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The peroxisomal Lon protease LonP2 in aging and disease: functions and comparisons with mitochondrial Lon protease LonP1

Laura C. D. Pomatto, Rachel Raynes, and Kelvin J. A. Davies*

Ethel Percy Andrus Gerontology Center of the Davis School of Gerontology and Division of Molecular & Computational Biology, Department of Biological Sciences of the College of Letters, Arts & Sciences, University of Southern California, 3715 McClintock Avenue, Los Angeles, CA 90089-0191, U.S.A

Abstract

Peroxisomes are ubiquitous eukaryotic organelles with the primary role of breaking down very long- and branched-chain fatty acids for subsequent β -oxidation in the mitochondrion. Like mitochondria, peroxisomes are major sites for oxygen utilization and potential contributors to cellular oxidative stress. The accumulation of oxidatively damaged proteins, which often develop into inclusion bodies (of oxidized, aggregated, and cross-linked proteins) within both mitochondria and peroxisomes, results in loss of organelle function that may contribute to the aging process. Both organelles possess an isoform of the Lon protease that is responsible for degrading proteins damaged by oxidation. While the importance of mitochondrial Lon (LonP1) in relation to oxidative stress and aging has been established, little is known regarding the role of LonP2 and aging-related changes in the peroxisome. Recently, peroxisome dysfunction has been associated with aging-related diseases indicating that peroxisome maintenance is a critical component of 'healthy aging'. Although mitochondria and peroxisomes are both needed for fatty acid metabolism, little work has focused on understanding the relationship between these two organelles including how age-dependent changes in one organelle may be detrimental for the other. Herein, we summarize findings that establish proteolytic degradation of damaged proteins by the Lon protease as a vital mechanism to maintain protein homeostasis within the peroxisome. Due to the metabolic coordination between peroxisomes and mitochondria, understanding the role of Lon in the aging peroxisome may help to elucidate cellular causes for both peroxisome and mitochondrial dysfunction.

Keywords

Lon protease; mitochondria; peroxisome; aging; mitochondrial DNA; hormesis; reactive oxygen species; oxidative stress; chaperone

*Address for correspondence (Tel: (213)740-8959; Kelvin@usc.edu).

I. INTRODUCTION

Peroxisomes are highly dynamic organelles that can modulate their size and number depending on the cell's metabolic demands (Lopez-Huertas *et al.*, 2000). While both mitochondria and peroxisomes are equally important in the β -oxidation pathway, much greater emphasis has been placed upon understanding the role of the mitochondria rather than peroxisomes. Thus, it is well established that mitochondria are critical for a number of cellular functions that include acting as metabolic signalling centres and contributing to cellular stress responses such as autophagy, redox signalling, oxidative stress and apoptosis (Lenaz *et al.*, 2002). Mitochondrial dysfunction has been shown to contribute to a number of diseases, including neurodegenerative and metabolic disorders (Cassarino & Bennett, 1999; Wallace, 2005).

Due to mitochondria being major sites for oxygen consumption, mitochondrial maintenance relies heavily on the proteolytic activity of Lon, the major protease in the mitochondrial matrix. The mitochondrial Lon protease (LonP1) degrades proteins damaged by reactive oxygen species generated by electron leakage from the mitochondrial respiratory chain, and also maintains the integrity of mitochondrial DNA (Bota & Davies, 2002; Matsushima, Goto & Kaguni, 2010). In the cytoplasm, nucleus and endoplasmic reticulum, the proteasome is responsible for selectively degrading oxidatively damaged proteins (Ullrich *et al.*, 1999; Davies, 2001; Pickering *et al.*, 2010, 2012) but in mitochondria, which have no proteasome, this task is accomplished by LonP1 (Bota & Davies, 2002). Thus mitochondrial LonP1, as might be expected of such an important, multi-functional enzyme, is highly conserved from prokaryotes to eukaryotes (Wang *et al.*, 1993). In addition to the mitochondrial isoform of Lon (LonP1), there also exists a peroxisome-specific isoform known as LonP2. While mitochondrial Lon has been associated with adaptation to acute oxidative stress and has been shown to decline with age (Ngo & Davies, 2009; Ngo *et al.*, 2011), both of which are attributes of the proteasome in other parts of the cell (Pickering *et al.*, 2010, 2012), the role of the peroxisomal isoform of Lon in adaptation is not as well understood. This limited insight into Lon's role within the peroxisome may be further restricted by an incomplete understanding of peroxisomal physiology, beyond its well-defined role in fatty acid metabolism.

The peroxisome is an abundant organelle, present in the majority of eukaryotic cells. Peroxisomes are self-replicating organelles that can also arise by budding off the endoplasmic reticulum (ER), and that are in dynamic flux to accommodate the metabolic needs of the cell. Unlike mitochondria, which have a double membrane, peroxisomes possess a single membrane with a crystalline core composed of urate oxidase (Lazarow, 1995). One of the primary functions of peroxisomes is their role in fatty acid metabolism, which also results in the synthesis of hydrogen peroxide (H_2O_2) as a by-product (Lazarow, 1995). Fortunately, peroxisomes contain enzymes, such as catalase and glutathione peroxidase, that can reduce H_2O_2 back to O_2 and H_2O ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$), because uncontrolled levels of H_2O_2 could instigate damage to various cellular constituents (Nordgren & Fransen, 2014). Additionally, LonP2 can degrade proteins damaged by H_2O_2 that escape reduction. Thus, the peroxisome-specific Lon protease plays a major role in

maintaining peroxisomal protein homeostasis, and provides a critical mechanism to avoid the accumulation of oxidative damage (Friguet, 2006).

Despite the widespread perception of H₂O₂ and other reactive oxygen species as merely toxic by-products of aerobic metabolism, increasing evidence indicates that peroxisome-generated H₂O₂ may act as a signalling molecule involved in the stimulation of various pathways, such as cell proliferation, differentiation, migration, and apoptosis (Veal, Day & Morgan, 2007; Titorenko & Terlecky, 2011). At low concentrations, H₂O₂ can function as a non-toxic signal due to its ability to stimulate stress-response pathways by triggering the increased expression of various repair proteins and chaperones that can mitigate against oxidative damage (Jamieson, 1998; Narasimhan, Yen & Tissenbaum, 2009). This type of signalling pathway has already been well documented in mitochondria, which can release a sub-lethal dose of H₂O₂ resulting in activation of oxidant-sensitive pathways (Finley & Haigis, 2009). Because peroxisomes have been shown to dissipate some 20–60% of the H₂O₂ they generate into the cytoplasm (Boveris, Oshino & Chance, 1972), they may potentially play a similar role as mitochondria in redox cell signalling. This hypothesis is further supported by the high abundance of peroxisomal enzymes, such as catalase and glutathione peroxidase, that control the degradation of H₂O₂ and may help maintain cellular redox homeostasis (Fig. 1) (Titorenko & Terlecky, 2011). Moreover, peroxisome dysfunction has been found to be associated not only with genetically based peroxisome disorders, but also with many chronic aging-related diseases such as Type II diabetes and cancer (Nordgren & Fransen, 2014), as well as aging itself (Perichon *et al.*, 1998). Since an imbalance between the production and removal of reactive oxygen is a hallmark of aging and aging-related diseases, increased understanding of peroxisome dysfunction and peroxisomal Lon may also shed light on the aging-related decline of peroxisome function.

