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REVIEW

Mitochondrial Lon protease at the crossroads of oxidative stress, ageing and cancer

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Abstract Lon protease is a nuclear DNA-encoded mitochondrial enzyme highly conserved throughout evolution, involved in the degradation of damaged and oxidized proteins of the mitochondrial matrix, in the correct folding of proteins imported in mitochondria, and in the maintenance of mitochondrial DNA. Lon expression is induced by various stimuli, including hypoxia and reactive oxygen species, and provides protection against cell stress. Lon down-regulation is associated with ageing and with cell senescence, while up-regulation is observed in tumour cells, and is correlated with a more aggressive phenotype of cancer. Lon up-regulation contributes to metabolic reprogramming observed in cancer, favours the switch from a respiratory to a glycolytic metabolism, helping cancer cell survival in the tumour microenvironment, and contributes to epithelial to mesenchymal transition. Silencing of Lon, or pharmacological inhibition of its activity, causes cell death in various cancer cells. Thus, Lon can be included in the growing class of proteins that are not responsible for oncogenic transformation, but that are essential for survival and proliferation of cancer cells, and that can be considered as a new target for development of anticancer drugs.

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Abbreviations

AAA+	ATPase associated with diverse cellular activities
Aco2	Aconitase 2
ALAS-1	5-aminolevulinic acid synthase 1
AOX	acyl-CoA oxidase
cART	Combined antiretroviral therapy
C/EBP	CCAAT-enhancer-binding protein
CBS	Cystathionine β-synthase
CDDO-Me	2-cyano-3, 12-dioxoo- leana-1,9(11)-dien-28-oic methyl ester
CDDO	2-cyano-3, 12-dioxoo- leana-1,9(11)-dien-28-oic acid
CHOP	C/EBP homology protein
Clp	Caseinolytic protease
ClpX	Caseinolytic protease X
cLβL	Clasto-lactacystin β-lactone
CODAS	Cerebral, ocular, dental, auricular and skeletal anomalies
COX	Cytochrome *c* oxidase
COX4-1	Cytochrome c oxidase, subunit 4, isoform 1
CR	Caloric restriction
EMT	Epithelial–mesenchymal transition
ER	Endoplasmic reticulum
ERK1	Extracellular-signal-regulated kinase 1
ERK2	Extracellular-signal-regulated kinase 2
FITC	Fluorescein isothiocyanate
GLS-1	Glutaminase C
HIF1-α	Hypoxia inducible factor 1 α
HIV	Human immunodeficiency virus
JNK	c-Jun N-terminal kinase

LONP1	Lon peptidase 1, mitochondrial
Lyf-1	Lymphoid transcription factor
m-AAA	Matrix-ATPase associated with diverse cellular activities
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged-red fibre
MMP-2	Matrix metalloproteinase-2
MnSOD	Manganese superoxide dismutase
mtDNA	Mitochondrial DNA
MTS	Mitochondrial-targeting sequence
NDFUS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3
NDFUS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7
NDFUS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8
NDUFB6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1
NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2
NF-kB	Nuclear factor kappa-B
Nkx2-5	NK2 homeobox 5
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
NRTI	Nucleotide analogue reverse transcriptase inhibitor
OA	Obtusilactone A
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PGC1 α	Peroxisome proliferator-activated receptor γ coactivator 1 α
Pim1	Proteolysis into mitochondria 1
PMP70	70-kDa peroxisomal membrane protein
PMSF	Phenylmethanesulphonylfluoride
POLDIP2	Polymerase (DNA-directed) delta interacting protein 2
PRSS15	Serine protease 15
PI	Protease inhibitor
PTS1	Peroxisomal targeting sequence type-1
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SDH5	Succinate dehydrogenase 5
SDHA	Succinate dehydrogenase complex, subunit A
SIRT1	Sirtuin 1
SIRT3	Sirtuin 3
StAR	Steroidogenic acute regulatory protein
TCA cycle	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
Tysnd1	Trypsin domain containing 1
VHL	Von Hippel Lindau tumour suppressor

Introduction

Proteins present in cells are continuously damaged by physical and chemical agents, such as heat, free radicals and toxic compounds. These agents can cause misfolding or aggregation of proteins, formation of adducts, and ultimately lead to cellular dysfunction. When damaged proteins accumulate in the cell, they are selectively degraded by different proteolytic systems. In the mitochondrial matrix, three proteases belonging to the ATPase associated with diverse cellular activities (AAA+) superfamily [1] are responsible for this function: ClpX, m-AAA and Lon.

Lon protease activity has been identified more than 30 years ago in *Escherichia coli* [2], and the gene encoding Lon was cloned in 1984; the human homologue (LONP1, best known as Lon or mitochondrial Lon) was identified in 1993 [3, 4], as a nuclear gene encoded mitochondrial protein of approximately 100 kDa [5], ubiquitously expressed in tissues and cells. Despite its strong conservation throughout evolution, relatively little attention has been paid to the role of Lon in the normal physiology of mitochondria, and to its possible involvement in the pathogenesis of human diseases. However, in the recent years Lon emerged as a fundamental component of mitochondrial quality control, and as an important factor in the mitochondrial alterations that can be observed in chronic diseases and cancer.

In this review, we will discuss the recent discoveries concerning Lon protease functions, with a major emphasis on the emerging role of this protein in cancer and in other human pathologies.

Lon protease throughout evolution

Lon, also known as protease La, is the first identified ATP-dependent protease with a Ser-Lys catalytic dyad from *E. coli*, and derives its name from the phenotype of *E. coli* mutants that lack *lon* gene and tend to grow in longer forms than their wild-type counterparts upon UV irradiation [6–9]. Lon is highly conserved throughout evolution, and its homologues were discovered in almost all organisms; in *eubacteria,* Lon protease is a soluble cytoplasmic protein, while in *archaea*, it is bound to the membrane, and in *eukarya* is localized in mitochondria and peroxisomes [10–12].

In *eubacteria*, Lon selectively degrades abnormal proteins, such as misfolded or mutant, as well as certain short-

lived regulatory proteins, including some antitoxins. Lon protease is essential for cellular homeostasis, for survival from DNA damage and developmental changes in response to numerous stresses, through the control of protein machinery and the regulation of metabolism [13]. Finally, bacterial Lon is able to bind DNA, in a sequence-specific manner [14].

In *eukarya*, mitochondrial Lon is encoded by nuclear genome, and was first identified in humans [3] and in the yeast *Saccharomyces cerevisiae* [15, 16]. Following translation in ribosomes as a precursor form that carries a mitochondrial targeting sequence (MTS) at N-terminal, the human and yeast Lon proteases are imported from cytosol to mitochondrial matrix where they are converted into the mature form after the cleavage of MTS. Mitochondrial Lon protease shares the majority of properties with bacterial Lon, including the capacity to bind DNA and the essential role in protein quality control [17]. It is still not clear whether mitochondrial Lon of *eukarya* also regulates the expression of specific proteins, as bacterial Lon does. However, observations made on the forms expressed by the filamentous fungus *Podospora anserina* (PaLon) and by the plant *Arabidopsis thaliana* (AtLon) suggest that Lon can exert some important regulatory functions in growth and development of organisms. In *Podospora anserina*, the deletion of PaLON1 leads to decreased lifespan in stressing conditions (such as growing at 11 or 36 °C, two temperatures far from the optimal growth temperature of 27 °C) and to alterations in ascospore germination and sexual reproduction, two important and finely regulated developmental steps [18]. In *Arabidopsis thaliana*, mutations of AtLON1 determine a delay in primary root elongation and a retardation of post-germinative growth that persists during the entire biological cycle of the plant [19].

