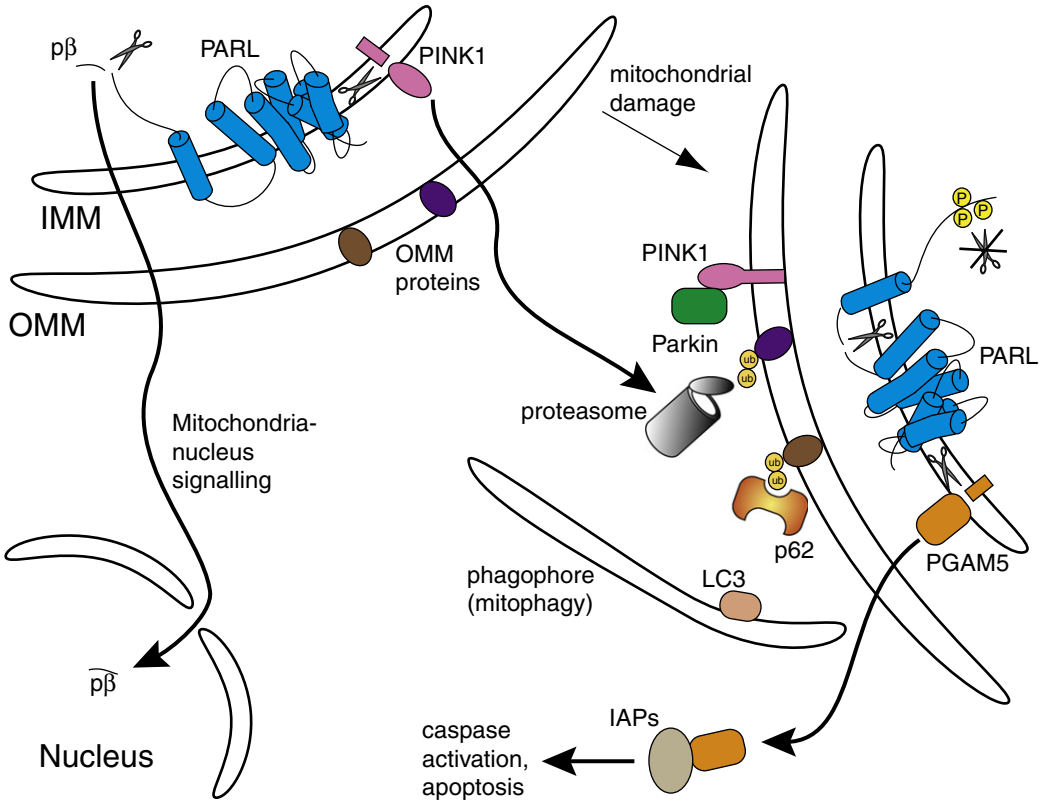


**Fig. 1.** Current knowledge on the substrates, regulation and downstream effects of PARL.

mechanism or substrate by which this occurs has not been well established [63]. More recent work has helped us to gain insight into the crucial role of PARL in apoptosis and mitophagy through the cleavage of its substrates (Section 4.2). Hence, it would appear that the regulation of this protein is critically important. In the next section, we review the current knowledge of how the mitochondrial rhomboid proteases are regulated.

## 3. Regulation of the mitochondrial rhomboid proteases

### 3.1. Composition of the lipid bilayer

Since rhomboid proteases are multi-pass TM proteins, it would not be surprising that their enzymatic activity can be regulated by the composition of the lipid bilayer. Indeed, *in vitro* reconstitution of purified rhomboid proteases indicates that their enzymatic activity can be influenced by their lipid environment [64]. The *Escherichia coli* rhomboid protease GlpG is more active when reconstituted in phosphatidylethanolamine (PE) than in phosphatidylcholine (PC). In support of this finding, molecular dynamics simulations indicate that GlpG forms hydrogen bonds with its lipid environment and induces lipid bilayer thinning [20]. Furthermore, studies examining the mitochondrial morphology of yeast and mammalian cells with reduced PE and cardiolipin (CL) indicate that these lipids are required for normal mitochondrial morphology [25,26,65–68]. These data suggest that the enzymatic activity of the mitochondrial rhomboid protease could be influenced by the composition of the mitochondrial membrane. However, the results from studies in yeast indicate that the activity of Rbd1 is unlikely to be affected by the mitochondrial lipid composition [26,68]. In comparison to the WT strain, yeast mutants with altered levels of mitochondrial CL and PE showed no detectable differences in Rbd1-dependent cleavage of Ccp1 [26,68–70]. Notably, the accumulation of s-Mgm1 was reduced in these mutants. However, it was shown that the alterations in the phospholipid composition impaired s-Mgm1 biogenesis in an Rbd1-independent manner rather than influencing Rbd1 enzymatic activity [26,88]. These results suggested that mitochondrial rhomboid proteases are unlikely to be regulated by the composition of the mitochondrial membrane. However, given that rhomboid proteases are very highly conserved, results indicating that the bacterial rhomboid proteases can be regulated by their lipid environment strongly suggest that the mitochondrial rhomboid proteases could also be regulated in the same way. A possible explanation for the lack of a detectable difference of Ccp1 cleavage by Rbd1 in the mitochondrial lipid mutants could be because Rbd1 cleaves Ccp1 much more efficiently than Mgm1, masking its reduced enzymatic activity. Hence, one cannot rule out the possibility that the mitochondrial rhomboid proteases are regulated by the composition of the mitochondrial membrane.

