Sajal Chakraborti · Naranjan S. Dhalla
Editors

Proteases in Physiology and Pathology

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*This book on **Proteases in Physiology and Pathology** is dedicated to Professor Kambadur Muralidhar, a distinguished biologist and an outstanding teacher/ educationist of our country. Professor Muralidhar was born in 1948 at Coimbatore, Tamil Nadu, India. He received his PhD from the Indian Institute of Science, Bangalore, in 1976. In the PhD work under Prof. N.R. Moudgal, he demonstrated that the beta subunit of luteinizing hormone can bind to ovarian receptors, which fetched him the Prof KV Giri Memorial Gold medal for the best PhD thesis of the year. He was selected for the first Lectureship in the School of Life*

Sciences at the newly founded Central University of Hyderabad in August 1976. During 1979–1981, he has been a Research Associate in the Department of Cell and Molecular Biology at SUNY-Buffalo, New York, USA, and worked with Prof. O.P. Bahl. His work led to the development of the most sensitive RIA for HCG, a pregnancy hormone. He also demonstrated the immuno-contraceptive vaccine potential of DS5-hCG beta subunit. He joined the University of Delhi in 1983 as Reader in Biochemistry, Department of Zoology, and became a Professor in Endocrinology and Biochemistry in 1988. He was Chairman of the Department during 2001–2004.

His laboratory discovered the presence of tyrosine-O-sulphate in sheep and buffalo PRL. Using biophysical techniques, his group has studied the protein unfolding and folding of buffalo GH. Naturally occurring size isoforms of buffalo prolactin, cathepsin derived peptides from buffalo prolactin, and a synthetic peptide based on the internal sequence of buffalo prolactin were discovered to be powerful inhibitors of angiogenesis stimulated by VEGF or bradykinin. His group has published over 140 articles in peer-reviewed journals and several books and chapters.

Professor Muralidhar was Chairman of the University Grants Commission's (Govt. of India) Curriculum Development Committee for Zoology (2001). He assisted University of Delhi in restructuring Undergraduate Science Education and introduced a new Honors course in Integrated Biology, which

is the first of its kind in the country. He was the Chairman of the PAC-Animal Science of the Department of Science and Technology (DST), Govt. of India, for three terms (2001–2004, 2004–2007, and 2010–2013) and nurtured research in this area. He assisted different funding agencies of the Govt. of India in different capacities. He also served as a member of Research Advisory Committees of several nationally important research institutions in India.

Professor Muralidhar is an elected Fellow of all the Science Academies of India, i.e., NASI, Allahabad; INSA, New Delhi; and IASc, Bangalore. He has delivered the Sadaksharaswami Endowment Lecture for SBC (I) (1996), US Srivastava Memorial Lecture for NASI (2007), V. Gopalakrishna Rao Endowment Lecture for Osmania University (2004), Y Subba Row Memorial Lecture for GGS Indraprastha University (2007), Prof MRN Prasad Memorial Lecture for INSA (2010), Hargobind Khorana Memorial Lecture for GGS IP University (2012), Distinguished Lecture for Kalyani University (2016), and GP Sharma Memorial Lecture for Punjab University (2011). He served as member of the Executive Council of NASI (2003–2005) and INSA (1999–2001). He was the Chief Editor of the Proceedings of Indian National Science Academy for over 6 years (2000–2005) when he transformed the journal and brought international recognition. He was Chairman of the INSA National Committee for Cooperation with IUBS for a term. He has recently become a JC Bose National Fellow (DST). Following superannuation in

December 2013, Kambadur Muralidhar has joined the South Asian University as an Honorary Professor in January, 2014.

Professor Kambadur Muralidhar undoubtedly is a legendary figure in Indian science. He has excellent ability to motivate young researchers. We feel honored to dedicate this book to Professor Kambadur Muralidhar and wish him good health and success in his long, fruitful activities.

Preface

I thought that my voyage was at its end at the last
limit of my power that the path before me was closed,
provisions were all exhausted and the time had come for me
to take shelter in a silent obscurity.
But, I find that thy will knows no end in me.
And, when old words die out on the tongue
new melodies break forth from the heart and where
the old tracks are all lost new country is unveiled
with its wonders.

(Rabindranath Tagore (Gitanjali: Song of offerings)

The history of research on proteases is relatively old, which has been initiated in the late eighteenth century, although in recent times it has gained a tremendous momentum because of their widespread applications, especially in biotechnology and medicine. There are many ways in which proteases elicit both the beneficial and detrimental effects on the functioning of living beings, and this has prompted researchers to study their roles in health and disease.