Structure and functions of the bacterial Lon protease

Lon protease can be divided into two subfamilies based on sequence homology and domain architecture: LonA (represented by *E. coli Lon, EcLon*) and LonB (mainly found in *archaea*) [20]. LonA proteases consist of three functional domains (Fig. 1): the N-terminal domain for substrate binding, the central ATPase domain, and the C-terminal containing the proteolytic active site with a Ser-Lys catalytic dyad [21, 22]. In contrast, *LonB* proteases are only composed of an ATPase domain and a protease domain (Fig. 1). In addition, *LonB* proteases contain a hydrophobic transmembrane inserted within the ATPase domain that contributes to their anchoring to the cytoplasmic face of the membrane [20]. Depending on the organism, 4–12 Lon polypeptides bind together and form a homo-oligomeric

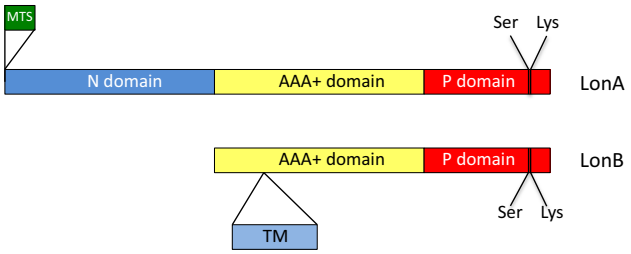

Fig. 1 Structure of Lon proteases. Lon proteases can be divided in two subfamilies, on the basis or their structure and sequence homology: LonA (its members are present in *eubacteria* and *eukarya*, which include human mitochondrial Lon) and LonB (present in *archea*). The N domain, present only in LonA subfamily, is involved in protein substrate binding and in the oligomerization of the enzyme; the AAA+ domain has an ATPase activity, and the carboxyl-terminal domain (P domain) contains the Ser-Lys dyad at the proteolytic active site. LonB subfamily members can have an additional transmembrane (TM) domain inserted in the AAA+ region, which anchors the protein to the membrane. A mitochondrial-targeting sequence (MTS) is present at the N term of the pre-protein synthesized in eukaryotic cells, which is removed by proteolytic cleavage after importing in mitochondria

Lon complex [22, 23]. The N terminus of Lon is the site of interaction with its protein substrate, and it has been shown that hydrophobic amino acids of protein substrates bind to this site. Once the substrate interaction is established, the hydrolysis of ATP translocates the substrate through the homo-oligomeric Lon complex, passing it through the proteolytic chamber where progressive protein cleavage then occurs [20, 23–25].

The EcLon is a 87-kDa ATP-dependent protease, and plays a major role in protein quality control, radiation resistance, cell division, filamentation, capsular polysaccharide production, lysogeny of certain bacteriophages, and survival under starvation conditions. EcLon forms homo-hexamers or dodecamers [23], degrades up to 50 % of aberrant proteins and is also responsible for the regulation of physiological events by targeting regulatory proteins for their degradation [9]. This function is conserved in other prokaryotes, such as in *Bacillus subtilis*, in which Lon regulates gene expression by controlling transcription factors' levels [26].

Lon-mediated proteolysis also plays a key role in pathogenic bacteria, such as *Brucella abortus* and *Pseudomonas aeruginosa*, where it is necessary to express the virulence genes and enhance the infection of mammalian cells [27, 28]. *Borrelia burgdorferi* has two ATP-dependent Lon proteases, Lon-1 and Lon-2. As the etiologic agent of Lyme disease, *Borrelia burgdorferi* is transmitted through the blood feeding of *Ixodes scapularis*. Interestingly, only *B. burgdorferi* Lon-1, but not Lon-2, level is up-regulated at the transcriptional level by in vitro exposure to blood. Thus, Lon-1 protease is essential for host adaptation from the arthropod to a warm-blooded host,

whereas Lon-2 protease functions as the canonical Lon and is important for cellular homeostasis [29].

So far, the oligomeric crystal structure of full-length Lon protein has not yet been solved and its crystallization is still a major challenge; however, the three dimensional structure of both the N (amino acids 8–117) and AAA+ domains of the EcLon protease has been successfully solved [20, 21, 25, 30, 31]. The EcLon protease forms a homo-oligomer to be a ring-shaped complex composed of six monomers with a central cavity, which has some similarity with the other ATP-dependent proteolytic machineries such as the 26S proteasome.

Unlike other two-component ATP-dependent proteases, both the proteolytic and ATPase activities are carried by the same polypeptide chain of Lon protease; it is very difficult, only on the basis of the structures of the two separate fragments, to understand how the ATPase domain and the protease domain communicate with each other and coordinate ATP hydrolysis with proteolysis [25, 30, 32–34]. The crystal structure of a truncated form of *Bacillus subtilis* Lon, BsLon-AP (which included both ATPase and protease domains) was solved, which is the first Lon fragment structure including both the ATPase and protease domains [21]. The structures of truncated Lon protease domains of several bacterial homologues reveal that the proteolytic active site contains at least a conserved catalytic Ser–Lys dyad. Mutation of either residue to Ala abolishes protease but not ATPase activity [14].

Concerning *archea*, the protease domains of *Archaeoglobus fulgidus* LonB (*Af*LonB) and *Methanococcus jannaschii* LonB (*Mj*LonB) have been successfully crystallized, and their structures solved [32, 33]. The *Brevibacillus thermoruber* Lon (Bt-Lon) sub-domain and DNA complex was crystallized recently; however, only the protein domain existed in the crystal, and the structure was resolved. The NMR technique was employed to achieve the detailed DNA-binding property of Bt-Lon, and the backbone resonance assignments of the sub-domain of Bt-Lon have been reported [35].

Pim1/Lon in yeast

As in human cells, a Lon-like, ATP-dependent serine protease has been identified in the matrix of *Saccharomyces cerevisiae* mitochondria. This protease, also known as Proteolysis into mitochondria 1 (Pim1), is required for selective proteolysis in the matrix, protection of mitochondrial proteins from stress-induced aggregation, and respiration-dependent growth [5, 16, 36]. Moreover, Pim1 is essential for the maintenance of the integrity of the mtDNA, as yeast cells depleted for Lon protease lose mtDNA integrity, contain inclusion bodies in the organelle and become respiratory deficient [10, 37]. The introduction of *E. coli* Lon into these Lon-lacking cells can partially restore mtDNA integrity, which demonstrates that there is remarkable functional conservation of Lon protease among distantly related organisms [38]. Cryoelectron microscopy shows that *S. cerevisiae* Lon is a heptameric ring-shaped protease [39]. By proteomic approach, several oxidized substrates (Hsp60, Sod2, Pdb1, Lpd1, Ald1, Ilv5 and Atp2, but not aconitase) of Pim1 have been identified [40, 41]. The majority of these substrates are involved in mtDNA maintenance, suggesting that they are involved in the protection of yeast mitochondrial genome.