II. THE ORIGIN OF PEROXISOMES

Organelles may be inherited from parental cells or synthesized *de novo* from the ER (Misteli, 2001). Neither mitochondria nor the ER can be generated by cells, therefore failure in their inheritance by daughter cells leads to apoptosis (Hoepfner *et al.*, 2005). When peroxisomes were discovered, they were originally suggested to be descendants of the ER (Novikoff & Shin, 1964) and it was hypothesized that they were inherited organelles (De Duve, 1996), in part because peroxisomes were considered to be precursors of the more efficient mitochondria, which were thought to act as both detoxifying agents and energy powerhouses (De Duve, 1996). This viewpoint, however, fails to explain the regenerative, ‘self-replicating’ capabilities of peroxisomes (Shio & Lazarow, 1981; Lazarow & Fujiki, 1985). Experiments in yeast and human fibroblasts lacking peroxisomes demonstrate the adeptness of these cells to regenerate peroxisomes (Hoepfner *et al.*, 2005). Cells lacking peroxisomes, due to the mutation of peroxisome genes, undergo *de novo* synthesis upon complementation of wild-type peroxisome genes, even after multiple passages of cells lacking peroxisomes (Subramani, 1998). Furthermore, electron microscopy has shown the physical connection between peroxisomes and ER membranes (Subramani, 1998).

The process of peroxisome formation from the ER, and the demonstration of cellular *de novo* synthesis of these organelles, was not fully accepted until the work of Hoepfner *et al.*

(2005). Utilizing two mutant *Saccharomyces cerevisiae* strains (*PEX3* and *PEX19*) they demonstrated the formation of peroxisomes from a specialized compartment within the ER. Peroxin 3 (Pex3) and Peroxin 19 (Pex19) are generated in the cytoplasm and first targeted to the ER before appearing in the peroxisome (Hoepfner *et al.*, 2005). Results from fluorescent imaging showed the initial interaction between Pex3 and Sec63, both critical membrane proteins, during peroxisome membrane formation within the ER. Budding of this membrane indicated that Pex3 becomes concentrated into structures that form adjacent to the ER, and that there is a subsequent migration of Pex19 to these structures (Hoepfner *et al.*, 2005). The physical interaction of Pex3 and Pex19 results in the import of proteins into the peroxisomal membrane (Hoepfner *et al.*, 2005). The subsequent incorporation of peroxisomal membrane proteins (PMPs) and the budding of the maturing peroxisome from the ER leads to the import of peroxisome matrix proteins, through Pex5 and Pex7 (Gould & Valle, 2000).

Recently, this foundational work in peroxisome biogenesis was challenged by Knoops *et al.* (2014). Their findings report that after reintroduction of Pex3 in a Pex3-deficient *Hansenula polymorpha* strain, it is not incorporated into the ER, but is instead collected in pre-peroxisomal structures that mature into normal peroxisomes. Interestingly, these pre-peroxisomal structures are present even upon the deletion of Pex3 (Knoops *et al.*, 2014). Therefore, there is currently evidence that peroxisomes use two mechanisms of proliferation: fission from pre-existing peroxisomes and budding from the ER, and that different organisms may display preference for one pathway over the other.

Recognition of peroxisomal enzymes is accomplished *via* two targeting sequences: peroxisome targeting signal 1 (PTS1) and peroxisome targeting signal 2 (PTS2). PTS1 is comprised of three amino acids (serine, lysine/arginine, leucine), and is the predominant targeting signal for most peroxisome proteins (Brocard & Hartig, 2006). Unlike other imported organelle proteins, the PTS1 signal is located at the C-terminus of target proteins and is recognized by Pex5 (Gould & Valle, 2000). This differs from other targeting signals located on the N-terminus of most imported proteins, which are typically removed prior to, or during, transport into the targeted organelle (Brocard & Hartig, 2006). As a result, proteins destined for the peroxisome must be fully synthesized, and proteolytically matured in the cytoplasm, before being imported into the peroxisome matrix (Brocard & Hartig, 2006). This mechanism may provide a protein quality-control measure, as peroxisome-targeted proteins can assume their mature and active states prior to import. This protein preassembly obviates the need for peroxisomes to import and maintain their own assortment of molecular chaperones to assist in the correct folding and assembly of peroxisome matrix proteins (Brocard & Hartig, 2006). However, the conjecture that imported proteins must be in a native conformational state prior to import was challenged by the import of denatured human serum albumin (HSA) incorporated with a PTS1 sequence, demonstrating that the predominant determinant for protein import is the presence of the PTS1 localization signal, rather than the state of protein folding (Brocard *et al.*, 2003).

The second peroxisome targeting signal, PTS2, is a less common localization sequence comprised of nine amino acids located on the N-terminus of proteins, which is recognized by Pex7 (Gould & Valle, 2000). When Pex7 and Pex5 are attached to a substrate, they are recognized and bound by Pex13 and Pex14 to generate a pore within the peroxisome

membrane, resulting in the subsequent release and import of the peroxisome-targeted protein (Gould & Valle, 2000).

III. THE DISCOVERY OF PEROXISOME-SPECIFIC LON

Peroxisomes were discovered in the mid-1950s and were initially characterized as ‘microbodies’ (Rhodin, 1954). Their actual metabolic functions were not uncovered until a decade later, at which time it was also found that peroxisomal enzymes generate H_2O_2 as a metabolic by-product of fatty-acid metabolism (Cooper & Beevers, 1969; Lazarow & De Duve, 1976), leading to the coining of the name ‘peroxisomes’ (De Duve & Baudhuin, 1966). The identification of which enzymes are actually targeted to the peroxisome was limited until the advent of mass spectrometry. Thus, the discovery of the peroxisomal isoform of Lon did not occur until 2004 (Kikuchi *et al.*, 2004). In order to identify organelle proteins, the organelles must first be purified from other cellular components. Due to their functional similarity, it is critical to ensure the complete removal of contaminating mitochondria when isolating peroxisomes. The method outlined by Ghosh & Hajra (1986) is still the standard approach for peroxisome isolation from rat liver, which relies upon the Nycodenz density gradient to remove the majority of other cellular components, with an additional purification step suggested by Kikuchi *et al.* (2004) that utilizes immunoprecipitation by targeting a peroxisomal membrane protein (PMP70).