Recent research revealed that about 2% of all gene products are proteases, indicating that it is one of the important functional groups of proteins. Notably, it seems difficult to know how a protease can be distinguished from another related one. On one side, scientists are engaged in understanding the basic mechanisms of the potentiality of different types of proteases in a variety of disease progression and evaluation of relevant therapeutics; on the other side, researchers are trying to answer two fundamental questions: How does knowledge of one protease help in the understanding of related proteases? How can a novel protease's role be truly ascertained?

Research on renin inhibitors as potential anti-hypertensive drugs started in the early 1970s. Some early peptide-like inhibitors showed significant inhibitory activity towards renin but lacked adequate bioavailability. After decades of research, the first bioavailable renin inhibitor aliskiren was approved and marketed in 2007; however it was discontinued in 2011 due to its side effects. Notably, the success of inhibitors of angiotensin-converting enzyme (ACE) and β-blockers for the treatment of hypertension have supported the concept that protease inhibitors can prove useful as successful drugs. Peptidomimetic inhibitors, which bind at the active site of matrix metalloproteases (MMPs), have been tested, and most MMP inhibitors in

clinical development are hydroxamate derivatives such as batimastat and marimastat, although nonpeptide MMP inhibitors such as AG3340 and Bay-12-9566 have also shown their efficacy in preventing different types of cancer. Despite the success achieved in understanding fundamental scientific information and in designing some highly valuable drugs by exploring active site targeted inhibitors, the limited number of protease inhibitors introduced during the past decade as well as several failures indicates a need for basic research on disease-causing proteases for more details. In this book, we believe that different authors in their respective chapters provided some novel information, which will eventually help to unravel many barriers that pharmacologists and drug designers are experiencing currently.

This book is intended to provide comprehensive treaties of physiological and pathological implications of some proteases. We would like to express our appreciation to all the contributors for their enthusiasm and perseverance in bringing this book to fruition. We wish to thank Dr. Madhurima Kahali (Biomedicine, Springer, New Delhi), Sowndarya Kumaravel and F. Avilapriya for all the very important initiating effort towards achieving this goal. Finally, we like to thank Prof. Sankar Kumar Ghosh (Vice Chancellor, University of Kalyani) for his encouragement.

Kalyani, West Bengal, India — Sajal Chakraborti
Manitoba, Winnipeg, Canada — Naranjan S. Dhalla

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About the Editors

Sajal Chakraborti is a Senior Professor of Biochemistry at the University of Kalyani, West Bengal, India. His research covers the role of proteases in regulating pulmonary vascular tone under oxidant and calcium signalling phenomena. He has been engaged in teaching and research in biochemistry for the past 40 years.

Naranjan S. Dhalla is a distinguished Professor at the University of Manitoba, Winnipeg, Canada. His expertise includes the subcellular and molecular basis of heart function in health and disease. He has been engaged in multidisciplinary research and education for promoting the scientific basis of cardiology, as well as training of professional manpower for combating heart disease for over 45 years.

Part I

Regulation of Proteases in Health and Disease

Physiological and Pathological Functions of Mitochondrial Proteases

1

Clea Bárcena, Pablo Mayoral, Pedro M. Quirós,
and Carlos López-Otín

Abstract
Mitoproteases display an essential role in the preservation of mitochondrial homeostasis under regular and stress conditions. These enzymes perform tightly regulated proteolytic reactions by which they participate in mitochondrial protein trafficking, processing and activation of proteins, protein quality control, regulation of mitochondrial biogenesis, control of mitochondrial dynamics, mitophagy, and apoptosis. In this chapter, we have revised the physiological functions of the intrinsic mitochondrial proteases, analyzing their roles in the different compartments of this organelle and their connection to human pathology, primarily cancer, neurodegenerative disorders, and multisystemic diseases.

Keywords
Mitochondria • Mitoproteases • Mitochondrial dynamics • Cancer • Aging • Neurodegenerative disorders

1.1 Introduction

Due to their prokaryotic origin, mitochondria possess some structural characteristics that make them remarkably different from other organelles of eukaryotic cells. They have a double membrane with an intermembrane space, being the inner membrane expanded by the formation of numerous foldings named *cristae*. Enclosed among these *cristae*, we can find the mitochondrial matrix, where several copies of mitochondrial DNA genome are contained. Mitochondria play essential biological

C. Bárcena • P. Mayoral • P.M. Quirós • C. López-Otín (✉)
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functions, fundamental for the generation of most of the cell supply of adenosine triphosphate (ATP), the source of chemical energy within the cells.