The identification of PE- and CL-rich microdomains within the bacterial membrane (although the same CL-specific assay might not be valid for yeast CL [71]) suggests that these phospholipids could also assemble into microdomains within eukaryotic mitochondria [72,73]. Perhaps the mitochondrial rhomboid proteases localize to such microdomains within the IM. In support of this, Mgm1 has been shown to localize to cristae folds, regions proposed to contain such lipid microdomains [74]. Given the nature of their multi-pass TM structure, it would be unlikely that the lipid environment does not influence the enzymatic activity of mitochondrial rhomboid proteases.

### 3.2. Proteolysis — β- and γ-cleavages

Being a typical mitochondrial protein encoded by the nuclear genome, the PARL protein sequence contains an N-terminal mitochondrial targeting sequence (MTS). The site of MTS cleavage in PARL is known as the α-site [38]. PARL also undergoes cleavage at two other sites known as the β- and γ-sites (Fig. 1) [19,38,63]. β-Cleavage is self-regulated *in trans* and occurs between S77 and A78, N-terminal to the first TMD. Mutating the catalytic serine results in impaired β-cleavage [38,63]. The product of β-cleavage is a small peptide, pβ, that possibly translocates from mitochondria to the nucleus, likely mediating mitochondria-nucleus signaling (Fig. 1) [38]. Expressing β-cleaved PARL in HeLa cells resulted in fragmented mitochondria, similar to that of cells expressing a modified form of WT PARL [63]. In contrast, abolishing β-cleavage by mutating S77 or the residues around it was associated with impaired PARL function, suggesting that β-cleavage is required for PARL activity [38,43,63]. It is intriguing that a PD-linked mutation in PARL, S77N, abolishes β-cleavage [43]. This strongly implies that β-cleavage is a very important regulatory mechanism of PARL. Further analysis of β-cleavage indicated that it is developmentally regulated. An antibody that specifically recognizes the N-terminus of PARL (including the pβ peptide) was mitochondrial-localized in mature neurons. However, in immature neurons, its staining was primarily nuclear. This result strongly suggested that β-cleavage occurs during cellular differentiation, likely regulating neuronal maturation [38].

In contrast to β-cleavage, γ-cleavage was shown to abolish PARL activity. Expressing γ-cleaved PARL resulted in elongated mitochondria similar to those of untransfected cells [19]. This phenotype is in stark contrast to the fragmented mitochondrial morphology observed when cells expressed WT or β-cleaved PARL, indicating that γ-cleavage abolishes PARL activity [19,63]. Although β- and γ-cleavages have opposing regulatory effects on PARL enzymatic function, γ-cleavage is mechanistically coupled to β-cleavage. Abolishing β-cleavage results in severely diminished γ-cleaved PARL [19]. All mitochondrial rhomboid proteases contain the "1 + 6" structural motif of TMDs [11,75]. γ-Cleavage occurs between the first and second TMDs of PARL and removes TMD 1 from the rest of the protein, resulting in a PARL protein with only the six core conserved rhomboid TMDs (Fig. 1). Homology modeling of the six core PARL TMDs based on the bacterial rhomboid protease GlpG suggests that disrupting the "1 + 6" PARL structural motif might alter the orientation of TMD 5, increasing the distance between the PARL catalytic site and the proposed catalytic aspartate in TMD 5, reducing PARL enzymatic activity [19]. Since the loop between TMDs 1 and 2 is highly conserved in vertebrates, it is proposed that the function of this loop is to allow for γ-cleavage, thereby regulating PARL enzymatic activity [19].