Localization of Lon to the peroxisome was demonstrated in both rat and human tissue through the use of an antibody generated against the LonP2 C-terminal sequence found in mice (Kikuchi *et al.*, 2004). Further studies, employing ultracentrifugation, indicated that LonP2 is not only an abundant enzyme in the peroxisome, but concentrates specifically within the dense crystalline core (Kikuchi *et al.*, 2004). Of note, the crystalline core found in rat liver peroxisomes is primarily comprised of urate oxidase and is nearly devoid of catalase and other oxidases, which may highlight a possible explanation for the concentration of LonP2 within the peroxisomal core (Hayashi *et al.*, 1976). As H_2O_2 cannot be degraded in this peroxisomal sub-compartment, it likely contributes to the rapid oxidation of proximal urate oxidase that may contribute to protein damage. Hence, it is logical that a proteolytic enzyme, such as LonP2, would also be in close proximity so as to prevent protein aggregation. Furthermore, the relative abundance of Lon within the highly oxidizing environment of the peroxisome’s core highlights a similarity shared with LonP1, the mitochondrial isoform of Lon. Both enzymes are in high concentration and close proximity to proteins that are at risk of oxidative damage and have the need for rapid degradation.

In mammals, it has since been found that peroxisomal Lon is ubiquitous throughout the body, with particularly high expression patterns in the pancreas, kidney, and liver (Omi *et al.*, 2008). In addition, although a peroxisome-specific isoform of Lon has been identified in many widely diverse organisms (rat, mouse, *Caenorhabditis elegans* and human), LonP2 may not be as highly conserved as the mitochondrial isoform, since it has not been physically detected in *Drosophila melanogaster* (Bartoszewska *et al.*, 2012).

IV. CHARACTERIZATION OF THE MITOCHONDRIAL (LONP1) AND PEROXISOMAL (LONP2) ISOFORMS OF LON

Both isoforms of Lon contain conserved domains that catalyse proteolytic activity within the peroxisome and the mitochondrion. Peroxisome-specific Lon, LonP2, is comprised of an 852 amino acid sequence belonging to the Lon A family, the primary form of Lon found in prokaryotes and eukaryotes (Altschul *et al.*, 1997; Bartoszewska *et al.*, 2012), and has a molecular weight of 95 kDa (Kikuchi *et al.*, 2004). In humans, LonP2 is encoded within the nucleus from chromosome 16, assembled in the cytoplasm, and targeted to the peroxisome matrix by the PTS1 targeting signal located on its C-terminus (Gould *et al.*, 1989). Prior to import, it is hypothesized that LonP2 folds into its catalytically active conformation consisting of three domains: the Lon N ('substrate recognition') domain, the ATPase domain, and the proteolytic domain (Fig. 2B). Substrates of LonP2 are recognized by a non-catalytically active serine residue within the Lon N domain that facilitates binding with substrates. LonP2 is an ATPase, which expedites degradation of oxidized proteins through the hydrolysis of ATP. The ATPase domain contains an AAA region consisting of the Walker A and B motifs, which are conserved three-dimensional protein structures characteristic of ATPases (Altschul *et al.*, 1997; Dougan *et al.*, 2002). Lastly, the proteolytic domain contains a catalytically active serine-lysine dyad for enzymatic substrate degradation (Stahlberg *et al.*, 1999; Bartoszewska *et al.*, 2012).

Similarly, mitochondrion-specific Lon, LonP1, is 959 amino acids in length with a molecular weight of 100 kDa and also contains the three characteristic domains of the Lon A family: a substrate-recognition 'N' region, an ATP-binding and hydrolysis region, and a serine-proteolytic site (Fig. 2C) (Ngo & Davies, 2007). In addition, human *lonP1* is a nuclear-encoded gene from chromosome 19 that is transported into mitochondria *via* a mitochondrial targeting sequence located at the N-terminus (Wang *et al.*, 1994). As LonP1 is imported into the mitochondrion, the mitochondrial targeting sequence is removed and it is folded into a catalytically active state, becoming either a six- or seven-membered, monomeric ring complex. Therefore, both the peroxisomal and mitochondrial isoforms of Lon demonstrate conserved domain similarity, but work is still needed to determine if these proteins evolved in parallel or from the same ancestral lineage.

The relationship between structure and function was further explored by statistical modelling (Bartoszewska *et al.*, 2012) using the yeast species *Penicillium chrysogenum*. Peroxisomal Lon assumes a heptameric ring structure consisting of seven monomers, which is consistent with the mitochondrial Lon homologue observed in *S. cerevisiae* (Stahlberg *et al.*, 1999). The similarity between the tertiary structures of yeast LonP1 and LonP2 is consistent with the overall structural conservation between the two isoforms, especially within the C-terminal region containing the ATPase domain (Dougan *et al.*, 2002). This conserved structural similarity, as well as the localization within the matrix of mitochondria or peroxisomes, further suggests the conserved role of peroxisomal Lon as a protease. Sequence comparison of the mitochondrial and peroxisomal isoforms of Lon revealed 39.6% of the amino acid sequence to be conserved (Fig. 2A) (Geneious Pro 5.1.7). This overall sequence similarity, although not high, sheds light upon key regions that contain critical

domains that are highly conserved. The regions that show the greatest overlap are within the conserved ATPase domain and the proteolytic domain. In addition, there is some similarity between the substrate recognition domains. The high amount of overlap within the functional domains of the two proteases suggests that peroxisomal Lon may have a similar function to mitochondrial Lon.

V. THE FUNCTIONAL ROLE OF PEROXISOMAL LON

Peroxisomes play a central role in cellular lipid metabolism and in the metabolism of oxidation by-products, evident from the variety of enzymes capable of generating or degrading H_2O_2 and superoxide (Nordgren & Fransen, 2014). However, scavenging of H_2O_2 and superoxide is not perfect, and some proteins do undergo oxidative modification. Because a degree of protein oxidation is an inevitable outcome of peroxisomal metabolism, the ability to degrade proximal proteins damaged through oxidation is critical for peroxisome homeostasis (Till *et al.*, 2012), as excessive protein oxidation can result in protein aggregation and eventual loss in peroxisome function (Terlecky, Koepke & Walton, 2006).

