Emerging role of Lon protease as a master regulator of mitochondrial functions

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Abstract

Lon protease is a nuclear-encoded, mitochondrial ATP-dependent protease highly conserved throughout the evolution, crucial for the maintenance of mitochondrial homeostasis. Lon acts as a chaperone of misfolded proteins, and is necessary for maintaining mitochondrial DNA. The impairment of these functions has a deep impact on mitochondrial functionality and morphology. An altered expression of Lon leads to a profound reprogramming of cell metabolism, with a switch from respiration to glycolysis, which is often observed in cancer cells. Mutations of Lon, which likely impair its chaperone properties, are at the basis of a genetic inherited disease named of the cerebral, ocular, dental, auricular, skeletal (CODAS) syndrome. This article is part of a Special Issue entitled "EBEC 2016: 19th European Bioenergetics Conference, Riva del Garda, Italy, July 2–6, 2016", edited by Prof. Paolo Bernardi.

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~~1.~~1 Introduction

Lon protease (Lon, also known as Lonp1) is a nuclear encoded, mitochondrial ATP-dependent serine peptidase, which mediates the selective degradation of mutant and abnormal proteins in the organelle, and helps in the maintenance of mitochondrial homeostasis. Proteins belonging to the family of Lon proteases are present virtually in all organisms. The interest in Lon has been rapidly increasing in the last years, as it turns out to be deeply involved in a plethora of biological processes, including (but not limited to) the turnover of mitochondrial proteins, the regulation of mitochondria DNA replication, cellular respiration and oxidative phosphorylation, and the maintenance of mitochondrial morphology and dynamics. Here, we review the most recent discoveries concerning the functions of Lon protease, paying particular attention to its role in the regulation of mitochondrial metabolism and carcinogenesis.

~~2.~~2 Structure and evolution of Lon protease

On the basis of sequence homology and structure, Lon proteases can be divided into two subfamilies: LonA (found in *eubacteria* and *eukarya*) and LonB (found in *archaea*) [1]. LonA proteases are formed by three functional domains: the

N-terminal, involved in substrate binding, the central AAA + domain, and the C-terminal domain (named P domain), which containing the Ser–Lys catalytic dyad for proteolytic activity [2]; LonB proteases are composed by an ATPase and a protease domain and a hydrophobic transmembrane region which anchors the protein to the internal face of cell membrane [1].

Lon is highly conserved throughout evolution, and its homologues have been found in all kingdoms [3]. Its high degree of conservation suggests that its presence is crucial for cell survival: its silencing leads to cell death *in vitro*, and its knock-out is embryonically lethal in mice. In *eubacteria*, members of Lon protease family are present as soluble cytoplasmic proteins, in *archaea* are bound to the cell membrane, and in *eukarya* are localized in mitochondria and peroxisomes [4,5].

The first Lon protease was identified in *Escherichia coli*, as an ATP-dependent protease with a Ser–Lys catalytic dyad form, and named “La” because *E. coli* mutants lacking *lon* gene tended to be longer than their wild-type counterparts upon UV irradiation [6]. This protein degrades misfolded or mutant proteins, is involved in radiation resistance, cell division, filamentation, capsular polysaccharide production, and survival under starvation conditions. Moreover, it selectively degrades some short-lived regulatory proteins [7] and binds DNA in a sequence specific manner [8]. The oligomeric crystal structure of a bacterial Lon protease has not yet been solved; however, the three dimensional structure of both the N (amino acids 8–117) and AAA + domains of the bacterial Lon protease haves been successfully obtained [9–11]. In *E. coli*, Lon protease forms an oligomer of six or twelve subunits, and creates a ring-shaped structure with a central cavity, which reminds the shape of the 26S proteasome [12].