### 3.3. Phosphorylation

Since β-cleavage is developmentally regulated, there must exist a molecular mechanism that regulates when it occurs. Indeed, S65, T69 and S70 have been shown to be phosphorylated, a mechanism that inhibits β-cleavage (Fig. 1). Mutating these residues to mimic phosphorylation resulted in severely diminished β-cleavage. Consistent with the role of phosphorylation on these residues being an inhibitor of PARL activity, PARL-induced mitochondrial fragmentation could not be detected in cells expressing the S65D/T69D/S70D triple phosphomimetic mutant. Interestingly, the inhibitory effect on β-cleavage was most pronounced with the S70 phosphomimetic [63]. This suggests that, although all three residues can be phosphorylated, phosphorylation on S70 has the greatest inhibitory effect on β-cleavage. One can speculate that since S70 is in closest proximity to S77 where β-cleavage occurs, it is possible that the presence of a phosphate group at S70 presents a steric hindrance, preventing β-cleavage. As previously discussed, β-cleavage has been shown to regulate PARL enzymatic activity. Thus, phosphorylation is yet another level at which PARL activity is regulated. Although impaired β-cleavage is associated with impaired PARL activity, we should point out that PARL mutants with reduced β-cleavage still retain enzymatic activity [63]. This strongly indicates that PARL is not simply a protease, but that it has other important regulatory functions such as the proposed mitochondria–nucleus signaling by pβ. Although the kinase(s) and/or phosphatase(s) that regulate PARL have not been identified, the very recent finding that PGAM5, a Ser/Thr phosphatase, is a novel substrate of PARL raises the possibility that PGAM5 might dephosphorylate

PARL, thereby regulating its enzymatic activity (implications detailed in Section 4.2.2).

## 4. PARL and human diseases

### 4.1. PARL and type 2 diabetes

PARL was first identified as a candidate gene for type 2 diabetes (T2D) in a study aimed at identifying genes that are differentially expressed in the skeletal muscle of lean, obese and type 2 diabetic Israeli sand rats. PARL mRNA expression was reduced in diabetic rats but was restored when they were exercised to successfully treat diabetes. Furthermore, a common polymorphism in exon 7 of PARL that results in an L262V amino acid substitution was associated with increased plasma insulin, a marker of insulin resistance. The plasma insulin concentrations of these subjects also increased more substantially with age than those of the control population [76].

The association between reduced PARL mRNA expression and diabetes was supported by similar observations in insulin-resistant rats and diabetic human subjects. Reduced PARL mRNA levels were associated with reduced mitochondrial mass, reduced mitochondrial activity and impaired insulin signaling. This correlated reduced PARL expression with mitochondrial dysfunction in insulin resistance and diabetes [77,78]. This is also in agreement with growing evidence that mitochondrial dysfunction could lead to insulin resistance [79–82]. These findings strongly implicate PARL, be it directly or indirectly, as a factor involved in T2D. In order to fully understand how reduced PARL levels contribute to insulin resistance, the pathways affected need to be better characterized. A screen to identify alterations in the proteome when PARL levels are reduced could be a start in determining the mechanism of PARL-mediated insulin signaling.

Given that obesity is a growing problem in developed countries, we feel that the role of PARL in diabetes warrants further study. Although the phenotypes associated with the loss of PARL are similar to those observed in T2D, the mechanism by which PARL mediates insulin resistance remains poorly understood. Mitochondrial mass and quality are maintained by balancing the constant biogenesis of new functional mitochondria with the degradation of damaged mitochondria by mitophagy. It is proposed that reduced PARL protein levels could contribute to metabolic defects associated with T2D by tipping this balance, resulting in reduced mitochondrial mass and altered mitochondrial dynamics required for mitophagy [78]. Although further research into the mechanism of PARL-mediated insulin resistance is required, the current findings strongly suggest that PARL could be a therapeutic target for treating T2D. However, we must stress that exercise should also be considered as a therapy for diabetes. Diabetic rats successfully treated with exercise had restored PARL mRNA levels along with reduced blood glucose levels and plasma insulin, indicators of improved insulin sensitivity [76]. Although the different diseases appear to be a result of defects in specific downstream effects of mitochondrial dysfunction, it appears that proper PARL function is central to maintaining mitochondrial quality, thereby preventing diseases such as T2D and Parkinson's disease.