The complex structure and the relevance of the mitochondrial function within the organisms justify the need to set an organization of the molecules that collaborate in the maintenance of the assembly and function of this energy machine, among which proteases are gaining an increasing attention. Proteases have been considered for many years just as performers of the catabolic reactions in the organisms; however, we are currently beholding a surprising and unexpected increment in the studies of these enzymes, positioning them as multifunctional molecules carrying essential functions in health and disease [1–3]. The study of proteases contained in the mitochondria, named *mitoproteases*, has also experienced an exciting expansion. Until very recently, they were seen only as members of the quality control system of mitochondria, in charge of the degradation of misfolded and damaged proteins or being responsible for the processing of proteins imported from cytosol into mitochondria [4]. Nevertheless, in recent years, we have witnessed an impressive progress in the knowledge of their functions, becoming increasingly evident that they are not merely actors of the catabolic functions of mitochondria. Instead, mitoproteases perform precise and tightly regulated proteolytic roles that determine time-specific functions of regulatory proteins.

Due to this increased knowledge and interest in the study of mitochondrial proteases, we have recently proposed the concept of *mitodegradome* to define the complete set of proteases and homologues that function in mitochondria from cells and tissues of an organism [5]. The mitodegradome would therefore modulate in an efficient and irreversible way the function of mitochondria in order to adapt these organelles to the diverse stress situations that they face, especially given the fact that there is an absence of de novo synthesis of mitochondria. This characteristic is of special relevance in post-mitotic tissues, endowing mitochondria and the mitodegradome an important role in health and aging. Consistent with this, malfunction or deregulation of mitochondrial proteases has been associated with numerous pathologies such as metabolic impairments, cancer, inflammatory diseases, and neurodegenerative disorders [6–8], as well as with the control of aging and longevity [9].

Mitoproteases have the common feature of their location in the mitochondria, which can be exclusive or shared with the cytosol. Beyond this characteristic, they form an assorted group attending to their proteolytic diversity. To set some clarity in this complex grouping, we have recently organized the mitoproteases according to their function, localization, and proteolytic nature in three groups: *intrinsic or resident mitoproteases*, which exert their function exclusively in this organelle regardless of the compartment in which they act; the *pseudo-mitoproteases*, which have a protease structure but are catalytically impaired; and *transient mitoproteases*, which are translocated into mitochondria only in some particular circumstances [5].

Among these three groups of defined mitoproteases, the *intrinsic* or *resident mitoproteases* are the most relevant, and it will be the main focus of this chapter. All the enzymes that belong to this group exert their function essentially in mitochondria, being considered as the bona fide mitochondrial proteases. In this group, we can find 20 enzymes, divided into 1 cysteine, 7 serine, and 12 metalloproteases.

The only member in the subgroup of cysteine proteases is the deubiquitinase USP30, which participates in the quality control system in the outer membrane [10]. By contrast, the metalloproteases are represented by members of seven different families, which exert a variety of functions in mitochondria. This subgroup includes the processing peptidases PMPCB, MIPEP, XPNPEP3, METAP1D, ATP23, and OMA1; the ATP-dependent proteases AFG3L2, SPG7, and YME1L1; the oligopeptidases NLN and PITRM1; and the relatively unknown enzyme OSGEPL1. Finally, the serine proteases are represented by beta-lactamase (LACTB); the processing peptidases IMMPL1, IMMPL2, and PARL; the ATP-dependent proteases LONP1 and CLPP; and the quality control protease HTRA2. Interestingly, many of these proteolytic enzymes are widely distributed from bacteria to mammals. In fact, there are bacterial orthologues of nearly all human mitochondrial proteases, including different oligopeptidases and aminopeptidases belonging to the M3 and M24 metalloprotease families; the highly conserved families of ATP-dependent proteases, such as Lon, ClpP, and FtsH proteases (orthologous of LONP1, CLPP, and the AAA proteases AFG3L2, SPG7, and YME1L1, respectively); the ATP-independent stress-response metalloprotease HtpX (orthologous of OMA1); and the serine proteases DegP and DegS (orthologous of HTRA2) (Table 1.1).