In *eukarya*, two Lon proteases are present: a mitochondrial and a peroxisomal form, encoded by two different genes. The mitochondrial Lon protease is encoded by the nuclear genome [13] and, after translation as a precursor form that carries a mitochondrial targeting sequence (MTS) at N-terminal, is imported into mitochondrial matrix where MTS is cleaved and the protein is converted into the mature form. Eukaryotic Lon shares the majority of properties with bacterial Lon, including the oligomerization in a ring-shaped form [14,15], the capacity to bind DNA, the essential role in protein quality control and in the protection of mitochondrial proteins from stress-induced aggregation [3,16].

The human mitochondrial Lon protease is encoded by the *LONP1* gene, which is approximately 29,000 base pair long. The main transcript variant of *LONP1* is about 3.4 Kb long, while two alternative transcripts encode Lon isoforms with shorter N-term coding region [13]. Lon produced as a preprotein of 959 aa with an N-term MTS that targets the protein to mitochondria [13]; the mature enzyme is a protein with a molecular weight of approximately 100 kDa [17]. Lon is located mainly in the matrix as a soluble protein, while a little fraction is associated with nucleoids in the inner membrane [18,19]. The 3D structure of the catalytic domain of human Lon is similar to those previously determined in other species [14]; crystal structure data suggest that the human Lon can form hexamers, which is the fully active form of the enzyme.

Lon is expressed ubiquitously in human tissues and organs, being present at high levels in the liver, brain, heart, skeletal muscle, placenta, and at low but detectable levels in kidney, lung, pancreas and immune cells [13].

3.3 Lon and mitochondrial metabolism

The critical role of Lon in mitochondrial protein homeostasis and cell metabolism has been demonstrated in a wide range of organisms across kingdoms. Over the last twenty years, several observations obtained by using a variety of experimental models have indicated that Lon is an important regulator of mitochondrial activity, and: i) acts as a multi-functional enzyme to remove misfolded and oxidatively-modified proteins; ii) supports cell viability during proteotoxic, hypoxic and endoplasmic reticulum stress; and iii) regulates mtDNA metabolism (Fig. 1). This enzyme has several functions, including that of a protease, a chaperone and a DNA-binding protein; its down-regulation has detrimental effects on mitochondrial respiration, mtDNA quantity and quality, and, ultimately, on cell metabolism.

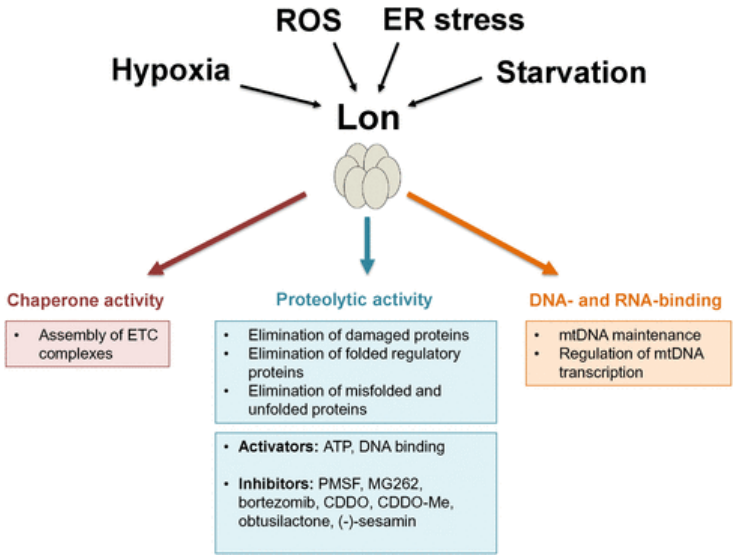

Fig. 1 Functions of Lon protease in human mitochondria; the stressors able to modulate Lon expression are indicated, as well as the molecules able to stimulate or inhibit Lon proteolytic activity. ETC, electron transport chain; PMSF, phenylmethanesulfonyl fluoride; CDDO, 2-cyano-3, 12-dioxoo-leana-1,9(11)-dien-28-oic acid; CDDO-Me, 2-cyano-3, 12-dioxoo-leana-1,9(11)-dien-28-oic methyl ester.