### 4.2. PARL and Parkinson's disease

#### 4.2.1. The link to apoptosis

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. It is characterized by the loss of dopaminergic neurons in the substantia nigra (SN) pars compacta. One of the key challenges in elucidating the mechanism of PD pathogenesis is understanding why neurons from the SN pars compacta are the only cells affected in this disease. Since neurons are energy demanding, it is possible that a slight impairment of mitochondrial function is sufficient to result in cell death. Mitochondria are not only involved in energy production; they are also crucial for calcium buffering. Recent studies indicate that dopaminergic neurons in the SN pars compacta are particularly sensitive to alterations in calcium homeostasis, suggesting that impaired calcium buffering could also contribute to the pathogenesis of PD [83,84] (reviewed in [85]). Although the etiology of PD remains unknown, several lines of evidence point towards the common theme of increased programmed cell death, also known as apoptosis, as a contributing factor to increased neuronal death (reviewed in [86]). Given that PARL has anti-apoptotic functions, it would not be surprising that altered PARL expression in PD patients might contribute to increased neuronal death. Indeed, several studies analyzing the transcriptional profiles of brain tissue (including the SN pars compacta) from healthy control and PD patients identified reduced PARL mRNA levels in tissues from PD patients [87–89]. These data are consistent with the role of PARL in protecting cells from apoptosis and further implicates PARL as a key player in the pathogenesis of PD.

Apoptosis is an evolutionarily conserved mechanism essential for development. Processes such as sexual differentiation and defense against infections are dependent on well-controlled apoptosis (reviewed in [90]). One of the hallmarks of apoptosis is the activation of a family of cysteine proteases known as the caspases. Upon activation, caspases cleave a broad spectrum of substrates, culminating in cell death. The activation of caspases is initiated by the release of cytochrome c from the mitochondrial IMS to the cytosol (reviewed in [91,92]). Upon treatment with $H_2O_2$, *Parl*$^{-/-}$ MEFs and primary myoblasts released cytochrome c more quickly than WT cells, suggesting that PARL negatively regulates apoptosis. Consistently, the loss of PARL resulted in massive apoptosis in mouse lymphocytes [39].

The Bcl-2 family of proteins is a key regulator of cytochrome c release during apoptosis and can be divided into three subgroups: (i) pro-apoptotic, (ii) anti-apoptotic and (iii) BH3-only (reviewed in [93,94]). Hax1, a protein bearing sequence similarities to Bcl-2 proteins, was demonstrated to have anti-apoptotic activity, suppressing apoptosis through its physical interaction with PARL to enhance the proteolytic processing of Omi. Although a physical interaction could be detected between Hax1 and PARL, such interaction was not detectable between Hax1 and Omi. This contradicts previous findings that Hax1 physically interacts with Omi and is a substrate of Omi [95]. Nevertheless, this led to the proposed model that the Hax1–PARL complex enhanced the recruitment of Omi to PARL, promoting its cleavage to suppress apoptosis. In support of this model, cells from *Hax1*-null mice and *Omi*-mutant mice expressed activated Bax, a pro-apoptotic Bcl-2 protein, earlier than cells from WT mice upon the induction of apoptosis [96]. This work was the first demonstration of a direct relationship between PARL and a Bcl-2 family-related protein, implicating the mitochondrial rhomboid protease as an important regulator of apoptosis.

Shortly after this study was published, another study provided evidence to the contrary, implying that the previously observed interaction between Hax1 and PARL was likely to be an artifact. Jeyaraju et al. showed that Hax1 is not localized to the mitochondrial IM or the IMS, and is therefore an unlikely interacting partner of the IM-localized PARL. Hax1 also interacted with PARL that was not imported into mitochondria, supporting the non-specific nature of their interaction. Multiple sequence alignments using Hax1 protein sequences from diverse organisms indicated that the previously believed Bcl-2 family-related domains in Hax1 are not conserved [57,97]. This argues against Hax1 as a Bcl-2 family-related protein. Although this study showed that Hax1 and PARL can interact non-specifically, one cannot rule out the possibility that they can also interact specifically with each other. The co-immunoprecipitations performed in this study were conducted using cultured cells over-expressing Hax1 and PARL or by mixing lysates from *Hax1*$^{-/-}$ MEFs with that from *Parl*$^{-/-}$ MEFs [57]. In contrast, the previous report showed the interaction between the endogenous proteins in mitochondria purified from mouse liver [96]. To draw a strong conclusion on the relationship between PARL and Hax1, a few outstanding questions need to be answered. Firstly, the most obvious