The peroxisomal isoform of Lon

In *eukarya*, a peroxisomal isoform of Lon (pLon) also exists. The presence of pLon has been reported in humans [42] and rats [43], as well as in the fungus *Penicillium chrysogenum* [44] and in the plant *Arabidopsis thaliana* [45, 46]. Although no studies have reported the presence of a peroxisomal form of Lon in *S. cerevisiae*, it has been identified in the yeast, *Hansenula polymorpha* [47]. The structure of pLon is strongly conserved among species, and is similar to that of the mitochondrial counterpart: it comprises the canonical three domains of Lon, and a peroxisomal-targeting sequence type-1 (PTS1) at the C-term. Its presence in yeasts, plants, fungi and vertebrates suggests that the divergence between the mitochondrial and peroxisomal forms of Lon occurred early in *eukarya*, and that both forms are strictly conserved throughout evolution.

Less is known concerning functions of pLon within the organelle. In *H. polymorpha*, it is involved in the degradation of unfolded and non-assembled peroxisomal matrix proteins, and assists in refolding incorrectly folded proteins; its inactivation determines an increase of intracellular ROS [44]. In *A. thaliana*, the peroxisomal form of Lon, called LON2, facilitates the degradation of peroxisomal matrix proteins, thus preventing the authophagic degradation of the organelle [48]. Studies on rats indicated that pLon plays a role in processing and activating a specific regulatory protein belonging to the peroxisome targeting signal (PTS)-1 containing proteins. It interacts with the 70-kDa peroxisomal membrane protein (PMP70), and with several enzymes involved in β-oxidation, including acyl-CoA oxidase (AOX) [49]. Finally, in human cells, pLon binds and degrades the self-cleaved form of Tysnd1, a protease responsible for degradation of several enzymes involved in peroxisomal β-oxidation pathway. Accordingly, knock down of pLon results in lowering β-oxidation of long fatty acid chains [42].

Taken together, these data indicate that pLon has basically maintained the same function (i.e. degradation of unfolded or non-assembled proteins within peroxisomes) throughout evolution of *eukarya*.

Lon protease in humans

Human Lon is encoded by the *LONP1* gene, also known as *PRSS15*, which is located in the region p13.2 of Chr 19 and is approximately 29,000 base pairs long. The main transcript variant of *LONP1* has been reported more than 20 years ago, as an mRNA of about 3.4 Kb [3]. This transcript variant, now reported in GenBank as transcript variant 1, is 3221 base pair long, and encodes for the most abundant isoform of the enzyme.

Two alternative transcripts have been reported: the transcript variant 2 (NM_001276479.1) is alternatively spliced at the 5′ end compared to the variant 1, uses the same translation start codon, but the encoded isoform lacks a 64 aa segment in the N-term coding region. The second alternative transcript (NM_001276480.1) contains an alternate 5′ terminal exon and uses an in-frame downstream start codon; the encoded isoform has a shorter N-terminus compared to isoform 1.

The encoded protein is produced as a preprotein of 959 aa with a predicted MW of about 107 kDa, and an N-term sequence targeting the protein to mitochondria [3]; the mature enzyme is a protein with a molecular weight of approximately 100 kDa [50]. The purification of mitochondrial matrix from bovine heart muscle, together with its fractionation by chromatography, revealed that Lon protease is located in the matrix [51]. The analysis of distribution of Lon in submitochondrial compartments of human cells confirmed that Lon is not present in the outer membrane fraction, nor is the intermembrane fraction; almost 90 % of Lon is soluble in the matrix, and the remaining part is associated with the inner membrane, where mitochondrial nucleoids are present [50–52].

The crystal structure of the catalytic domain of human Lon has been recently obtained, at 2 Å resolution [34]. Human Lon has an overall fold similar to that of Lon proteases whose crystal structures have been previously determined [32, 33, 53], with major differences limited at the loops connecting the various secondary structural elements. Despite not formally proved, crystal structure data strongly suggest that the human form of Lon is inactive as a monomer, but can form hexamers; the oligomerization causes a conformational change that makes the enzyme fully active.

Lon is expressed ubiquitously in human tissues and organs [3]. No quantitative data concerning Lon mRNA levels have been reported in this study; however, northern blot analysis revealed that high levels of Lon mRNA are present in the liver, brain, heart, skeletal muscle and placenta, and lower but clearly detectable levels in lung, kidney and pancreas [3, 4].

Lon and mitochondrial metabolism

In human cells, Lon is involved in several aspects of mitochondrial biology (Fig. 2), by interacting with proteins involved in various functions of the organelle (Table 1). Lon belongs to the mitochondrial proteolytic systems, even if it is not part of the mitochondrial unfolded protein response. Several reports indicate that Lon expression and/or activity increase in the presence of high levels of carbonylated proteins, as well as proteins modified by peroxidation and glycation [54, 55]. Indeed, Lon primarily degrades misfolded and damaged proteins, including mitochondrial aconitase (Aco2), glutaminase C (GLC), steroidogenic acute regulatory protein (StAR), 5-aminolevulinic acid synthase (ALAS-1), and cystathionine β-synthase (CBS), and its down-regulation leads to profound alteration of mitochondrial proteome [56]. However, the direct involvement of Lon in the degradation of putative proteins has been demonstrated only for Aco2 [51], ALAS-1 [57], StAR [58], the mitochondrial transcription factor A (TFAM) [59], GLC [60] and CBS [61]. Interestingly, two of these proteins participate directly or indirectly, in tricarboxylic acid (TCA) cycle: Aco2 catalyses the isomerization of citrate to isocitrate, whereas GLC catalyses the conversion of glutamine to glutamate, which is not an intermediate of TCA cycle but can be converted to α-ketoglutarate. By silencing Lon and analysing mitochondrial proteome in colon carcinoma cells, it was found that the percentage of modulated proteins accounted for oxidative phosphorylation (OXPHOS) and TCA cycle proteins was 12.7 and 8.5, respectively [56]. In addition, it was found that Lon down-regulation affects the levels of several other proteins in the mitochondrial matrix, including those involved in energetic metabolism, mitochondrial architecture, ribosome assembly, mtDNA metabolism and stress response [56]. Cells deficient for Lon display low levels of complex I, II and IV. This leads to a remarkable respiratory defect which is associated with altered mitochondrial morphology, the accumulation of electron-dense bodies in the matrix, and cell death by apoptosis. High-throughput quantitative proteomics of enriched mitochondrial preparations from B16F10 melanoma cells revealed that the expression levels of several subunits of complex I and complex V were deeply altered when Lon was knocked down [62]; this observation indicates that Lon modulation leads to changes in cell bioenergetics through the remodelling of OXPHOS subunits. In HeLa cells, Lon down-regulation does not affect the steady state levels of mitochondrial OXPHOS proteins, nor it modifies basal or maximal respiration; no changes in the expression of several mitochondrial stress proteins have been reported [63]. Accordingly, no mitochondrial proteotoxic stress response was noted. However,