Early studies that focused on determining the functional role of Lon in the peroxisome utilized the yeast strain, *H. polymorpha*. To understand if peroxisomal Lon played a role in degrading misfolded proteins, investigators created a mutant dihydrofolate reductase (DHFR) incapable of correctly folding, which, upon transport into the peroxisome, was subsequently degraded. Yeast strains lacking LonP2 showed stabilization of the mutated form of DHFR. In addition, a mutant form of alcohol oxidase, which is misfolded upon peroxisome import indicated that in the absence of LonP2, the misassembled protein was not degraded (Aksam *et al.*, 2007). Together, these findings support the role of peroxisomal Lon as a critical protease to degrade peroxisome matrix proteins (Aksam *et al.*, 2007).

Further studies have examined the role of Lon in the quality control of proteins in the peroxisome. Similar to its mitochondrial isoform, peroxisome-specific Lon is an ATP-stimulated enzyme (Bartoszewska *et al.*, 2012). The ATP-stimulated activity of LonP2 was discovered from studies using the fungus, *P. chrysogenum*. In *in vitro* assays, isolated LonP2 rapidly degraded misfolded α - or β -casein in the presence of ATP (Bartoszewska *et al.*, 2012). In addition, quantitative measurement of the proteolytic degradation of resorufin-labelled β -casein demonstrated high proteolytic activity of LonP2 only upon the addition of ATP (Bartoszewska *et al.*, 2012). As proof of principle, a mutation at the catalytically active serine residue rendered the enzyme non-functional and in the presence of either α - or β -casein and ATP, the substrate could not be degraded (Bartoszewska *et al.*, 2012). We suggest that future studies should delve further into understanding the specific role of ATP in the enzymatic activity of LonP2. This is important because LonP1 utilizes ATP hydrolysis not for substrate degradation, but rather for a conformational change in its proteolytic site to allow for proteolysis (Patterson-Ward, Huang & Lee, 2007). The inclusion of other ATP variants, specifically non-hydrolysable ATP analogs, would inform whether LonP2 utilizes ATP in a similar or different manner from that of LonP1. We feel that such initial *in-vitro* studies will be important in determining whether peroxisomal Lon can function as an ATP-stimulated protease, mirroring the role of mitochondrial Lon.

Peroxisome-specific Lon has also been demonstrated to be necessary for cell viability. Preliminary studies, conducted in yeast, assessed cell survival by deleting the homolog for peroxisomal Lon, *pln*. In addition, a critical yeast signalling molecule for autophagy, *atg1*, was also deleted, forming *pln.atg1* double mutants (Aksam *et al.*, 2007). Singular deletion of peroxisomal Lon had little effect on cell viability, but did contribute to the elevation of reactive oxygen species, whereas the loss of *atg1* significantly decreased cell survival. In the double mutant, loss of both *pln* and *atg1* showed a synergistic effect, which further decreased cell survival (Aksam *et al.*, 2007). This suggests that the proteolytic role of Lon is important for general protein maintenance of the peroxisome. However, the accumulation of high levels of protein damage may exceed the enzymatic degradation rate of Lon, making autophagy of the peroxisome the cell's most efficient mechanism to control protein damage. The cumulative loss of both *pln* and *atg1* greatly mitigated cell survival due to the increase in protein damage and inability to degrade the peroxisome when the damage became too extensive (Aksam *et al.*, 2007).

In addition, *pln* and *pln.atg1* deletion mutants had an increase in the accumulation of protein aggregates, as well as increased peroxisome number and size in *P. chrysogenum* (Aksam *et al.*, 2007; Bartoszewska *et al.*, 2012). The protein aggregates were hypothesized to be substrates for peroxisomal Lon, suggesting that LonP2 is necessary to prevent the accumulation of misfolded proteins in the peroxisome matrix. This downstream effect resulting from *pln* deletion is reminiscent of the protein aggregates that arise in *C. elegans* mutants lacking the mitochondrial Lon homologue (PIM1) (Erjavec *et al.*, 2013). Protein aggregates are also a prevalent marker in senescent cells lacking mitochondrial Lon (Bota, Ngo & Davies, 2005). Together, this indicates that loss of peroxisomal Lon, coupled with the cell's inability to degrade damaged proteins *via* pexophagy, results in electron-dense aggregates (Bartoszewska *et al.*, 2012) which presumably accelerate cellular dysfunction.

Optimal protein quality control is crucial to counteract the continual damage resulting from reactive oxygen species generation, a trait common to both peroxisomes and mitochondria (Bulteau, Szweda & Friguet, 2006; Bonekamp *et al.*, 2009). In *P. chrysogenum*, mutants lacking LonP2 showed increased DNA damage compared to their wild-type counterparts (Bartoszewska *et al.*, 2012). DNA damage was further exacerbated when these mutants were grown in an environment that restricted access to a carbon source, through the breakdown of oleic acid *via* the β -oxidation pathway (Poirier *et al.*, 2006). This restrictive approach greatly increased peroxisome metabolic activity and accelerated the generation of reactive oxygen species and subsequent oxidative damage (Bartoszewska *et al.*, 2012). A similar trend was detected in the yeast strain, *H. polymorpha* where *pln.atg1* double mutants exhibited a significant increase in intracellular levels of reactive oxygen species (Aksam *et al.*, 2007). This demonstrates the connection between the metabolic state of peroxisomes, a major generator of H₂O₂, and the consequences of uncontrolled peroxisome protein maintenance.

Due to LonP2's role in maintaining the peroxisome proteome during normal conditions, it is important to determine if its expression is stress dependent. This is plausible because mitochondrial-specific LonP1 expression is rapidly elevated following multiple types of stressors including heat shock, serum starvation and H₂O₂ exposure (Ngo & Davies, 2009). In addition, ER stress has been implicated in causing increased transcription of the

mitochondrial Lon protease (Hori *et al.*, 2002). Interestingly, peroxisomes proliferate in response to various forms of oxidative stress such as hypoxia/reoxygenation (also known as reperfusion injury) ultraviolet (UV) irradiation and H₂O₂ exposure, with increased expression of peroxisome Pex family import machinery proteins (Lopez-Huertas *et al.*, 2000). Interestingly, in a glucose-rich environment these stressors do not appear to increase peroxisome-specific Lon protein levels in yeast (Aksam *et al.*, 2007). However, for methylotrophic yeast strains cultured in methanol-enriched media, which is a documented trigger for peroxisome biogenesis, peroxisomal Lon expression was found to increase (Aksam *et al.*, 2007). This may occur because methanol, as the sole carbon source, can only be broken down in peroxisomes, thereby resulting in peroxisome biogenesis and increased H₂O₂ production (van der Klei *et al.*, 2006). Therefore it is difficult to distinguish if LonP2 levels increase simply because there are more peroxisomes or if peroxisomal LonP2 is induced to a higher extent than other peroxisomal enzymes. More work is needed to understand if peroxisomal Lon has a similar stress-response role to that exhibited by mitochondrial Lon.