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~~3.1.~~3.1 Proteolytic activity of Lon

Although Lon is a multifunctional enzyme, it was first identified as a serine protease, with an active site containing the highly conserved Ser–Lys catalytic dyad localized in the P domain at the C-terminus. In ~~*Escherichia* *E. coli* Lon is one of the main soluble proteolytic enzymes responsible for degradation of abnormal proteins, and is part of the heat shock regulon [20]. The ~~homolog~~homologue of bacterial protease La in *Saccharomyces cerevisiae* is localized in mitochondria and is known as proteolysis in mitochondria (Pim1) or Lon [21]. Yeasts lacking Pim1 display a non-functional mitochondrial genome, are respiratory-deficient, and loose the ATP-dependent proteolytic activity [21]. Complementation analysis revealed that *E. coli* protease La and yeast Pim1 were functionally equivalent, and that the expression of a chimeric protein bearing a mitochondrial targeting sequence at the N-terminus of protease La in yeasts lacking Pim1 restored proteolytic activity and stabilized mitochondrial genome [22]. Presuming that in the absence of Pim1 proteins that accumulate in mitochondria are substrates of its proteolytic activity, a number of targets of such function have been identified so far, and include proteins involved in ribosome assembly, energetic metabolism, and mtDNA metabolism [23]. The ability of Pim1 to degrade proteins modified by oxidative stress has also been reported, and strengthens the idea that lack of Pim1 in yeasts contribute to the aging phenotype and predispose the organism to age-related conditions [24].

Lon proteolytic activity has been maintained throughout the evolution, and, in mammals, is crucial for the maintenance of mitochondrial function and integrity through the elimination of proteins damaged by oxidative stress and/or the selective degradation of key proteins in response to multiple stresses or metabolic changes [3]. In humans, Lon exists as oligomers and, in particular, is catalytically active in the form of a hexameric ring [14]. The catalytic dyad required for peptide-bond hydrolysis is localized at Ser885–Lys896. Mechanistic studies have demonstrated that Lon cleavages generally occurred between hydrophobic amino acids positioned at internal sites within the primary sequence of the target protein, and consequential cleavages occurred processively along the polypeptide chains [25].

Modifications able to modulate Lon proteolytic activity have not yet been identified. Nonetheless, human Lon is target of sirtuin 3 (SIRT3), likely at Lys-917, which is near the catalytic dyad, thus suggesting that Lon proteolytic activity can be regulated *via* deacetylation [26]. In humans, Lon is responsible for the degradation of: i) stably folded proteins, including 5-aminolevulinic acid synthase, steroidogenic acute regulatory protein and mitochondrial transcription factor A (TFAM) and cytochrome *c* oxidase 4 isoform 1 (COX4-1) [27–30]; ii) misfolded and unfolded proteins, including glutaminase C [31]; and iii) oxidatively-modified proteins, including mitochondrial aconitase and cystathionine beta-synthase [18,32]. Recent studies that used a folding-incompetent form of the mitochondrial ornithine transcarbamylase (OTC), named OTC-Δ, to assess the ability of Lon to degrade misfolded proteins, revealed that Lon efficiency in proteolysis could vary according to the status of its targets. Interestingly, the rate of degradation of unfolded OTC-Δ was much faster than the rate of unfolded OTC, likely because of the exposure of different hydrophobic residues in the unfolded state of these proteins [33]. Conversely, aggregated proteins are not targets of Lon proteolytic activity.