question is the authenticity of the physical interaction between PARL and Hax1. Secondly, the strong similarity in the phenotypes between *Parl*-null mice, *Omi*-mutant mice and *Hax1*-null mice (such as severe lymphocyte loss and increased apoptotic cell death [39,96,98,99]) suggests that the three genes might interact. A critically important difference between *Parl*-null mice and the *Omi*-mutant and *Hax1*-null mice is the apparent lack of neuronal cell death in *Parl*-null mice [39]. This would suggest an unlikely genetic interaction between the three genes. Perhaps determining whether these genes truly interact could shed light on whether these relationships warrant further study. Additional analysis is required to elucidate this potentially interesting and important aspect of PARL-mediated biology.

PGAM5 was very recently shown to undergo PARL-dependent cleavage at its N-terminus between amino acids 24 and 25 [17]. Interestingly, it was more recently discovered that a cleaved C-terminal fragment of PGAM5 (after amino acid 24, Δ24 PGAM5) accumulates in the cytosol as a substrate of the inhibitor of apoptosis proteins (IAPs). This was concomitant with an increase in the expression of active caspase 3, promoting apoptosis [100]. Although the protease responsible for the formation of Δ24 PGAM5 has not been identified, evidence points towards PARL as a very likely candidate. Firstly, PARL-dependent cleavage of PGAM5 increases with a loss in mitochondrial membrane potential [17]; treating cells with staurosporine, a compound previously shown to dissipate the mitochondrial membrane potential, increased the accumulation of Δ24 PGAM5 [100,101]. Secondly, the PARL cleavage site in PGAM5 coincides with that of the protease that generates the pro-apoptotic form [17,100]. Together, these data strongly implicate PARL as the protease that cleaves PGAM5 upon mitochondrial damage, promoting caspase-dependent apoptosis.

PARL-dependent cleavage of PGAM5 suggests that PARL is pro-apoptotic, whereas the evidence that MEFs lacking PARL undergo increased apoptosis suggests that PARL is anti-apoptotic [17,39]. However, the observed increase in cell death could be due to severe mitochondrial dysfunction in these cells [39]. It was previously observed in *Drosophila* that the loss of Rhomboid-7 results in severely reduced lifespan and impaired mitochondrial dynamics required for proper mitochondrial function [15]. Furthermore, in the absence of PARL, PINK1 accumulates in mitochondria and is associated with reduced mitochondrial mass, reduced membrane potential and increased reactive oxygen species (ROS) [43,54,55]. It would not be surprising that the severe mitochondrial dysfunction associated with the loss of PARL resulted in increased apoptosis through PARL-independent mechanisms. The use of PARL knock-out organisms has proved to be invaluable in the study of PARL-mediated apoptosis. However, these studies are complicated by the overlapping functions of mitochondria in different responses to stress. Yet, one must recognize that PARL-mediated stress response warrants further study as the loss of PARL results in such dramatic phenotypes. The challenge in future studies is in associating the observed phenotypes with the appropriate pathways that are impaired.

#### 4.2.2. Mitophagy — a new paradigm in the onset of Parkinson's disease

The results from numerous studies have indicated that increased apoptosis could contribute to the pathology of PD [86]. However, in recent years, impaired mitophagy has also been implicated in the pathogenesis of PD [59]. Indeed, for several years, many in the field have focused on how PARL could regulate apoptosis. Consistent with the recent implications of impaired mitophagy on PD, in the last 5 years, the role of PARL in regulating mitophagy has been the focus of intense research. PARL was first implicated in PD when the *Drosophila* mitochondrial rhomboid protease, *rhomboid-7*, was found to interact genetically with *pink1*, *parkin* and *omi* — genes whose mammalian homologs are PD-linked [28]. Within months of this discovery, Parkin was found to be selectively recruited to damaged mitochondria, promoting their clearance by mitophagy [102]. More importantly, knocking down PINK1 impaired Parkin recruitment to damaged mitochondria [58]. Since these discoveries, many groups in the field have focused on the role of PARL and its substrate, PINK1, in Parkin-mediated mitophagy. Our work and work of others have shed much-needed light on the mechanism of PINK1/Parkin-dependent mitophagy.