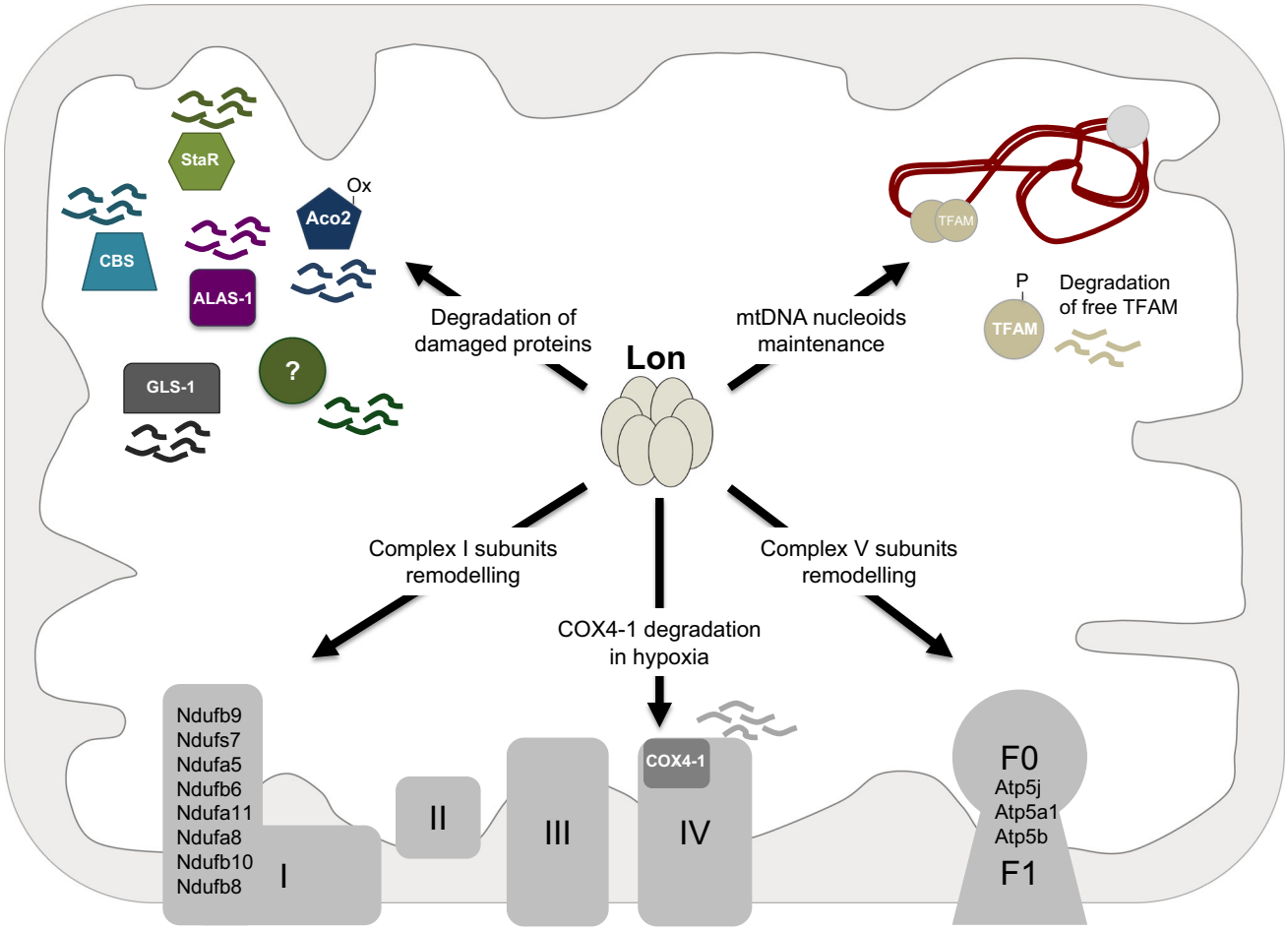

Fig. 2 Lon functions in mitochondria of human cells. Lon exerts three main functions in the matrix of human mitochondria: (*i*) it degrades damaged or oxidized proteins, such as aconitase (Aco2), glutaminase C (GLS-1), steroidogenic acute regulatory protein (StAR), 5-aminolevulinic acid synthase (ALAS-1), Cystathionine β-synthase (CBS); (*ii*) it regulates mitochondrial metabolism by selective degradation of, and interaction with some subunits of OXPHOS complexes; (*iii*) it regulates mtDNA replication and maintenance by degrading the phosphorylated form of TFAM

a threefold increase of ATP-stimulated caseinolytic protease (Clp) activity was observed in protein extracts of these cells, even if the protein levels of Clp remained constant; no increased activity was observed in the absence of ATP. These observation suggests that, in this cell model, a compensatory mechanism exists at post-translational levels (maybe mediated by ATP levels) that could maintain a normal turnover of matrix proteins, and counteract the toxic effects of Lon down-regulation [63]. Collectively, these data indicate that Lon activity is essential in the large part of, but not all cells.

The link between Lon proteolytic activity and mitochondrial respiration is important also in the presence of hypoxia and oxidative stress. Under hypoxic conditions, Lon is required for the degradation of cytochrome *c* oxidase (COX), subunit 4, isoform 1 (COX4-1) to optimize the efficiency of respiration at low O_2 concentration; this phenomenon has been observed in different human cell lines including Hep3B (liver), HeLa (uterus), Hct116 (colon), A594 (lung) cells, 293 (embryonic kidney), RCC4 (renal carcinoma) [64, 65]. Mild oxidative modification in the aconitase results in increased exposure of hydrophobic residues and increased susceptibility to proteolysis by Lon [51]. In parallel, LONP1 knock down is associated with an increase in lactate concentration in cell medium [56]. This in not surprising if we consider that in the absence of Lon cells have reduced respiratory capacity. Taken together, these examples demonstrate that Lon plays a role in the regulation of mitochondrial and OXPHOS functions.

Lon also regulates mitochondrial biology by intimately balancing TFAM:mtDNA ratio, both in the replication and transcription of mtDNA. As a unique feature that distinguishes Lon from other matrix proteases, it is a component of mitochondrial nucleoids, and interacts with many of the proteins that form them [66], including TFAM [59], the helicase Twinkle, the DNA polymerase-γ (the enzyme

Table 1 Proteins interacting with Lon in mitochondria of human cells

Protein	Interaction with Lon	References
TCA cycle		
Aconitase	Degraded by Lon when oxidized	[51]
Glutaminase c	Degraded by Lon	[60]
Oxidative phosphorylation		
COX4-1	Degraded by Lon in hypoxic conditions	[64, 65]
NDFUS8	Stabilized and up-regulated by Lon	[123]
SDH5	Degraded by Lon when not interacting with SDHA	[97]
Maintenance of mtDNA		
mtDNA pol-γ	Physically interacts with Lon in nucleoids	[66]
PDIP38	Physically interacts with Lon in nucleoids	[67]
TFAM	Degraded by Lon when phosphorylated	[59]
Twinkle	Physically interacts with Lon in nucleoids	[66]
Other processes		
5-aminolevulinic acid synthase (ALAS-1)	Degraded by Lon	[57]
Cystathionine β-synthase (CBS)	Degraded by Lon	[61]
SIRT3	Physically interacts with and deacetylates Lon	[82]
StAR	Degraded by Lon	[58]