Lon proteolytic activity plays a role at different stages in the mitochondrial stress response [34]. Although Lon cannot yet be considered as a key player in the mitochondrial unfolded protein response (UPR^{mt}), a recent multi-layered genetic and proteomic dissection of mitochondrial activity in the liver proteome from 40 strains of BXD mice revealed that Lon expression highly correlates with expression of heat shock 60 kDa protein-1 (HSPD1), heat shock 10 kDa protein-1 (HSPE1), heat shock 70 kDa

protein-9 (HSPA9), and caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP), which are all involved in the UPR^{mt} [35]. It has also been reported that, upon mitochondrial proteotoxic stress, C/EBP homology protein (best known as CHOP) activates heat shock proteins as well as several proteases, including Lon, which collectively prevent the accumulation of misfolded proteins in the mitochondria [36]. In addition, in human cells, Lon is up-regulated after serum starvation [37], and its down-regulation leads to increased starvation-induced autophagy [38], and accumulation of PTEN-induced putative kinase-1 (PINK1), an essential regulator of mitophagy [39]. Similarly, in *Drosophila*, Lon promotes the degradation of PINK1 within the mitochondrial matrix to prevent healthy mitochondria to be targeted for mitophagy [40]. These observations indicate that Lon could somehow participate in the regulation of mitophagy, which represents one of the late-stage quality control mechanisms in mitochondrial stress response.

3.2. 3.2 Chaperone activity of Lon

Molecular chaperones essentially act to *de novo* fold proteins or to refold misfolded proteins to their native state [41]. Lon merges proteolytic and chaperone activities on a single polypeptide chain, but while proteolytic activity is restricted at the P domain, chaperone activity is mediated by the ATP-binding domain and the N-terminal domain. The 2.0-A° resolution crystal structure of *Thermococcus onnurineus* NA1 Lon (TonLon) revealed that the chaperone and degradation chambers are contiguous and there is virtually no constriction of the chamber between the chaperone domain and the protease active sites [42]. Chaperone-like functions of Lon are involved in the assembly of mitochondrial membrane complexes in yeast and in humans, and, at least in yeast, these functions are maintained after inactivation of proteolytic site and are prevented when ATP-binding site is mutated [43,44]. Interestingly, in yeasts lacking Afg3p and Rca1p ATP-dependent metalloproteases, growth deficiency and defects in the assembly of the inner membrane complexes can be partially rescued by overexpression of Pim1. However, a cooperation of both activities is required for the full function of Pim1 [44]. Targets of Lon chaperone activity are still not known. However, using a proteomic approach, one of the first attempts to identify such targets created a list of 76 candidate proteins that, at the end, allowed to identify as Lon binding partners NADH dehydrogenase ubiquinone iron–sulfur protein 8 (NDUFS8), heat shock protein (Hsp)-60, and mtHsp70 [45]. Nevertheless, whether or not Lon assists folding and/or refolding of these proteins is still unclear.

3.3. 3.3 Lon as a mtDNA-binding protein

Together with its proteolytic and chaperone activities, Lon ability to bind mtDNA is conserved from bacteria to mammalian mitochondria [46,47]. *E. coli* Lon binds both single stranded DNA (ssDNA) and RNA (ssRNA), and double stranded DNA (dsDNA) in a non-specific manner, and this interaction enhances Lon ATPase and proteolytic activities [46,48–50]. Unlike bacterial Lon, human Lon binds specific ssDNA [47]. Specifically, it binds sequences contained in the light chain promoter (LSP) noncoding DNA and in the heavy chain promoter (HSP) coding DNA, which are both sites where replication and transcription of mtDNA are initiated [47]. *In vitro* and bioinformatics analyses revealed that Lon binds G-rich DNA consensus sequences that have the propensity to form G-quartets or G-quadruplex, as well as GU-rich RNA sequences [19,47]. Even if G-quartets structure could potentially form in several regions along mtDNA, yet not all of these regions are actually bound by Lon. Indeed, the single- or double-stranded state of mtDNA, together with the presence of other proteins that can restrain Lon interaction, or the G-quartets flanking sequences are factors affecting Lon binding to G-quartets. Moreover, Lon ability to bind to DNA needs conformational changes in Lon itself, and such changes are inhibited by ATP, and are stimulated by a protein substrate. Interestingly, Lon S855A mutant, which lacks both ATPase and proteolytic activity, still maintains DNA binding activity but, in this case, does not undergo conformational changes [19].