During periods of decreased peroxisome content, removal of peroxisome proliferation signals causes Lon to degrade β -oxidation enzymes, a potential consequence of the decline in demand for peroxisome fatty-acid metabolism (Yokota, Haraguchi & Oda, 2008). Phthalate and adipate ester plasticizers, such as di(2-ethylhexyl)phthalate (DEHP) and di(2-ethylhexyl)adipate, are used to chemically induce peroxisome proliferation (Lock, Mitchell & Elcombe, 1989). Rats treated with DEHP for a period of 2 weeks exhibited an increase in peroxisome proliferation markers, specifically β -oxidation enzymes and acyl-CoA oxidase, in hepatic tissue. Lon levels were also increased during the 2-week drug regimen and reached maximal expression upon discontinuation of DEHP (Yokota *et al.*, 2008). By contrast, the β -oxidation enzymes acyl-CoA oxidase and thiolase showed immediate decline following removal of DEHP, whereas Lon levels did not subside until 6 days following the termination of treatment (Yokota *et al.*, 2008). Therefore, due to the lag in the reduction of Lon levels following discontinuation of DEHP and the concurrent immediate decline of β -oxidation enzymes, it is likely that peroxisome-specific Lon is necessary for the degradation of β -oxidation enzymes (Yokota *et al.*, 2008). These results further support Lon's integral role in maintaining overall peroxisome homeostasis, in addition to its role as a protease to degrade damaged proteins.

VI. LONP1'S ROLE IN MITOCHONDRIAL DNA INTEGRITY

LonP1 has been shown to interact directly with mitochondrial DNA (mtDNA) (Fu, Smith & Markovitz, 1997). High binding affinity of the prokaryotic Lon found in *Escherichia coli* showed binding to sequences similar to those found in the small non-coding region of the human mitochondrial displacement loop (D-loop) (Fu & Markovitz, 1998). Additional studies of *E. coli* Lon (La) found that during periods of stress, La blocks DNA replication through degradation of the transcriptional initiator, DnaA, halting division and allowing for repair (Jonas *et al.*, 2013). Point-mutation within the ATPase-domain of La blocks protein degradation, but not DNA binding (Amerik *et al.*, 1991; Fischer & Glockshuber, 1994). In a conserved manner, human LonP1 preferentially binds only to single-stranded mitochondrial DNA within the D-loop (Fu & Markovitz, 1998; Lu *et al.*, 2003). The fact that LonP1 has no

preference for binding to double-stranded DNA could suggest a potential role as a transcriptional regulator. This was confirmed by LonP1's proteolytic regulation of mitochondrial transcription factor A (TFAM), which binds directly to the D-loop and is required for mtDNA integrity (Matsushima *et al.*, 2010). Phosphorylation of TFAM prevents its binding to mtDNA and its subsequent degradation by LonP1 (Lu *et al.*, 2013). Conversely, loss of LonP1 leads to the accumulation of TFAM. LonP1 has also been shown to regulate mitochondrial transcription factor B2 (mtTFB2), as loss of Lon leads to the accumulation of mtTFB2, a critical regulator of mitochondrial DNA transcription (Matsushima *et al.*, 2010). In addition, loss of Lon has been linked to decreased respiration and lesions to mtDNA (van Dyck, Pearce & Sherman, 1994; van Dyck, Neupert & Langer, 1998; Guha *et al.*, 2011). While the predominant role of Lon is the proteolytic degradation of oxidized proteins, it is clear that LonP1 may also contribute, albeit indirectly, to transcriptional regulation within the mitochondria.

VII. BIOLOGICAL SUBSTRATES FOR PEROXISOMAL LON

The electron-dense inclusion bodies that developed in the *P. chrysogenum* mutant with peroxisomes lacking Lon were primarily composed of normal Lon substrates that were unable to be degraded and, thus, accumulated and aggregated (Bartoszewska *et al.*, 2012). Mass spectrometry revealed that a catalase-peroxidase was the major component of these peroxisome protein aggregates (Bartoszewska *et al.*, 2012). Catalase-peroxidase is the most abundant H₂O₂ decomposing enzyme of the peroxisome. It is estimated that for every one molecule of H₂O₂-producing enzyme there is one molecule of catalase to counteract it, demonstrating the importance of catalase as a detoxifying enzyme in the peroxisome (Antonenkova *et al.*, 2010). To investigate the proteolytic relationship between catalase-peroxidase and Lon, purified catalase was subjected to increasing concentrations of H₂O₂ until loss of protein conformation led to loss of enzymatic activity, and then was subsequently exposed to peroxisome-specific Lon. Without H₂O₂ pre-treatment, catalase-peroxidase was resistant to proteolysis by Lon. However, following exposure to low concentrations of H₂O₂, significant catalase degradation by LonP2 was observed (Bartoszewska *et al.*, 2012). This finding demonstrates that oxidatively misfolded catalase-peroxidase, but not its native counterpart, is a good substrate for peroxisome-specific Lon. In a subsequent study, HEK293 cells stably overexpressing LonP2 caused the mislocalization of catalase and its accumulation in the cytoplasm. Moreover, catalase activity was greatly decreased, highlighting the additional role that peroxisomal Lon may play in catalase import (Omi *et al.*, 2008).