Functionally, the mitoproteases include the classical proteases involved in the import of proteins to mitochondria and in the protein quality control system. However, it is now recognized that mitoproteases exert a variety of functions within mitochondria, including mitochondrial protein trafficking, processing and activation of proteins, protein quality control, regulation of mitochondrial biogenesis, control of mitochondrial dynamics, mitophagy, and apoptosis [5].

The present chapter discusses the different proteolytic functions of mitoproteases in the mitochondrial compartments, focusing on recent advances of the study of this group of enzymes. Additionally, we present an overview of the role of these enzymes in human pathology.

1.2 Physiological Roles of Mitoproteases

1.2.1 Proteolysis in the Outer Membrane

The mitochondrial outer membrane plays essential roles in mitochondrial biogenesis, as well as in the control of mitochondrial dynamics and mitophagy. Since the amount of proteins in the outer membrane is lower compared with the inner membrane, the function of mitoproteases in the outer membrane is to regulate the fusion and fission machinery in order to maintain the integrity of the mitochondrial network. Apart from the intrinsic mitoproteases, transient proteases and the cytosolic ubiquitin proteasome system also collaborate in the regulation of these processes, maintaining the quality control in this membrane [11]. Actually, the only mitochondrial protease that exerts its biological function in the outer membrane is the deubiquitinating enzyme USP30. The role of this enzyme is to inhibit the function of mitofusin 1 and mitofusin 2 by specifically removing the non-degradative

Table 1.1 Intrinsic mitochondrial proteases

Symbol	Class	Localization	Mitochondrial function	Associated pathology in humans
OMA1	Metallo	Inner membrane	Mitochondrial dynamics, mitophagy, and apoptosis	Improves cisplatin chemosensitivity
PARL	Ser	Inner membrane	Mitophagy and apoptosis	Type 2 diabetes, Parkinson's-like disease, neuronal injury, and cerebral ischemia
AFG3L2	Metallo	Inner membrane	PQC and mitochondrial biogenesis	Dementia, ataxia spinocerebellar, spastic paraplegia
SPG7	Metallo	Inner membrane	PQC and mitochondrial biogenesis	Spastic paraplegia, ataxia, chronic ophthalmoplegia, type 2 diabetes, coronary artery disease
YME1L1	Metallo	Inner membrane	PQC and mitochondrial biogenesis	–
IMMP1L	Ser	Inner membrane	Protein import and activation	–
IMMP2L	Ser	Inner membrane	Protein import and activation	Thyroid cancer, Tourette syndrome
LACTB	Ser	Intermembrane space	Mitochondrial biogenesis	–
NLN	Metallo	Intermembrane space	PQC	–
ATP23	Metallo	Intermembrane space	PQC and processing peptidase	–
HTRA2	Ser	Intermembrane space	PQC, mitophagy, and apoptosis	Cancer, Parkinson's disease, essential tremor, neuronal injury, and cerebral ischemia
OSGEPL1	Metallo	Matrix	Mitochondrial biogenesis	–
CLPP	Ser	Matrix	PQC	Acute myeloid leukemias, Perrault syndrome
PITRM1	Metallo	Matrix	PQC	Alzheimer's disease
LONP	Ser	Matrix	PQC and mitochondrial biogenesis	Cancer, CODAS syndrome
METAP1D	Metallo	Matrix	Protein import and activation	Colon cancer
MIPEP	Metallo	Matrix	Protein import and activation	–

(continued)

Table 1.1 (continued)

Symbol	Class	Localization	Mitochondrial function	Associated pathology in humans
PMPCB	Metallo	Matrix	Protein import and activation	–
XPNPEP3	Metallo	Matrix	Protein import and activation	Nephropathy
USP30	Cys	Outer membrane	Mitochondrial dynamics and mitophagy	Parkinson's disease

PQC protein quality control

ubiquitylation marks [12]. Consequently, the inhibition or depletion of USP30 in human cells induces fusion, with a concomitant increment in interconnected mitochondria [10]. Recently, it has been observed that this effect is maintained in cells deficient in mitofusins [12], suggesting a regulation independent of these mitoproteins. Additionally, it has been described that USP30 also blocks parkin-mediated mitophagy through the removal of ubiquitin moieties from damaged mitochondria [13].

1.2.2 Proteolysis in the Intermembrane Space

Mitoproteases located at the intermembrane space mainly exert quality control functions. All the proteases associated with this region are ATP-independent enzymes, which is probably due to the compartmentalization of the intermembrane space, keeping it separated from the ATP production area. Among these proteases, we can find HTRA2 and ATP23, which participate in protein quality control; NLN, an oligopeptidase; and LACTB, whose function is still unknown.