responsible for mtDNA replication [52]) and the Polymerase (DNA-Directed), Delta Interacting Protein 2 (POLDIP2, also known as PDIP38) [67]. Lon is a protein able to bind DNA, but the putative binding domain is still unknown. Unlike bacterial Lon, human Lon recognizes single-stranded DNA (ssDNA) but not double-stranded DNA, and specifically binds in vivo to a G-rich consensus sequence in the light-strand promoter, as well as to the RNA transcribed from that [68, 69]. The binding to ssDNA is stimulated by the presence of protein substrates, and is inhibited by ATP binding [52]. The role of Lon in the regulation of mtDNA replication has been recently elucidated, and is tightly linked to TFAM phosphorylation. In vitro experiments with purified TFAM demonstrated that, when phosphorylated, its ability to bind mtDNA is impaired, the DNA-free TFAM is degraded by Lon protease and the mtDNA is unpacked [59]. Interestingly, a similar mechanism has been observed in *Drosophila melanogaster*, suggesting that mtDNA regulation through selective degradation of TFAM evolved early in animal cells [70]. Lon down-regulation leads to mtDNA and mtRNA depletion, and Lon down-regulation in cells with severe mtDNA deficits blocks TFAM degradation and increases mtDNA content [62]. The role of Lon in mtDNA maintenance is not limited to the copy number regulation: binding of Lon increases the sensitivity of mtDNA to oxidative stress damage, probably by stabilizing ssDNA (which is more exposed to ROS) or by blocking the proteins responsible for DNA repair [69]. If Lon's binding capacity to mtDNA is related to its role in OXPHOS function, or represents a function that evolved independently, is still not clear. The crucial role of Lon in maintaining mtDNA copy number has been also proven in vivo. Indeed, attempts to get a Lonp1$^{-/-}$mice failed, as Lon inactivation is lethal at embryonic stage [62]. Analysis of mtDNA levels in embryos have demonstrated that inactivation of Lon determines a progressive loss of mtDNA during gestation, which in turn determines growth arrest 7.5 days post coitum and leads to death within 8.5 days. It is interesting, however, to note that, Lonp1$^{+/-}$ mice develop normally, are fertile, and do not show any obvious pathological alteration—including any reduction of mtDNA levels—despite a 50 % reduction in the levels of Lon RNA and protein in any of the tissues analysed [62].

Regulation of Lon expression: a stress response protein

The mechanisms regulating Lon transcription and expression in human cells have been only partially elucidated. Lon levels are increased by different stress stimuli, including endoplasmic reticulum (ER)-stress, high levels of reactive oxygen species (ROS) and hypoxia, in various cell lines and conditions [6–9]. Lon has not a direct anti-oxidant activity, but rather contributes to reduce the effects of oxidative stress by selectively eliminating proteins that have been damaged by oxidative stress [51]. Thus, it is not surprising that its up-regulation in the presence of oxidative stress has been observed in many different models. In rhabdomyosarcoma cells, oxidative stress induced by H_2O_2

resulted in a modest (about 2-fold), time dependent increase of Lon mRNA [55]; in the same conditions a more marked increase (up to 8-folds) of the protein level has been observed. In SW872 preadipocytic cell line, oxidative stress induced by deoxythymidine, an antiretroviral drug, as well as by D-deoxyribose, can double Lon levels [71]. Interestingly, in yeast strains SC0437, Lon levels appears not regulated by mtDNA copy number, even if Lon can bind and is involved in maintenance of mtDNA [71]. Serum starvation and heat stress—though to a lesser extent—have also been shown to increase Lon protein levels, at least in rhabdomyosarcoma cells [55]. In this model, heat stress was induced by exposing cells to 45 °C for one hour, whereas serum starvation was obtained by incubating cells for one hour in serum-free medium and then adding 2 % horse serum-supplemented medium for cell recovery. A similar increase has been observed in the presence of ER stress in HeLa cells [72].

A more in-depth analysis of the transcriptional regulation of *LONP1* has been performed in HepG2 (hepatosarcoma), WI-38 (lung fibroblast) and SW872 human cell lines [73]. In these cells, oxidative stress induced by increasing concentrations of hydrogen peroxide determines an increase up to fivefolds of Lon mRNA levels. Other molecules known to determine oxidative stress, such as d-deoxyribose or stavudine, have similar effects. The analysis of DNA region upstream of transcriptional start site of LONP1 has evidenced the presence of putative binding sites for the transcription factors NRF-2, Nkx-2, and NF-kB, and Lyf-1. Deletions of the DNA region −623/+1 (which contains consensus sequences for Nrf2 and NF-kB) followed by luciferase assays showed that this DNA sequence is essential for response to oxidative stress, and that NRF2 and NF-kb are likely involved in the regulation of LONP1 transcription [73]. More recently, the analysis of transcriptional regulation of *LONP1* has been also performed in HEK293 cells, and three binding sites for NRF2 have been identified at the position −196, −183 and −150 of *LONP1* promoter [74]. Finally, a Chip-Seq-based pathway analysis of NRF-1, a transcription factor crucial for mitochondrial biogenesis and function, identified Lon promoter as one of its targets [75].

Hypoxia is another major inducer of Lon. The up-regulation of Lon in hypoxic conditions has been observed in THP-1 (monocytic cancer), HeLa (cervical cancer) and RKO (colon cancer) cells [64, 65], and is determined by the stabilization of HIF-1α and its binding on the promoter of *LONP1*; accordingly, VHL-deficient cancer cell lines such as RCC4, showed very high levels of HIF-1α and a consequent, constant up-regulation of Lon [64]. The observation that Lon is up-regulated by hypoxia at the mRNA and protein level has been further confirmed in an animal model, i.e. in rat astrocytes cultured in hypoxic conditions, and in rat ischaemic brain [72]. Finally, although no data are available in human cells, observations in rat Zajdela hepatoma cells, as well as in hyperthyroid rats suggest that Lon up-regulation is correlated with enhanced mitochondrial biogenesis, even if the molecular mechanisms underlying this phenomenon are not clear [76]. Taken together, these data indicate that Lon can be considered a stress response protein, enhanced expression of which in stressing conditions helps to overcome the accumulation of damaged (oxidized or carbonylated) proteins, and adapts cells to survive in a more challenging environment [55, 65, 77–80].

Finally, Lon was up-regulated—either at the mRNA or protein levels—as a result of steroidogenic differentiation in rat ovary. In vitro experiments in HEK293 cells showed that this response is due to an increased activity of the promoter, which is dependent on the presence of StAR, even if the factors linking StAR and Lon transcription have not been identified yet [81]. Interestingly, StAR is a substrate of Lon [58], and so it promotes the expression of the protease involved in its own degradation.

All these studies pointed out clear discrepancies among changes in *LONP1* transcription, levels of Lon mRNA, and the amount of proteins detected in mitochondria, suggesting that a fine tuning in Lon expression exists at multiple levels [82], and that mechanisms related with protein stability are involved in Lon regulation. In particular, several studies showed that changes in protein levels are higher than changes in mRNA levels in the same cell model and conditions [55, 71, 73]. This phenomenon has been observed in a number of stress-responsive proteins, as a mechanism to rapidly increase protein content without the considerable time lag associated with synthesis, export and translation of de novo synthesized mRNA [83].