The necessity to degrade nonfunctioning catalase is critical for peroxisome function. As catalase is a major peroxisomal enzyme, any loss of function can dramatically disregulate the balance between H₂O₂ generation and degradation (Antonenkova *et al.*, 2010). This is important due to the large amount of H₂O₂ generated by peroxisomes. Studies using rat liver homogenates found the overall rate of H₂O₂ production to be 38 nmol min⁻¹ g⁻¹ of liver protein (Boveris *et al.*, 1972). However, isolated peroxisomes are estimated to generate approximately 44–172 nmol min⁻¹ g⁻¹ protein. Fortunately, only some 30% of peroxisome-generated H₂O₂ actually appears to diffuse into the cytoplasm (approximately 13.2–51.6 nmol min⁻¹ g⁻¹ protein), where it is primarily broken down by glutathione peroxidase, and

the remainder is degraded by catalase (Boveris *et al.*, 1972). The relationship of H₂O₂ breakdown between glutathione peroxidase and catalase appears to be sigmoidal (Jones *et al.*, 1981). At low homeostatic concentrations, catalase is the dominant catabolic enzyme, whereas at higher concentrations, glutathione peroxidase becomes the predominant means of breaking down H₂O₂ (Jones *et al.*, 1981). This relationship demonstrates that at physiologically normal concentrations, the majority of H₂O₂ degradation is contained within the peroxisome. However, excess levels of H₂O₂ generation may result in greater cytoplasmic H₂O₂ leakage and the necessity of cytoplasmic glutathione peroxidase activity as a secondary defence.

It is important to note that even at low concentrations of H₂O₂ (100 μ M), catalase can undergo approximately 40% loss of enzymatic function (Bartoszewska *et al.*, 2012). Higher levels of H₂O₂ exacerbate the loss of catalase function and the accumulation of nonfunctioning proteins. To mitigate loss, the peroxisome may compensate by maintaining a high concentration of catalase and its reliance upon LonP2 to degrade dysfunctional catalase efficiently.

The delicate balance between protein damage and protein degradation in peroxisomes may well parallel mitochondrial protein homeostasis. Mitochondrial Lon plays a critical role in preventing the accumulation of oxidized proteins that will diminish mitochondrial function. However, if the protein damage is too extensive, mitochondrial Lon can no longer maintain protein homeostasis, resulting in increased mitochondrial damage and cellular apoptosis (Bota & Davies, 2002). Although this has not yet been shown to occur in peroxisomes, this mechanism of oxidative dysfunction may also arise when LonP2 proteolytic capacity is overwhelmed in the presence of increased damage to proximal peroxisomal proteins.

Recently, LonP2 has been implicated in the degradation of the serine protease, trypsin domain-containing 1 (Tysnd1). Similar to Lon, Tysnd1 has been shown to degrade multiple PTS1-containing proteins and remove the PTS2 targeting sequences from protein precursors (Okumoto, Kametani & Fujiki, 2011). Loss of PTS1 results in the accumulation of immature β -oxidation enzymes and in diminished processing of long-chain fatty acids. Upon self-cleavage of Tysnd1, the inactive subunits are further degraded by peroxisomal Lon. The proteolysis of Tysnd1 by LonP2 may not be due to a specific relationship between these enzymes, but simply the affinity of Lon to degrade damaged proteins marked by the exposure of hydrophobic residues (Gur & Sauer, 2008). Therefore, the capability of the peroxisome to remove unnecessary and nonfunctioning proteins from its matrix is critical to ensure continued function.

VIII. THE CHAPERONE-LIKE ACTIVITY OF PEROXISOMAL LON

Protein chaperones are an important feature of the mitochondrial protein quality-control system. They are critical in helping to ensure correct protein folding during periods of cellular stress. Various synergistic partnerships are formed between multiple ATP-dependent and independent chaperones to promote native folding of proteins (Fink, 1999). In *C. elegans*, the coordinated biochemical roles of heat shock protein 78 (Hsp78), a chaperone-like enzyme, and PIM1, a mitochondria-specific yeast Lon homolog, are important in

preventing the accumulation of protein aggregates (Leidhold *et al.*, 2006; Voos, 2009). However, unlike mitochondria, peroxisomes do not have protein-folding machinery such as heat shock response proteins, potentially because proteins are imported into peroxisomes as fully folded entities (Gould *et al.*, 1989). Despite the fact that peroxisomes appear not to have a dedicated protein machinery, it has been suggested that LonP2 has chaperone-like activities in addition to its role as a protease. To explore the potential role of Lon as a chaperone protein, purified peroxisome-specific Lon was incubated with denatured citrate synthase to determine if the enzyme could prevent protein aggregation. In the presence of Lon, independent of ATP, protein aggregation was decreased by 40% in comparison to control samples (Bartoszewska *et al.*, 2012). Complete inhibition of protein aggregation occurred with increasing amounts of Lon and enzymatic activity of the denatured protein was re-established with the addition of ATP, thus indicating that prevention of aggregation was not through a proteolytic pathway (Bartoszewska *et al.*, 2012). Thus, these findings add to the known multifunctional characteristics of peroxisomal Lon.

Based on the current studies focused on the role of Lon in the peroxisome, LonP2 has consistently been shown to be a protease that mirrors the biological function of the mitochondrial isoform, LonP1. The conserved structure and domains between peroxisomal and mitochondrial Lon emphasize how configuration facilitates their function as serine proteases. However, only peroxisome-specific Lon has been directly shown to have chaperone-like activity. LonP1's role as a chaperone has only been hypothesized, but further work in a newly discovered class of Lon-like proteases (LonC), which lack the canonical ATPase domain, has hinted that they act as chaperones (Li *et al.*, 2013). In addition, unlike mitochondria, which possess an additional chaperone system, peroxisomes do not contain heat shock response proteins to assist in proper protein folding. Instead, peroxisomal Lon appears to have evolved into a multifaceted protein that is capable of acting as both an ATP-dependent protease and a chaperone. The relationship between these two activities was shown to be modulated depending on cellular requirements. During peroxisome biogenesis, Lon's chaperone-like activity may assist in the correct folding and normal assembly of imported proteins. During periods of decreased metabolic needs, the proteolytic activity of Lon hinders its chaperone-like activity, which allows for the removal of unnecessary peroxisome proteins through a combination of proteolysis and activation of autophagy pathways (Goto-Yamada *et al.*, 2014). Thus, the dynamic role of peroxisomal Lon is dependent upon the biological needs of the peroxisome.