Co-immunoprecipitation studies also revealed that Lon interacts with Twinkle, polymerase γ, TFAM, which are major constituents of mtDNA nucleoids, and with mitochondrial heat shock protein-70 (mtHSP70), and heat shock protein-60 (Hsp60), which also have a role in mtDNA metabolism [19,45]. Although the physical interaction between Lon and these proteins have has been demonstrated, the functional outcome of such interactions is still unknown for almost all proteins, except for TFAM, which is the major mtDNA packaging protein and the most important regulator of mtDNA transcription [51,52]. Indeed, the number of TFAM molecules per mitochondrial genome, *i.e.* the ratio TFAM/mtDNA, determines the compaction of mtDNA thus affecting accessibility to transcription and replication factors [52]. The higher is the ratio, the tighter is mtDNA compaction and the lower is the accessibility to mtDNA. In *Drosophila* Schneider cells, overexpression of Lon reduced TFAM levels, and, conversely, Lon down-regulation is associated with increased levels of TFAM and mtDNA, increased mitochondrial transcription, and unchanged levels of other mtDNA nucleoid proteins, including helicase, DNA polymerase γ, and mitochondrial single strand binding protein (mtSSB). By selectively degrading TFAM and controlling TFAM/mtDNA ratio, Lon is responsible for mitochondrial transcription maintenance [53]. Recent studies reported that, in human cells, phosphorylation of TFAM at Ser 55 and 56 at the high-mobility group box-1 (HMG1) impairs its ability to bind mtDNA and promotes its degradation by Lon [29].

Yeast cells lacking Pim1 displayed higher frequency of large mtDNA deletions that lead to defects in respiration [21,54]. A similar phenotype has been observed in yeast cells missing Ump1, which has a key role in the assembly of 20S proteasome, thus suggesting that the role of Lon in mtDNA maintenance should depend on the integrity of Lon proteolytic activity, among other factors [55]. Lon knockdown in B16F10 melanoma cells is associated with decreased mtDNA levels, and Lon-null mouse embryos, which are not viable, also display a decrease in mtDNA copy number if compared to heterozygous or wild-type embryos [43]. These reports highlight the importance of Lon in the maintenance of mtDNA levels. Nonetheless, other data show that levels of Lon binding to mtDNA may vary depending on: i) a specific intracellular setting, ii) a specific cell type, and iii) the absence or presence of stressors. On one hand, at the single cell level, specific mtDNA polymorphisms could affect Lon binding capacity. On the other, the specific mitochondrial status of different cell types could impact the kinetics of the single-stranded *versus* double-stranded state of mtDNA thus altering binding of Lon and other proteins [56]. Among stressors, reactive oxygen species (ROS) deeply influences Lon binding to mtDNA. In LS174T human colon adenocarcinoma cells, the exposure to H_2O_2 significantly reduced Lon binding to mtDNA. It has been hypothesized that the reduced binding can occur at least for three non-mutually exclusive reasons: i) repair and replication proteins are recruited to mtDNA and compete with Lon binding; ii) Lon itself is recruited away from mitochondrial genome; iii) oxidatively modified Lon is not able to bind mtDNA [56].

Although there is certainly evidence that Lon has a role in the maintenance of mtDNA and in the regulation of its copy number, the precise function still remains unclear. For instance, a crucial point is to clarify whether or not Lon has a direct role in mtDNA replication and/or transcription or acts to degrade and regulate proteins that form nucleoids is still not known [57].

4.4 Lon in genetic diseases

Together with ClpXP, m-AAA and i-AAA, Lon is one of the major enzymes with proteolytic activity acting in the mitochondrial matrix. Very recently, mutations in *LONP1* gene have been mapped and identified as causative of the cerebral, ocular, dental, auricular, skeletal (CODAS) syndrome, a multisystem developmental disease characterized by a wide variety of clinical manifestations, including hypotonia, ptosis, motor delay, hearing loss, postnatal cataracts and skeletal and dental abnormalities [58,59]. Eleven missense mutations and one nonsense mutation have been identified in eleven patients [58,59]. The majority of these mutations are localized in the AAA+ domain, and result in amino acids substitutions. *In vitro* analysis of Lon variants revealed that: i) proteolytic activity is maintained for certain Lon substrate but not for other; ii) localization is still mitochondrial; and iii) the ability to form homo-oligomers is impaired [59].