HTRA2 is a trimeric serine protease with crucial roles in the degradation of oxidized proteins [14] and in the inhibition of mitophagy. This last function can be exerted under stress conditions through the degradation of parkin when liberated to the cytosol or by processing the intramitochondrial Mulan E3 ubiquitin ligase [15, 16]. Mouse cells deficient for this enzyme accumulate mutations in the mtDNA, which has led to suggest that this mitoprotease also collaborates in the maintenance of mtDNA integrity [17]. The decline in mitochondrial mass and membrane potential after loss of HTRA2, resulting in ATP depletion, is also remarkable [18]. HTRA2 also regulates cell death through different pathways. First, it seems to stimulate TNF-induced necroptosis as well as a secondary germ cell death pathway in *Drosophila melanogaster* [19, 20]. However, its most studied role in cell death is the promotion of apoptosis, through two different and somewhat opposing ways. Thus, some studies suggest that its activation by PARL avoids aggregation of pro-apoptotic proteins, as BAX, in the outer membrane [21]. Conversely, it has also been suggested that, during apoptosis, HTRA2 autoactivates itself before being released to the cytoplasm, where it cleaves and inactivates anti-apoptotic proteins, such as X-linked inhibitor of apoptosis protein (XIAP) and the tumor-suppressor WT1 [22, 23], which finally results in the promotion of apoptosis.

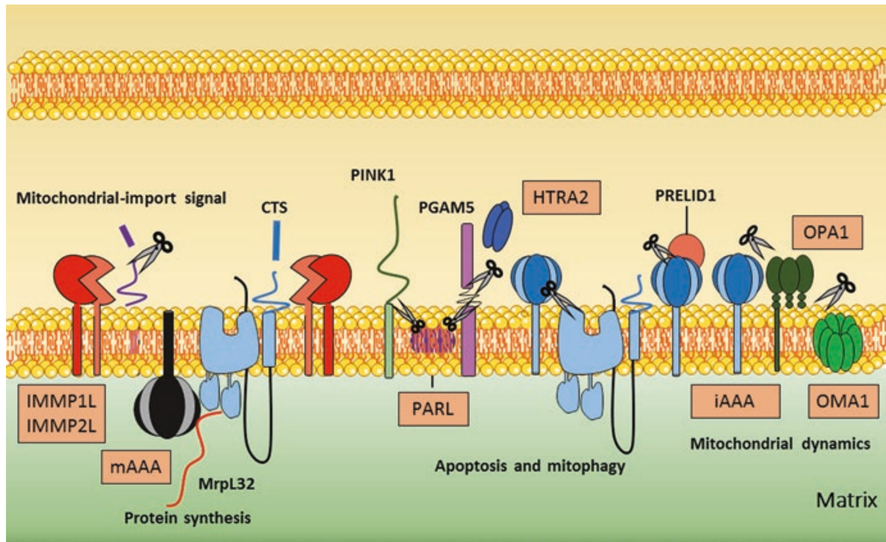

Fig. 1.1 Mitochondrial proteases in the inner membrane. Intrinsic mitochondrial proteases that exert their role in the inner membrane. The proteolytic activities of mitoproteases in this compartment are vastly diverse, participating in protein import, mitochondrial dynamics, and regulation of apoptosis and mitophagy

Among the quality control proteases, we can also find ATP23 (XRCC6BP1) [24, 25]. Although the function of this metalloprotease in mammals has not been confirmed yet, its yeast orthologue participates in the quality control of mitochondria through the degradation of lipid transfer proteins that are highly conserved in the course of evolution. The high functional conservation that is usually found among mitoproteases from yeast to mammals suggests that ATP23 might play a similar role in mammalian mitochondria [25].

NLN is supposed to be an oligopeptidase that functions in the intermembrane space. It is also known as *neurolysin* or *mitochondrial oligopeptidase M* and can be found both in the cytosol and inside of mitochondria [26].

Finally, among the mitoproteases from the intermembrane space, we can find LACTB, whose function has not been completely defined yet. However, as it is known to form internal filaments, it has been proposed to possess a structural function through the compartmentalization and structuration of the intramitochondrial membrane [27].