IX. PEROXISOMES, LONP2, AGING AND DISEASE

Similar to other organelles, peroxisomes are impacted by aging, as identified through changes in metabolism and biogenesis (Titorenko & Terlecky, 2011). One such characteristic is the overall decline in peroxisome protein import, with accumulation of 'older' peroxisomal proteins, as demonstrated through immunocytochemical analysis of middle- to late-passage human fibroblasts (Terlecky *et al.*, 2006). In response to the natural decline in peroxisome import, the senescent cell compensates by inducing peroxisome biogenesis, resulting in a twofold increase in peroxisome volume compared to early- and middle-passage cells (Terlecky *et al.*, 2006). However, an increase in peroxisome number does not equate to an overall increase in peroxisome activity. This is partly due to the primary

mechanism of peroxisome proliferation, which arises from cellular fission and division. Peroxisome fission is an asymmetric process, resulting in the nascent portion receiving the majority of newly synthesized matrix proteins (Delille *et al.*, 2010). This results in the formation of a seemingly healthier peroxisome at the expense of a more highly damaged one. Consequently, the division of an already damaged organelle may temporarily alleviate cellular stress, but eventually the damaged peroxisomes, if not cleared, will outweigh the formation of healthy ones (Manivannan *et al.*, 2012).

As previously described, one of the main H₂O₂ detoxifying enzymes of the peroxisome is catalase. Without this enzyme, the capacity to degrade major toxic by-products of peroxisome metabolism would be significantly diminished resulting in the accumulation of damage. Importantly, one hallmark of cellular senescence is a marked decline in the peroxisomal import of catalase (Terlecky *et al.*, 2006). This results in the accumulation of catalase as a nonfunctioning entity within the cytoplasm, paralleled by the peroxisome's diminished capacity to degrade H₂O₂ (Legakis *et al.*, 2002). This aging-dependent decline has been hypothesized to result from the low affinity between catalase's targeting sequence, which contains a divergent form of the PTS1 signal (lysine–alanine–asparagine–leucine), and the ability of Pex5 to recognize and target catalase to the peroxisome, even in low-passage cells (Legakis *et al.*, 2002). With age, the import efficiency of catalase further declines because of an increased 'sticking' of Pex5 to the peroxisome membrane, thereby reducing the cytoplasmic pool of available Pex5 that can be used to import peroxisomal proteins (Legakis *et al.*, 2002). Loss of Pex5 import efficiency may be partially explained by its high redox sensitivity, resulting in loss of functionality (Apanasets *et al.*, 2014), which further contributes to the cytoplasmic retention of catalase in aged cells.

The lack of catalase import results in a redox imbalance within the peroxisome which can no longer properly compensate for H₂O₂-generating reactions, transitioning the organelle into a major producer of H₂O₂ (Walton & Pizzitelli, 2012). Furthermore, the process of catalase mislocalization has been found to begin in as early as middle-passage cells and contributes to the gradual increase in reactive oxygen species, which is a hallmark of cellular senescence (Terlecky *et al.*, 2006). This progressive decline in activity and mislocalization of catalase was substantiated by the comparison of liver tissue from young and old rats (Beier, Völkl & Fahimi, 1993). In aged rats, there was a substantial decrease in catalase expression and a 30–40% loss of enzymatic activity. In addition, the decline in catalase levels was coupled with an increase in peroxisome size and heterogeneity within the organelle's population (Beier *et al.*, 1993). This may be partly due to the asymmetric fission pattern of peroxisomes. The asymmetrical division causes the formation of two pools of peroxisomes: slightly enlarged and potentially dysfunctional peroxisomes and smaller, enzymatically active peroxisomes. Thus, peroxisome heterogeneity may be the cell's attempt at restoring declining peroxisome activity (Huber *et al.*, 2012). Further research is needed to determine whether loss of catalase leads to peroxisome dysfunction or is an inherent characteristic of these oxidant-producing organelles.

Interestingly, no work of which we are aware has focused on the relationship between mammalian LonP2 and aging. As catalase has been identified as the primary peroxisome-specific protein for which loss is the most detrimental to organelle function, it is important to

understand the relationship between the aging-dependent decline of catalase and peroxisomal Lon. We can hypothesize, because the proteolytic activity between LonP1 and LonP2 appears to be conserved, that loss of LonP2 activity may lead to an accumulation of nonfunctioning catalase, which may accelerate loss of overall peroxisome function.

In an initial study focused on the age-related changes of mitochondrial Lon, a fourfold decrease in *lonP1* mRNA expression was found in the skeletal muscle of aged mice compared to young control mice (Lee *et al.*, 1999). Subsequently, it was shown that muscle LonP1 protein and enzymatic activity exhibit a similar decline with age, and that this is exacerbated in manganese superoxide dismutase heterozygotes which suffer a lifelong oxidative stress (Bota, Van Remmen & Davies, 2002). However, the impact of an age-associated decrease of mitochondrial Lon was not fully understood until studies focused on the differences in young *versus* senescent human fibroblasts (Bota *et al.*, 2005; Ngo *et al.*, 2011). These studies demonstrated the consequences of mitigated LonP1 expression, which results in the accumulation of oxidized proteins, inability to adapt to oxidative stress, decreased mitochondrial membrane potential, and increased mitochondrial mass (Ngo *et al.*, 2011). Furthermore, the adaptability of Lon is greatly diminished in senescent cells compared to their younger counterparts (Ngo *et al.*, 2011). As Lon is a stress-response protein, the necessity to respond rapidly to oxidative demands is crucial in maintaining mitochondrial function. In a similar manner, peroxisomal Lon expression is dependent on peroxisome biogenesis. The rapid import of LonP2 into peroxisomes is necessary to ensure the proper folding and removal of nonfunctional enzymes in order to maintain peroxisome function.

In addition, aconitase, an iron–sulfur protein that plays a critical role in energy production *via* the tricarboxylic acid (TCA) cycle, is a target substrate for mitochondrial Lon (Bota & Davies, 2002) and has been shown to have decreased activity with age. This decline in enzymatic function has been associated with a reduction in lifespan in *Drosophila melanogaster* (Yan, Levine & Sohal, 1997). Furthermore, aconitase was identified as one of the predominant proteins subject to oxidative damage as a result of aging (Yan *et al.*, 1997). Mice lacking the mitochondrial form of superoxide dismutase (SOD2) were more susceptible to oxidative stress due to the consequent decrease in the rate of superoxide dismutation to H₂O₂ (Bota *et al.*, 2002). In consequence, these animals provided a good model to understand Lon's role in protein removal and accumulation of damaged proteins with age. Young animals were found to have higher basal levels of Lon in contrast to either the wild-type older animals or their aged SOD2^{-/+} counterparts. Furthermore, though aconitase protein levels did not change, its activity substantially decreased in aged animals (Bota *et al.*, 2002). Thus the link between mitochondrial Lon and its substrate, aconitase, may lend to speculation that a similar relationship exists between peroxisomal Lon and catalase.