To better understand which are the consequences of Lon mutations *in vivo*, immortalized CODAS lymphoblast cell lines have been generated [59]. In such cells, while mtDNA copy number was unchanged, mitochondrial ultrastructure was abnormal, showing electron-dense aggregations, and reduction in the maximal oxygen consumption. Interestingly, recently a clinical condition that shares the skeletal features of the CODAS syndrome has been described, that includes a complex mixture of other manifestations that have been collected under the name epiphyseal, vertebral, ear, nose, plus associated findings (EVEN-PLUS) syndrome [60]. Three out of three patients had recessive mutations in the heat shock 70-kDa protein 9 (HSPA9) gene which encodes for the mitochondrial chaperone HSPA, also known as mortalin or 75-kDa glucose-regulated protein (GRP75). The fact that mutations in two distinct proteins like Lon and HSPA9 are associated with a similar phenotype suggests that these two molecules might be functionally linked in the network of mitochondrial chaperone–protease, and that a new family of diseases, namely mitochondrial chaperonopathies, likely exists and has to be further characterized.

Many of the CODAS-associated mutations involve residues located on the hexamer external surface. Fig. 2 provides insight into the weak interaction involving some of the residues that were found mutated in CODAS-affected patients. As anticipated by Dikoglu et al. [58] some of the interactions involving the residues in the wild type form will be lost upon mutation. This is the case of Glu476, forming a salt bridge with Arg542 that cannot be restored upon substitution to an arginine; similar conclusions can be drawn for Arg672, interacting with Glu488. Panel B of Fig. 2 displays the surface electrostatic potential of the Lon hexamer: despite the limitations imposed by having performed the calculation on a structure obtained by homology modelling, it is worth noting that its distribution on the molecular surface is characterized by the presence of large patches of negative potential, but also of some well defined spots displaying markedly positive potential values, located in the internal cavity formed by the six monomers. Electrostatic forces are known to play crucial roles in protein–protein interactions and substrate recognition [61]; therefore, the presence of well defined positive and negative regions is likely to be involved in the recognition of partners displaying regions of opposite surface electrostatic potential, as might be the case of mtDNA.