Lon in human diseases and in ageing

Excluding cancer, a link between Lon and human diseases has been reported in few cases (Table 2). Mutations of Lon have been shown to cause CODAS syndrome—an inherited, extremely rare developmental disorder characterized by cerebral, ocular, dental, auricular, and skeletal anomalies—by two independent groups [84, 85]. Strauss and colleagues have shown that CODAS is caused by four pathogenic amino acid substitutions in the AAA+ domain of Lon, which cause substrate-specific defects of proteolytic activity and alteration in the morphology and functionality of mitochondria [84]. Dikoglu and collaborators further expanded the list of mutations of *LONP1* causing CODAS syndrome, and identified six new missense mutations, one non-sense mutation and one in-frame deletion; the predicted aminoacid substitutions are

Table 2 List of diseases in which Lon expression is modified

Disease	Effects observed	References
Cerebral, ocular, dental, auricular, skeletal (CODAS) syndrome	Aminoacid substitutions in primary sequence of Lon impairs its proteolytic activity and oligomerization. Cells with mutated Lon have swollen mitochondria and reduced spare respiratory capacity	[84, 85]
Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS)	Lon levels are increased in mitochondria holding the A3243G mutation in mtDNA	[89]
Myoclonic epilepsy with ragged-red fibres (MERRFs)	Lon expression is higher; Lon enzymatic activity is lower	[90]
Hereditary spastic paraplegia	Lon expression is lower	[91]
Friedrich ataxia	Frataxin deficiency causes increased Lon expression	[92]
Hereditary paraganglioma	SDH5, not complexed with SDH5A, is rapidly degraded by Lon	[97]
HAART related lipodystrophy	Lon expression is increased by oxidative stress, but not by low mtDNA copy number	[71]

The main alterations observed are reported

distributed mostly at the interface of the domains that form the protein [85]. Apart from CODAS syndrome, no other disease caused by mutations of Lon has been reported. However, modifications in Lon expression and activity have been reported in myoclonic epilepsy with ragged-red fibres (MERRFs) and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) [86–88]. MELAS is a genetically heterogeneous disorder with a variable clinical phenotype, caused by mutations of several genes encoded by mtDNA. Immortalized B-cells harbouring the A3243G mutation—the most common mutation causing the disease—in mtDNA display increased levels of Lon protein that specifically accumulates in mitochondria holding the mutated genome and missing complex I subunit NDUFS3 [89]. Similarly, MERRF cybrids carrying the A8344G mutation in the tRNA(Lys) gene of mtDNA—which causes more the 80 % of MERRF cases—are characterized by increased Lon expression, but lower Lon activity, compared to wild-type cybrids [90]. Lon accumulation in the presence of a reduced activity can be linked to the fact that Lon is somehow inhibited, and increasing its expression could represent an attempt to stabilize mitochondrial respiratory chain complexes. In cultured skin fibroblast cells from patients with hereditary spastic paraplegia, Lon mRNA and protein levels are lower compared to control cells [91]. Frataxin deficiency causes a significant up-regulation of Lon in the cardiac mouse model of Friedrich ataxia [92]. In this case, the increase of Lon is accompanied by the increased proteolytic activity and a concomitant decrease in Fe-S subunits of complex I (NDUFS3), complex II (SDHB), and complex III (Rieske), which is related to the enzymatic deficiency of the respiratory chain [92]. Finally, Lon plays a crucial role in the pathogenesis of hereditary paraganglioma, a rare, benign tumour caused by mutations in the succinate dehydrogenase (SDH) complex that impair its functions [93–96]. In particular, it has been shown that SDH5, an assembly factor necessary for flavinylation of the SDHA subunit of SDH complex, is a substrate of Lon, and that is normally protected from Lon-mediated degradation by its stable interaction with SDHA. In cells harbouring the G78R mutation of SDH5, SDH5/SDHA interaction is dysregulated, the mutated form of SDH5 is rapidly degraded by Lon, and flavinylation of SDHA is severely reduced [97].

Alterations of Lon levels have been also reported in some acquired diseases, such as lipodystrophy. This disorder can be observed in patients infected by the human immunodeficiency virus (HIV) assuming combined antiretroviral therapy (cART), usually a combination of two nucleotide analogue reverse transcriptase inhibitors (NRTI) and one protease inhibitor (PI), for long periods. The disease is characterized by fat redistribution, metabolic alterations, quantitative and qualitative alterations of mtDNA, and chronic oxidative stress [98]. Fat is usually accumulated in one or more anatomical sites (abdomen, dorsocervical spine, breasts) and/or lost on the face, buttocks, legs and arms. Fat redistribution observed in cART-related lipodystrophy is often associated with other secondary effects, including (but not limited to) hepatotoxicity, pancreatitis, peripheral neuropathy, ataxia, stroke-like episodes, hypertrophic cardiomyopathy and hypogonadism [98, 99]. In adipose tissue specimens obtained from lipodystrophic patients, levels of Lon mRNA and protein were up-regulated, and such up-regulation was linked to oxidative stress but not to the mtDNA depletion caused by antiretroviral drugs, despite the crucial role of Lon in mtDNA maintenance [71]. Since cART is a very complex therapy, which can differ from patient to patient, it is difficult to attribute the effect observed on Lon expression to a single drug. However, in vitro data obtained by our group indicate that NRTIs, and in particular stavudine, determine Lon up-regulation in SW872

liposarcoma cells; this increase is caused by higher levels of ROS [71]. Furthermore, Polo and colleagues have recently shown that the non-nucleoside analogue reverse transcriptase inhibitor efavirenz is able to increase the expression of Lon in HepaRG hepatoma cells [100].

As far as the role of Lon during cellular ageing is concerned, it has been shown that yeast cells lacking Pim1 age prematurely, display a shorter replicative life span, increased cytosolic levels of oxidized and aggregated proteins as well as decreased proteasome activity [101]. In *Podospora anserina*, the constitutive overexpression of PaLon leads to lower levels of carbonylated and carboxymethylated proteins, higher resistance to exogenous stresses and extended lifespan [102]. Overexpression of PaLon has a beneficial effect on respiration, and indeed middle-aged and senescent PaLon-overexpressing strain respires more efficiently than the wild-type. In a human melanoma cell line, Lon silencing in vitro leads to a senescent phenotype, and a significant increase of β-galactosidase activity (a marker of cell senescence) while its overexpression causes a reduction of β-galactosidase [62].

Accordingly, Lon expression and activity decrease in aged rats and mice, and are accompanied by the accumulation of oxidized and carbonylated proteins in the matrix together with a decrease in the activity of mitochondrial aconitase [77, 103]. Of note, such a reduction is partially rescued with exercise training [104], in a process that is strictly associated with mitochondrial biogenesis [105], and completely rescued by caloric restriction (CR) in muscle cells [106]. The fact that decrease of Lon during ageing can be prevented by CR likely places Lon itself in the downstream pathways involved in energy metabolism, including the sirtuin 1 (SIRT1)—peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α axis [107]. In human embryonic fibroblasts, even in the absence of increased Lon protein level, an increase in ATP-dependent Lon-like proteolytic activity has been observed in senescent mitochondrial fraction compared to the young counterpart [54].

Ageing is characterized by a exponential decline of mitochondrial capacity, which is linked to decreased mtDNA levels, mtRNAs and protein expression, increased oxidative stress, and increased accumulation of oxidatively modified proteins [108–110]. Mitochondrial quality control, which operates to maintain mitochondrial quality, is also reduced with ageing [111]. Indeed, old mice show reduced Lon levels and activity in skeletal muscle compared to young animals [77], an effect that is exacerbated in MnSOD heterozygous mice. Lon levels increase with age in rat hearts, but Lon activity does not change, suggesting that inactive Lon is progressively accumulated with ageing [112], probably because of oxidative damage of the enzyme that reduces its activity [113].

Lon and cancer

Cancer cells exhibit different metabolic requirements from the most normal differentiated cells, as they must generate the energy required to support rapid cell division [114, 115], in addition to the ATP that is required to maintain normal cellular processes. Cancer cells secrete most of the glucose-derived carbons as lactate rather than oxidizing it completely by OXPHOS in mitochondria, a phenomenon known as the "Warburg effect" [116]. This high glycolytic activity produces ATP faster, even if with a lower efficiency, and provides precursors needed for the biosynthesis of macromolecules [117, 118] and for the generation of reducing equivalents as antioxidant defences [119]. Thus, cancer cells are often characterized by a profound metabolic reprogramming [120], which involves changes in the regulation of glycolysis and of the mitochondrial functions. An increasing amount of data indicate that Lon participates in this process.