1.2.3 Proteolysis in the Inner Membrane

The mitoproteases located in the inner membrane are diverse and participate in several if not all mitochondrial processes that occur in this compartment (Fig. 1.1). Among them, we can find the ATP-dependent proteases mAAA (SPG7 y AFG3L2)

and iAAA (YME1L1), which participate in the quality control of the inner membrane, in the mitochondrial biogenesis, and in the regulation of mitochondrial dynamics. iAAA, and its only subunit YME1L1 protease, has its active site oriented to the intermembrane space, whereas mAAA, composed in humans of AFG3L2 and SPG7 (or paraplegin), has its active site oriented to the matrix [28, 29]. mAAA and iAAA proteases exert their quality control role through different mechanisms. First of all, these proteases degrade damaged or non-assembled subunits of the electron transport chain, so their absence provokes the malfunction of oxidative phosphorylation (OXPHOS) caused by the accumulation of defective complexes [29–31]. Besides, mAAA subunit AFG3L2 is critical to the maintenance of inner membrane integrity under aberrant protein accumulation caused by the loss of temporal and spatial coordination in the assembly of the oxidative phosphorylation complexes [32]. AAA proteases also have a role in mitochondrial biogenesis. Some years ago, it was observed that yeast cells without mAAA had a respiratory deficiency as a consequence of damaged mitochondrial translation as well as a defective processing of the ribosomal subunit MrpL32 [33]. It was later defined that this protease controls the assembly of mitochondrial ribosomes and the synthesis of mitochondrially encoded respiratory chain subunits [32]. mAAA protease, and in particular its subunit AFG3L2, also has a role in calcium homeostasis as it induces mitochondrial fragmentation through the processing of OPA1, thereby decreasing calcium uptake [34]. Also, in the case of iAAA (YME1L1), it degrades the translocase subunit TIM17A as a consequence of the stress response, decreasing protein import into mitochondria [35]. Additionally, YME1L1 protease also modulates cardiolipin levels and the resistance to apoptosis by degrading PRELID1 (known as Ups1 in yeast), a protein that prevents apoptosis by complexing to TRIAP1, a p53-regulated protein, and mediating this way the intramitochondrial transport of phosphatidic acid, necessary for cardiolipin synthesis and consequently apoptosis resistance [36].

Mitoproteases in the inner membrane also have a role in mitochondrial dynamics (Fig. 1.1). OMA1, an ATP-independent protease, is a stress-response protease that functions together with the AAA proteases. It is activated under different stress conditions, such as oxidative and heat stress, and membrane depolarization [37]. As a result, it carries the proteolytic cleavage of all long forms of OPA1, inhibiting this way the fusion process [38–40]. OMA1 negatively regulates itself by autoprocessing in order to control and limit the stress response [37]. The processing of the inner membrane fusion protein OPA1 by OMA1 and/or YME1L1, as well as the regulation of the abundance of mitofusins and DRP1 at the outer membrane, serves to regulate mitochondrial dynamics. In particular, the processing of OPA1 by YME1L1 seems to provide a connection between mitochondrial dynamics and OXPHOS function dependent on the metabolic state [41]. Actually, it has been recently reported that depolarization of the mitochondrial membrane leads to OMA1 activation, and depending on the energy status of the cell (ATP levels), YME1L1 will degrade OMA1 (high levels) or OMA1 will degrade YME1L1 (low levels), allowing cells to adapt mitochondrial dynamics to distinct cellular insults [42]. It has also been described that a third and unknown cysteine protease is able to cleave OPA1 in the C-terminal region, apparently an event that occurs in the liver after a meal.

The exact nature of this additional processing of OPA1 is still unknown; however, it seems to be dependent on mitofusin 2 and independent of OMA1 [43]. In consonance to this, cells from *Oma1*-deficient mice are unable to process and inactivate OPA1. For this reason, these cells are protected against mitochondrial fragmentation, and consequently they show an increase in highly connected mitochondria [39]. On the other hand, *Yme1l1*-deficient mice cells have constantly activated OMA1, so they show fragmented mitochondria [44]. As a consequence of this regulation of mitochondrial dynamics by OMA1 and AAA proteases, *Oma1*-null mice have an unbalanced OPA1 processing, being unable to adapt mitochondrial dynamics to stress conditions in the cells. As a result, these mutant mice are obese and have a defective thermogenesis, proving the connection between the control of metabolic homeostasis and the regulation of mitochondrial dynamics [39, 45]. Very recently, it has also been described that loss of Oma1 in yeast cells leads to elevated ROS levels and activation of stress survival responses in a TORC1-mediated way, linking mitochondrial quality control and TOR signaling in the response to stress stimuli [46].