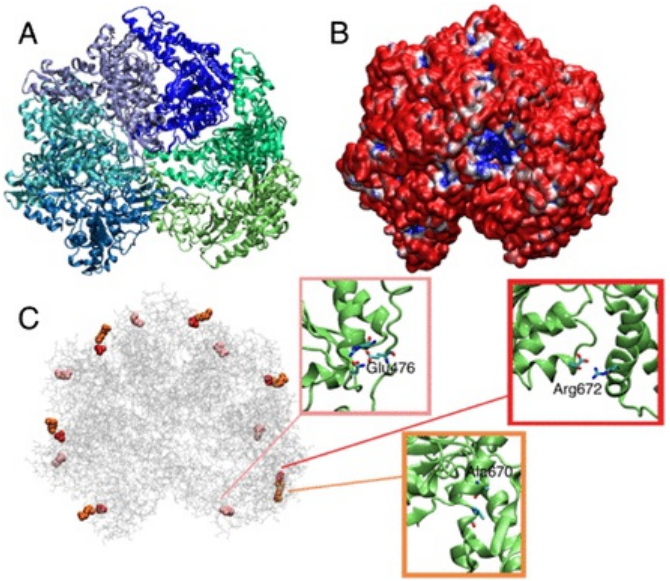

Fig. 2 A) The structure of the human mitochondrial Lon hexamer, without its amino-terminal mitochondrial-targeting sequence (MTS), calculated by homology modelling by Swiss-Model [72–74], using the crystal structure of the Lon hexamer from *Bacillus subtilis* [75] as template. Each monomer is displayed in a cartoon representation and coloured differently. B) Electrostatic potential mapped on the molecular surface of human Lon hexamer. Potentials less than – 3 kT/e are ~~colored~~coloured in red, and those greater than + 3 kT/e are depicted in blue. The electrostatic potential was calculated using the APBS software [76] The .pqr input file required to run APBS was prepared using PDB2PQR [77], on the Lon hexamer model calculated by homology modelling. C) The location of some of the CODAS-associated mutations on the three-dimensional structure of human Lon is shown. The hexamer structure is represented as lines; Glu476, Ala670 and Arg672 are represented as spheres and coloured in pink, orange and red, respectively. A zoom on the residues mutated in patients affected by CODAS and their interaction with neighbouring amino acids is also provided. All the panels

in this figure were prepared using VMD software [78].

alt-text: Fig. 2

5.5 Lon and cancer

Lon is not an oncoprotein *stricto sensu*. However, several lines of evidence point to Lon as a protein deeply involved in the metabolic reprogramming observed in neoplastic transformation. First, upregulation of Lon protease has been observed different types of human cancer, including (but not limited to) different malignant B-cell lymphoma, mammary epithelial, cervical cancer, non-small-cell lung cancer cells, bladder cancer cells, oral squamous cell carcinoma, and colorectal, head-and-neck, and metastatic prostate cancer [38,43,62–65].

Second, Lon protease plays a crucial role in the process of cell adaptation to a hypoxic environment, a condition often observed in solid tumours [66]. *LON* gene expression is induced in response to hypoxia, through a direct effect of HIF-1α, the master regulator of oxygen homeostasis, on *LON* gene promoter [27,67]. Several HIF-1α binding sites have been detected in the 5′-flanking region of the human *LON* gene, at positions −29, −67, −192 and −506 from transcriptional start site [27]. When upregulated by HIF-1α, Lon protease degrades the cytochrome *c* oxidase 4-1 subunit (COX4-1), and allows the assembly of the alternative subunit COX4-2, which in turn confers optimal enzyme activity in condition of low oxygen tension and adapts cancer cells to hypoxic conditions [27,37]. Hypoxia leads to Lon upregulation in several cell types in humans, including monocytic acute myelogeneous ~~leukemia~~leukaemia (THP-1), cardiomyocytes, embryonic kidney (293T) cells, rhabdomyosarcoma cells, renal cell carcinoma (RCC4) stably expressing Von Hippel–Lindau protein (VHL) [27,37,63,68,69]. Moreover, Lon overexpression impairs Complex I assembly in melanoma cells, causing up-regulation of NDUFB6, 8, 10 and 11, and downregulation of NDUFV1, NDUFV2, NDFUS3 and NDFUS7. These changes lead to a reduction of respiration through Complex I, an overall downregulation of respiration and upregulation of glycolysis [43]. *In vivo*, Lon overexpression favours glycolysis, facilitates proliferation, and capability to migrate and form metastasis of melanoma cells in nude mice [43].

Third, Lon$^{+/-}$ mouse model, in which the expression of Lon is halved, is characterized by a lower tendency to develop cancer and a higher resistance to carcinogenic compounds than wild type counterparts. Accordingly, growth of Lon-silenced cancer cells in xenograft model is significantly reduced if compared to control cells, while cells overexpressing Lon grow more rapidly [43].

Fourth, clinical data strongly suggest that a higher expression of Lon is associated with higher aggressiveness of different types of cancer, and short survival of patients ha~~ve~~s been observed in metastatic melanoma expressing high levels of Lon and, in agreement with such observation, overexpression of Lon in melanoma increases experimental metastasis formation, while knockdown decreases cell proliferation and lung metastasis [43]. Moreover, a retrospective immunohistochemical analysis on paraffin embedded tissues of bladder cancer showed that patients with high Lon expression had lower overall survival rates than those with low Lon expression [64].