Several groups, including ours, have observed an up-regulation of Lon protease in different types of human cancer, including (but not limited to) different malignant B cell lymphoma from patients [121], mammary epithelial, cervical cancer [122], non-small-cell lung cancer cells [122, 123], bladder cancer cells [124], oral squamous cell carcinoma, and colon cancer, either at RNA or protein level [56, 62]. In silico analysis further expanded the spectra of tumour cells characterized by high levels of Lon expression, by including lung, colorectal, head-and-neck, and metastatic prostate cancer [67, 124, 125]. It has been observed that such up-regulation can significantly increase colony formation activity in some cancer cell lines compared with controls, probably leading to a higher proliferative rate [67].

Given the multiple roles of Lon in mitochondria, it is not surprising that effects of higher expression levels of this protein in tumour cells are very complex, and their consequences not fully understood. At mitochondrial level, a higher expression of Lon determines deep changes in organelle architecture and functionality, which in turn makes cancer cell more resistant to stress conditions observed in tumour microenvironment. During hypoxia—often observed in solid tumours—Lon is up-regulated by HIF-1α, and degrades the cytochrome c oxidase 4–1 subunit (COX4-1), thereby permitting the assembly of another subunit COX4-2, which confers optimized enzyme activity for low oxygen and adapts cancer cells to the hypoxic environment [47]. Ectopic overexpression of Lon has been shown to cause a switch from respiration to glycolysis, and to facilitate the proliferation and transformation of cells, as well as their capability to migrate and form metastasis in nude mice [62]. Despite the fact that Lon is involved in

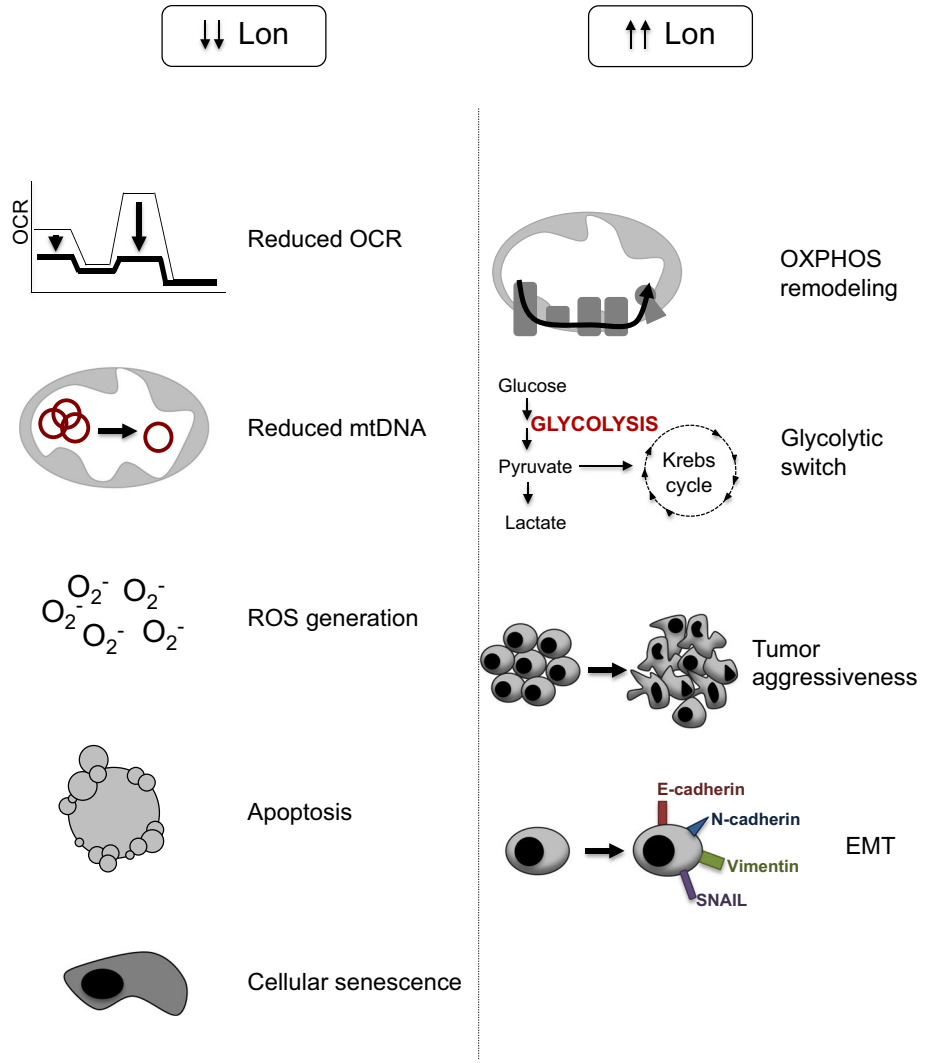

Fig. 3 Effects of changes of Lon expression in cancer cells. Changes in Lon expression have several effects in cancer cells. Lon knockdown leads to: reduction of oxygen consumption rate (OCR), decrease of mitochondrial DNA content (mtDNA), increase of reactive oxygen species (ROS), increased susceptibility to undergo apoptosis and cellular senescence. Lon overexpression affects tumour bioenergetics by inducing OXPHOS remodelling and by shifting energy production to glycolysis. This leads to increased stress adaptations that favours tumour aggressiveness and epithelial–mesenchymal transition (EMT)

antioxidant response, and is up-regulated by oxidative stress [71, 73], its overexpression has been shown to cause mitochondrial ROS generation, in different cell models [62, 123]. In 293T, OEC-M1, SCC-15 and FADU cell lines, Lon overexpression causes the stabilization and up-regulation of NDFUS8 subunit of complex I, which in turn determines an increase in mitochondrial ROS production, probably because of an impairment in the complex I assembly [123].

An impairment in Complex I assembly and functionality caused by Lon overexpression has been also observed in B16F10 melanoma cells. In this model, Lon overexpression causes the up-regulation of NDUFB6, 8, 10 and 11, which are associated with the assembly and stability of complex I membrane domains, and the down-regulation of NDUFV1, NDUFV2, NDFUS3 and NDFUS7. These changes cause a reduction of respiration through complex I—while respiration through complex II is maintained—and lead overall to the down-regulation of respiration and up-regulation of glycolytic pathway. In this model, no significant increase of ROS production has been observed after Lon overexpression [62].

The biological significance of these phenomena is still debated. By using 293T cells as a model, Cheng et al. proposed that ROS produced by Complex I promote cell proliferation by activating MAPK (p38, JNK, and ERK1/2) and Ras-ERK signalling, pointing out the overexpression of Lon as the driving force the promote oncogenic

transformation. Interestingly, Lon overexpression also promotes the expression of markers related with epithelial–mesenchymal transition (EMT), such as E-cadherin, N-cadherin, Vimentin, and Snail, and promotes cell migration via up-regulation of MMP-2, while Lon down-regulation impairs cell migration. Since EMT is inhibited by antioxidant molecules such as N-acetyl cysteine, Lon-induced EMT process is suggested to be dependent on ROS generation [123].