Mitoproteases of the inner membrane also participate in the regulation of apoptosis by different mechanisms (Fig. 1.1). For example, mitochondrial fusion serves to maintain the integrity of cristae, avoiding this way the release of pro-apoptotic proteins and protecting against apoptosis. Probably for this reason, the absence of YME1L1 leads the processing of OPA1 by OMA1, causing a loss of the cristae integrity and mitochondrial fragmentation and eventually an increased susceptibility to apoptosis [44]. OMA1 also has a role in the regulation of mitophagy, as alteration of its activity stabilizes OPA1 and prevents mitochondrial fragmentation, a process that is required in order to elicit mitophagy [47]. On the contrary, it can be activated by the pro-apoptotic proteins BAX and BAK [48] and by other stress stimuli [37]. As a result of the degradation of OPA1, cytochrome c is released promoting apoptosis. Consequently, deficiency in this metalloprotease protects against apoptotic stimuli [39, 49].

PARL is a rhomboid intramembrane protease located in the inner membrane and with known roles in mitophagy and autophagy. In the first case, its function is related to the degradation of the mitochondrial kinase PINK1, which triggers the binding of parkin protein to depolarized mitochondria in order to induce mitophagy. Other mitoproteases participate in the processing of PINK1. Thus, under normal conditions, PINK1 enters the mitochondria, and once there, it is processed by MPP [50]. In addition, it is cleaved and consequently destabilized by PARL, leading to its release from mitochondria in order to be degraded by the ubiquitin-proteasome system in the cytosol [51]. Afterwards, PINK1 is recycled in a process still poorly understood involving other mitoproteases such as mAAA, CLPP, and LONP1 [50]. PARL also regulates mitophagy by participating in the degradation of PGAM5 [52], a phosphatase that activates mitophagy and necrosis under stress conditions. Its degradation by PARL would therefore prevent mitophagy [53, 54]. Finally, PARL also has a role in the regulation of apoptosis in a HTRA2-independent manner, through the remodeling of cristae and the control of cytochrome c release during apoptosis [55, 56]. Accordingly, mice deficient in PARL show an increased apoptosis that leads to a premature death [55].

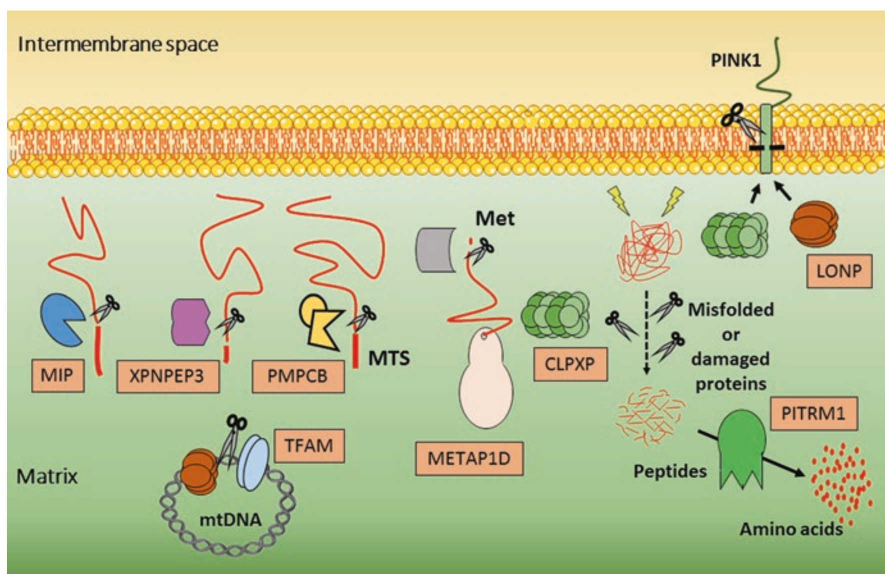

Fig. 1.2 **Matrix mitochondrial proteases**. Intrinsic mitochondrial proteases situated mainly in the matrix. Their roles in this compartment are predominantly the processing of imported peptides, maintenance of mtDNA, and degradation of misfolded or damaged proteins

The last mitoproteases from the inner membrane are IMMP1L and IMMP2L, processing peptidases that eliminate hydrophobic signals from proteins that have been imported into the intermembrane space after being processed by MPP [57]. These two mitoproteases also promote the assembly of yeast translocase inner membrane (TIM) complexes through the cleavage of specific carboxy-terminal subunits [58].