Fifth, compounds able to inhibit Lon protease activity have the capability to slow down cancer cell growth. To date, few molecules targeting Lon protease activity are known; some of them, such as MG132 and *clasto*-lactacystin ~~b~~-lactone (cL~~b~~L) are proteasome inhibitors that can partially act on Lon protease [28]; others, such as the synthetic triterpenoid CDDO and its methyl ester derivative (Me-CDDO), are more specific for Lon protease. These latter molecules can block Lon activity in lymphoma B cells, leading to apoptotic cell death ~~apoptotic cell death~~, similarly to what observed in Lon knockdown cells [62]. This effect can be observed in other cancer cell lines, including RKO colon cancer cells and hepatocarcinoma HepG2 cells [70], but is not present in normal cells such as fibroblasts, suggesting that neoplastic cells are more sensitive to Lon inhibition than normal, non-transformed cells.

Although the role of Lon protease in carcinogenesis has been partially elucidated, some aspects remain to be clarified. In particular, it is not clear which is, among the different functions of Lon protease, the one(s) that favour tumour survival and proliferation. As it is very difficult to separately analyse the functions of Lon, the effects of its higher expression and the consequence of its upregulation at organelle and cellular levels in cancer cells are far from being clear. So far, changes observed in mitochondria of cancer cells overexpressing Lon indicate that a prominent role is played by the proteolytic and chaperon functions of Lon, while the functions related with mtDNA regulation are less relevant. When Lon expression is altered, changes in mitochondrial architecture and functionality seem to be related to a profound alteration of mitochondrial proteome, not limited to protein encoded by mtDNA, which in turn makes cancer cells more resistant to the stress conditions typically observed in tumour microenvironment [38]. However, a definitive response could be obtained only with mutants of Lon in which the single functions of the protein will be abolished.

Another crucial aspect concerning the role of Lon in carcinogenesis that has to be better clarified is whether its upregulation that can directly promote neoplastic transformation, or is just a secondary adaptation that favours cancer cell growth. Some data seem to indicate that the overexpression of Lon protease could play a direct role in cancer cell proliferation and tumour aggressiveness. As stated above, Lon overexpression causes Complex I impairment, through the stabilization and upregulation of NDUFS8. As a consequence, ROS produced by Complex I promote cell proliferation through the activation of p38, JNK, and ERK1/2, and Ras–ERK signalling, which in turn favour cell proliferation. Moreover, Lon overexpression promotes epithelial mesenchymal transition (EMT), by increasing the expression of E-cadherin, N-cadherin, vimentin, and Snai1, and favours cell migration and invasion by up-regulating MMP-2. Conversely, Lon down-regulation impairs cell migration. Since antioxidant molecules arrest Lon-induced EMT, it is likely that this process is induced by Complex I-dependent increase of ROS generation [63].

Studies in non-transformed cells could provide further insights on the role of Lon in carcinogenesis. The observation that, in normal human fibroblasts, silencing of Lon leads to abnormal mitochondrial function and morphology, switch to anaerobic metabolism and apoptosis suggests that the effects of Lon silencing observed in most cell lines could be a general phenomenon, not limited to cancer cells [71].

6. Conclusions

Despite the notable progression in the knowledge of Lon biology in the last years, several aspects of its role remains to be addressed. In particular, it is not clear which is the relative contribution of the different activities of Lon to mitochondrial homeostasis. Elucidating this aspect could be extremely helpful for designing drugs able to target specific Lon activities, without affecting the others.

At this regard, although Lon protease knock-down is embryonically lethal and causes *in vitro* cell death, the recent association of Lon mutations with CODAS syndrome clearly indicates that a partial alteration of the functionality of the enzyme is compatible with life. Thus, it will be extremely important to understand whether different mutants of Lon observed in CODAS patients are characterized by the impairment of just one of the multiple functions of Lon.

Transparency Document

The Transparency Document associated with this article can be found, in the online version.

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- Lon displays proteolytic activity, chaperone activity and mtDNA-binding activity.
- Lon dysregulation is involved in cancer and in CODAS syndrome.

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