Reprogramming mitochondrial metabolism in cancer cells could also be obtained by post transcriptional and post translational mechanisms. We have recently shown that in colorectal cancer cells Lon is deacetylated by SIRT3 [82]. This protein is a mitochondrial-specific sirtuin, which can regulate several metabolic pathways in mitochondria, and act both on pro- or anti- oncogenic targets [126, 127]. The ability to regulate the activity of Lon likely represents one of the mechanism used by SIRT3 to regulate and reprogram mitochondrial functions in cancer cells.

The analysis of the effects of Lon silencing or overexpression in normal, non-transformed cells could provide further insights on the role of Lon in the process of cancerogenesis. However, few data are available on this point so far. In WI-38 VA13 normal human fibroblasts, silencing of Lon leads to early apoptotic cell death, highly abnormal mitochondrial function and morphology, and a switch to an almost exclusive anaerobic metabolism [128]; these observations suggest that the effects of Lon silencing observed in most cancer cell lines could be general phenomena, not limited to malignant cells. The $Lon^{+/-}$ mouse model—in which the expression of Lon is halved in all tissues analysed—has provided further interesting insights into the role of Lon in tumourigenesis. When treated with cancerogenic compounds known to induce colorectal and skin cancer, $Lon^{+/-}$ mice are more resistant to develop cancer and have fewer tumours than WT mice, indicating that the expression of Lon can favour tumour development and growth. These results are in perfect agreement with the observation that, in xenograft model, the growth of Lon-silenced cancer cells is significantly reduced compared to control cells, while cells overexpressing Lon grow more rapidly [62].

Finally, higher expression of Lon is associated with short survival in patients with metastatic melanoma, and its overexpression in melanoma cells increases experimental metastasis formation, whereas knockdown of this protease decreases cell proliferation and lung metastasis [62]; higher level of Lon mRNA has been observed in HB2 human breast cancer cells overexpressing ErbB2 [129], a condition found in some breast carcinomas and associated with tumour aggressiveness and poor prognosis [130]. 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 [124]. Taken together, these data clearly indicate that Lon is a protein higher expression of which can favour tumour growth, while its down-regulation is related to slow proliferation rate, or even death, of cancer cells.

Inhibitors of Lon

Since down-regulation of Lon leads to loss of mitochondrial function, the reduced cell proliferation capacity, and apoptosis in cancer cells, Lon is gaining attention as a potential target in cancer therapy.

In *E. coli*, ADP acts as an inhibitor of Lon protease indicating regulation in response to changes in energy charge [131]. The bacterial Lon activity can be inhibited by T4 phage infection through the T4-encoded PinA protein a few minutes after infection [132].

To date, no data linking modification of Lon expression with specific pharmacological agents used in the clinics have been reported, and a few inhibitors of mitochondrial Lon protease have been identified; all these compounds have been tested only at pre-clinical level. Not surprisingly, the serine protease inhibitor phenylmethanesulphonylfluoride (PMSF) can inhibit Lon [121]. Certain proteasome inhibitors such as MG132 and *clasto*-lactacystin β-lactone (cLβL) can diffuse into the mitochondria and inhibit the degradation of the StAR, an endogenous substrate of mammalian Lon, suggesting that the proteasome and Lon share some similarities in their proteolytic mechanism [58, 133]. Moreover, we found that some other proteasome inhibitors, such as MG262 and Bortezomib (velcade), can also inhibit Lon protease activity [51, 108].

Lon has been shown to be directly and selectively inhibited by the synthetic triterpenoid CDDO and its methyl ester derivative (Me-CDDO) in vivo and in vitro, and such an inhibition is associated with cell death in B cell lymphoma; both molecules have no effects on 20S proteasome [121]. By using a biotinylated-CDDO as a probe, the mechanism of inhibition was elucidated. CDDO forms a conjugate with Lon protease which blocks the activity of Lon. Lymphoma B cells treated with CDDO exhibit the accumulation of electron-dense aggregates within mitochondria matrix and apoptotic cell death, in a way similar to that observed in Lon knockdown cells. Thus, Lon protease activity inhibition may play a contributory role in CDDO-induced lymphoma cell death, which further supports that mitochondrial Lon is a novel anticancer drug target. We have observed the same effects on other cancer cell lines, including RKO colon cancer cells and hepatocarcinoma HepG2 cells (Gibellini et al., Oncotarget, in press). The same molecules have negligible effects on normal fibroblasts, suggesting that a different sensitivity to

these drugs exist between normal, non-transformed and malignant cells.

The coumarinic derivatives were demonstrated to be efficient, low-molecular-weight, non-peptidic and versatile protease inhibitors. By using casein-FITC assay, certain coumarinic compounds were found to have inhibition activity to the human Lon protease, but they have no effects to yeast proteasome [134].

Finally, two small-molecule compounds, obtusilactone A (OA) and (−)-sesamin from *C. kotoense* (a small evergreen tree) were identified as potent Lon protease inhibitors through enzyme-based screening. In vitro, both molecules inhibit the activity of purified recombinant Lon. In cultured cells, OA causes a significant accumulation of aconitase in a time- and dose-dependent manner. According to molecular docking analysis, OA and (−)-sesamin interact with Ser855 and Lys898 residues in the active site of Lon protease, likely inhibiting Lon activity [135]. Recently, we identified a natural small molecular compound from Chinese medicine Chuanxiong, which blocks specifically the Lon-mediated TFAM degradation (Liu & Lu, unpublished data).

Taken together, these observations indicate that Lon could be a promising target for developing anticancer drugs. However, some obstacles for developing an effective drug targeting Lon currently exist. First, it is quite difficult to target compounds to the mitochondrial matrix, as the IMM is highly impermeable. Second, Lon inhibitors available so far have a low grade of specificity, and the enzymatic activity of peroxisomal isoform of Lon, which displays a high degree of similarity with mitochondrial Lon [42, 43, 49], might be theoretically inhibited by drugs designed for Lon.

Conclusions

Lon protease is a major controller of several mitochondrial functions. Thus, it is not surprising that Lon is emerging as one of the non-oncogenic proteins that is essential for the survival and proliferation of cancer cells, and able to promote neoplastic transformation. This capability is due to both quantitative and qualitative effects on mitochondria: Lon is able to increase the biogenesis of these organelles, to regulate the replication and transcription of mtDNA, and to reprogram mitochondrial metabolism by altering the relative composition of OXPHOS complexes (Fig. 3).

Many aspects of the role on Lon in cancer biology remain to be elucidated. First, little is known about the signalling pathways that lead to higher Lon levels in cancer cells. The recent observation that in Ramos cells the up-regulation of CHOP and CEB/P, two proteins characteristic of the mitochondrial unfolded response, is followed by up-regulation of Lon but not other mitochondrial chaperones or proteases could indicate that these factors are specifically involved in Lon regulation [136].

Second, the regulation of Lon at posttranscriptional level is still largely unknown. As stated above, it is well known that Lon is not simply regulated by changes in mRNA levels, but that different isoforms of Lon, obtained by alternative splicing, exist. Once within mitochondria, Lon is a target of acetylation and deacetylation modifications [82, 137, 138], and the enzyme activity is regulated by binding to DNA, by ATP and ADP, and by its oxidation levels. However, little is known on the regulation of all these mechanisms, and on their involvement in the up-regulation of Lon during cancerogenesis.

Third, in vivo data are still limited. The availability of a mouse model has shed some light on the role of Lon in malignant transformation in vivo; however, data in humans are still lacking, and a systematic analysis of Lon expression in large cohort of patients with different types of cancer, along with the correlation with clinical outcome, is urgently needed.

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