1.2.4 Proteolysis in the Mitochondrial Matrix

Among the ATP-dependent proteases that exert their function in the mitochondrial matrix, we can mention LONP1 and CLPP, which participate in quality control, mitochondrial biogenesis, and mitochondrial stress response (Fig. 1.2). LONP1 is a serine protease highly conserved through evolution. It participates in the quality control of the matrix by degrading oxidized, misfolded or mutated proteins, ensuring cell viability. Therefore, it can respond to different stress stimuli that can be potentially harmful [59, 60], as well as to normal conditions that require a reconditioning of the protein homeostasis in mitochondria. LONP1 has been shown to degrade several proteins in mammals, including succinate dehydrogenase subunit 5 (SDH5), aconitase, glutaminase C, cytochrome *c* oxidase isoform COX4–1, steroidogenic acute regulatory protein (StAR), mitochondrial transcription factor A (TFAM), cystathionine-β-synthase (CBS), heme oxygenase-1, and 5-aminolevulinic

acid synthase (ALAS1) [61–68]. Probably due to this wide spectrum of action, mice *knockout* for LONP1 exhibit an early embryonic lethality. However, mice heterozygous for the deletion of *Lonp1* show alterations in mitochondrial respiration and in the OXPHOS system, probably causing an inability for metabolic reconversion in malignant cells and thereby showing a decreased tumoral susceptibility. This study has clearly demonstrated the indispensable role of LONP1 in life and disease [69]. This mitoprotease also has a role in mitochondrial biogenesis, as it functions as a DNA-binding protein that upon stress conditions is released from mtDNA and degrades the transcription factor TFAM. This way, LONP1 controls mtDNA maintenance as well as mitochondrial gene expression [68, 70, 71].

The other ATP-dependent protease with proteolytic function in the mitochondrial matrix is the serine protease CLPP (Fig. 1.2). It is known that CLPP forms the complex CLPXP together with the chaperone CLPX; however, its function is still not completely clear. It has been related to the degradation of misfolded proteins and, consequently, to the mitochondrial unfolded protein response (UPR^{mt}) in *Caenorhabditis elegans* [72]. However, it has been recently reported that its deletion in DARS2-deficient mice, a mouse model of UPR^{mt}, alleviates their mitochondrial cardiomyopathy, suggesting that CLPP does not participate in mammalian UPR^{mt} [73]. It has also been described that CLPP absence in mice provokes hearing loss, infertility, and growth retardation, probably through the accumulation of CLPX subunits and mtDNA [74].

Apart from these two ATP-dependent proteases, mitochondrial matrix also needs processing peptidases engaged in the import of proteins to mitochondria and oligopeptidases that deal with the peptides that result from the degradation of damaged proteins by LONP1, CLPP, and mAAA proteases (Fig. 1.2). Among them we find PITRM1, an oligopeptidase from the pitrilysin family. This metalloprotease also shows a presequence processing role that is critical for correct mitochondrial function [75–77]. PITRM1 degrades the mitochondrial amyloid β-protein in human cells [78] and, in yeast, it has also been observed that amyloid β-protein can in turn inhibit the yeast orthologue of PITRM1, impairing this way the processing activity of MPP. This would trigger the accumulation of preproteins in mitochondria, linking accumulation of amyloid β-protein to mitochondrial dysfunction [77].

As explained before, most of mitochondrial proteins are encoded in the nucleus DNA. In order to be translocated into the mitochondria after being synthesized in the cytosol, these proteins carry mitochondrial import signals that, once inside of the organelle, need to be removed. This action is performed by the so-called mitochondrial processing peptidases, a group of enzymes that includes PMPCB, MIPEP, METAP1D, and XPNPEP3 [79]. PMPCB (also known as β-MPP) is responsible for the processing of the majority of mitochondrial proteins, thus being the most important mitochondrial protein peptidase [80]. It forms a heterodimer with its non-protease homologue PMPCA (α-MPP), resulting in the MPP complex [81]. PMPCA, although without protease activity, facilitates the proteolytic processing of PMPCB by recognizing and binding to the mitochondrial targeting presequences from the imported proteins [82]. As already stated, PMPCB is the major mitochondrial processing peptidase. However, there are other processing peptidases with more defined