Mitophagy is a form of macroautophagy where damaged mitochondria are sequestered into a double-membrane structure known as the autophagosome. Subsequent fusion of the autophagosome with the lysosome results in the degradation of its contents by the lysosomal hydrolytic enzymes. In mammalian cells, mitophagy occurs in different pathways (autophagy reviewed in [103,104], mitophagy reviewed in [105]). In this review, we focus on the PINK1/Parkin-mediated mitophagy pathway.

As briefly described above in Section 2.3, PINK1 is cleaved by PARL upon its import into mitochondria [43,55]. This cleavage allows it to be exported to the cytoplasm where it is rapidly degraded in a proteasome-dependent manner [43,55,106]. In healthy cells with properly functioning mitochondria, the rapid turnover of PINK1 results in low basal levels of the protein [106]. Disrupting the mitochondrial membrane potential impairs PINK1 import and PARL-dependent cleavage, resulting in the mitochondrial accumulation of full-length PINK1 [53,55,106]. Under these conditions, PINK1 can selectively recruit Parkin, an E3 ubiquitin ligase, to mitochondria, catalyzing the ubiquitination of OMM proteins (Fig. 1). To date, several mitochondrial substrates of Parkin have been identified, including mitofusins 1 and 2 (Mfn1/2), voltage-dependent anion channel (VDAC) and Tom20 [107–110]. It is proposed that Parkin-mediated ubiquitination of OMM proteins leads to their proteasomal degradation and the recruitment of the autophagic machinery [110]. Since increased mitochondrial fission has been shown to promote mitophagy in mammalian cells, it is not surprising that the pro-fusion proteins, Mfn1/2, are substrates of Parkin and are degraded in a proteasome-dependent fashion [107,111,112]. Interestingly, a recent study found that rapamycin-induced mitophagy in yeast is independent of mitochondrial fission, suggesting that the mechanism of mitophagy is not conserved from yeast to mammals [113]. Indeed, to date, there are no known homologs of PINK1 or Parkin in yeast. A more recent study found that damaged mitochondria form spheroids that colocalize with lysosomal markers in an Mfn1/2 (fusion)-dependent but Parkin- and autophagy-independent manner [114]. This suggests that the requirement for mitochondrial fission might be specific to Parkin-mediated mitophagy that is not conserved in yeast. Perhaps mitophagy in yeast occurs in a similar PINK1/Parkin-independent pathway. The finding that mitochondrial spheroid formation requires Mfn1/2, and that the over-expression of Parkin inhibits spheroid formation in favor of mitophagy suggests that mitochondrial fusion promotes spheroid formation whereas increased fission (or reduced fusion) promotes Parkin-dependent mitophagy in mammalian cells upon mitochondrial damage, consistent with recent findings [111,112,114].

In addition to altering mitochondrial dynamics, Parkin-mediated ubiquitination of OMM proteins also leads to the recruitment of p62, a protein that binds to ubiquitin and LC3, a component of autophagosomes (Fig. 1) [115]. Activated LC3 is tightly associated with membranes and is required for the growth of autophagosomes. These results strongly suggest that Parkin uses both the ubiquitin–proteasome system (UPS) and the autophagic pathway to promote the clearance of damaged mitochondria (Fig. 1).

The requirement for PARL in Parkin-mediated mitophagy and PD is clear from studies demonstrating that cells lacking PARL have impaired Parkin recruitment to damaged mitochondria, and consequently, impaired mitophagy. More importantly, Shi et al. identified a novel mutation in PARL associated with PD. The PD-linked S77N mutation in PARL results in reduced β-cleavage, and accordingly, impaired PARL-mediated mitophagy [43]. The current model in the field places PARL as an important regulator of mitophagy in response to mitochondrial damage. In healthy mitochondria, PARL constitutively cleaves PINK1 as it gets imported, allowing its export and rapid proteasomal degradation, indicating that mitochondria are healthy. However, when mitochondria are damaged, the loss of mitochondrial membrane potential

impairs PINK1 import, resulting in the accumulation of full-length PINK1 on the OMM, serving as an anchor for Parkin recruitment and subsequent mitochondrial clearance. Although the PINK1/Parkin mitophagy pathway is rapidly gaining acceptance as a PD-associated pathway, it is important to note that many of these studies that contributed significantly to the understanding of this pathway were performed in cells over-expressing Parkin. A few recent studies have shown that endogenous levels of Parkin may not be sufficient to induce mitochondrial clearance, suggesting that previous results may be artifacts of Parkin over-expression [116,117]. However, previous studies have detected mitophagy in cells with endogenous Parkin, albeit at low levels [118]. It would not be surprising that, in the absence of Parkin over-expression, cells commit to apoptosis much more quickly. Furthermore, it would be very unlikely that cells degrade the entire mitochondrial network rather than commit to cell death, as they would lose their primary energy source. Hence, while systems that over-express Parkin do not necessarily represent the amount of endogenous mitochondrial clearance, these tools have helped us to better understand the role of various PD-linked mutations in the progression of mitophagy. However, the role of these studied proteins needs to be verified, especially if we are to apply our knowledge based on these research conditions to the pathogenesis of PD. In the next part of this review, we provide our perspectives on how impaired PARL function might contribute to the pathogenesis of PD.

## 5. Concluding perspectives — PARL, the master regulator of mitochondria-mediated stress response?

Combining the current findings, we propose that PARL-dependent cleavage of its substrates serves as an important form of signaling. Depending on the substrate (PINK1 or PGAM5), the message might signal healthy or damaged mitochondria. In healthy cells, the constant dephosphorylation of PARL (perhaps by its substrate PGAM5) maintains its enzymatic activity, promoting its self-regulated β-cleavage. This, in turn, allows PARL-dependent constitutive cleavage of PINK1, allowing PINK1 export to the cytoplasm and rapid proteasome-dependent degradation. Upon mitochondrial damage, the loss of mitochondrial membrane potential disrupts PINK1 import into mitochondria, resulting in the accumulation of full-length PINK1 on the OMM and subsequent recruitment of Parkin to inhibit mitochondrial fusion and promote mitochondrial degradation by mitophagy (Fig. 1).

At the same time, by an as yet unknown mechanism, PARL selectively cleaves PGAM5, resulting in the efficient export of PGAM5 to the cytosol to promote apoptosis. The reduction in mitochondrial PGAM5 could result in an increase in phosphorylated PARL, inhibiting PARL enzymatic activity, contributing to further mitochondrial damage to potentiate cell death by apoptosis (Fig. 1).

The role of PARL in promoting mitophagy and apoptosis seems counter-intuitive since mitophagy has been proposed to mitigate the extent of cellular damage induced by mitochondrial dysfunction, thereby promoting cell survival. However, in the case that only a small pool of mitochondria is damaged, their efficient clearance by mitophagy could be sufficient to maintain cell survival as mitophagy is induced more quickly than apoptosis. The increase in the pro-apoptotic form of PGAM5 and active caspase 3 can only be detected 1 h after treatment with STS, whereas Parkin recruitment to mitochondria can be detected as early as 20 min after the induction of mitophagy [100,119]. However, in the event of prolonged or extensive mitochondrial damage beyond the capacity of mitophagy, PARL might help to ensure the health of the multi-cellular organism by promoting apoptosis.

Although research has shown that increased apoptosis could contribute to increased neuronal death leading to PD, recent evidence to the contrary has puzzled the PD community, shifting the focus of research to the role of impaired mitophagy in the pathogenesis of PD. For a period of time, it seemed as if the roles of impaired apoptosis and mitophagy in PD were mutually exclusive. These most recent findings shed new light on the role of PARL in mitophagy and apoptosis, suggesting that impaired PARL function could be the missing link between mitophagy and apoptosis in the pathogenesis of PD. Given the importance of PARL in these quality control mechanisms, one must wonder whether other substrates of PARL exist and their role(s), if any, in these pathways. A future challenge for the field would be to identify more substrates of PARL and how PARL is regulated beyond what is already known. Evidently, phosphorylation plays a crucial role in regulating PARL activity. However, the kinase(s) and/or phosphatase(s) responsible for this form of regulation have not been identified. As previously discussed, current data suggests that PARL is not only a protease, but it has additional functions. Cells expressing PARL mutants with impaired β-cleavage have phenotypes similar to that of cells lacking PARL, however, these mutants still retain enzymatic activity [38,43,63]. Better understanding the many functions of PARL and how it is regulated will reveal the intricacies of cellular quality control and shed more light on the pathogenesis of diseases such as T2D and PD. In sum, it is clear that the mitochondrial-localized rhomboid proteases are critical regulators of the life and death of the cell, and we look forward to additional discoveries of this important intramembrane protease family.

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