LonP1 plays an important role in maintaining mitochondrial integrity during aging and aging-related diseases. A study focused on identifying DNA variants associated with mitochondrial disorders uncovered multiple small nucleotide polymorphisms (SNPs) in the *lonP1* gene (Wang *et al.*, 2010). While these SNPs were benign, a later study demonstrated a much more deleterious impact of SNP variation in the *lonP1* gene. Specifically, four

mutations in *LonP1* were found directly to cause the development of CODAS syndrome, a rare multi-congenital disorder characterized by cerebral, ocular, dental, auricular, and skeletal abnormalities. All four mutations were found to be clustered within LonP1's ATPase domain, thus altering Lon's ability to utilize ATP efficiently (Strauss Kevin *et al.*, 2015). Although CODAS is a very rare genetic disease, this finding highlights that loss of Lon function can be detrimental to organismal well-being. In a similar study conducted in mice, researchers discovered that the homozygous loss of Lon results in embryonic lethality (Quirós Pedro *et al.*, 2014). Interestingly, heterozygous mice developed normally, further demonstrating that a dose-dependent threshold of Lon is necessary for survival (Quirós Pedro *et al.*, 2014).

While LonP1 is critical for mitochondrial protein maintenance, chronic upregulation of LonP1 has been associated with tumorigenesis. Various tumour cell lines, such as lymphoma cells and hepatocyte carcinomas, have been shown to have higher levels of Lon expression and activity compared to non-cancerous tissue (Bernstein *et al.*, 2012; Kita, Suzuki & Ochi, 2012). Cervical cancer has been found to have a twofold higher Lon expression compared to healthy cervical tissue. Moreover, the proliferation of tumour cells was abrogated by knockdown of Lon, which lowered cellular respiration and further limited tumorigenesis (Nie *et al.*, 2013). Similarly, silencing of Lon in colon cancer promotes cell death, alters mitochondrial proteome expression, and decreases mtDNA transcripts and oxidative phosphorylation complexes, thus contributing to lowered mitochondrial efficiency (Gibellini *et al.*, 2014). Overall, dysregulation of LonP1 is highly detrimental to cellular homeostasis and is a potential contributing factor to various chronic and developmental diseases. Much less is known about the impact of peroxisomal Lon during disease states, indicating that further work is needed to explore how changes in LonP2 expression can impact chronic disease progression.

X. THE INTERACTION BETWEEN MITOCHONDRIA AND PEROXISOMES

Peroxisomes and mitochondria have an important role in redox metabolism. During aging, abnormalities in mitochondria can cause them greatly to increase their basal production of oxidants (Fig. 3). As a result, the damage that occurs within the mitochondrion is not isolated, but impacts the rest of the cell by increasing the overall amount of reactive oxygen species, protein oxidation, lipid peroxidation, and DNA damage (Balaban, Nemoto & Finkel, 2005). A similar relationship, involving the spread of oxidative damage, is believed to occur between peroxisomes and the cell (Manivannan *et al.*, 2012).

The ability of oxidative damage generated in one organelle to affect the whole cell emphasizes another similarity between mitochondria and peroxisomes. To investigate this potential relationship, a study was conducted using mice that lacked the peroxisome import protein, Pex5. This deletion resulted in animals with nonfunctional peroxisomes and an inability to provide mitochondria with fatty substrates for β -oxidation (Baumgart *et al.*, 2001). This led to a decrease in the cellular availability of acetyl-CoA, the starting point for the first phase of mitochondrial TCA cycle energy conversion (Cohen *et al.*, 2014). Interestingly, peroxisome dysfunction was not isolated to the peroxisome but also resulted in deleterious alterations in the mitochondrial electron transport chain. This region of the

mitochondrion is considered one of the major areas of superoxide and H₂O₂ production. In addition, a parallel increase in the levels of manganese superoxide dismutase, an enzyme which converts superoxide into H₂O₂ and oxygen ($2\text{O}_2^{\bullet -} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$), was observed in these animals (Baumgart *et al.*, 2001). Further evidence for a decline in mitochondrial respiratory function included an increase in the population-wide heterogeneity of mitochondrial membrane potential, indicative of mitochondrial impairment (Chen, Chomyn & Chan, 2005). In a possibly looped signalling mechanism, damaged mitochondria cause cells to activate the mitochondrial retrograde signalling pathway (RTG) (Liu & Butow, 2006). Further evidence suggests that peroxisomal reactive oxygen species influence mitochondrial redox levels and may lead to apoptosis *via* the mitochondrial pathway (Ivashchenko *et al.*, 2011; Wang *et al.*, 2013). In this respect, peroxisomes may have a direct impact on the oxidation status of mitochondrial proteins, potentially tethering the role of LonP2 to mitochondrial metabolism.

In response to mitochondrial dysfunction, a series of stress-response transcription signals are activated, leading to an increase in peroxisome proliferation. Interestingly, chronic activation of the mitochondrial RTG, and the associated increase in peroxisome biogenesis, has been shown to be a predominant indicator for yeast longevity (Borghouts *et al.*, 2004). The metabolic product, acetyl CoA is both an output molecule of peroxisome fatty acid metabolism, and an input molecule for the mitochondrial TCA cycle. The importance of acetyl CoA in mitochondrial respiration is an obvious point of coordination between the metabolic processes of peroxisomes and mitochondria. The first evidence of communication between mitochondria and peroxisomes was the discovery of mitochondria-derived vesicles (MDVs) expressing mitochondria-anchored protein ligase (MAPL), a mitochondrial outer-membrane protein, shown to fuse with a subset of peroxisomes (Neuspiel *et al.*, 2008). In addition, peroxisomes have been shown to localize at the ER–mitochondria junction, rather than sporadically throughout the cell. This close proximity between mitochondria and peroxisomes may allow for improved synchronization between their respective metabolic processes (Cohen *et al.*, 2014).

Overall, the ability of peroxisome dysfunction to cause mitochondrial changes, and *vice versa*, indicates a strong link between these organelles. However, this link may only become obvious in either disease states or in aging, when mitochondrial or peroxisome impairment is most pronounced. Multiple studies have examined how mitochondria and peroxisomes become susceptible to protein damage from reactive oxygen species, but little work has focused on the initial triggers of organelle abnormality.

Additionally, no work of which we are aware has examined the potential link between the proteostasis machinery of mitochondria (LonP1) and peroxisomes (LonP2), or the consequences of losing either on the other organelle. So far, it has been shown that mitochondrial Lon levels are mitigated with age, and that its inducibility upon oxidative stress also declines. A similar response may occur for peroxisome-specific Lon, resulting in the peroxisome's inability to remove damaged proteins (i.e. catalase), thus tipping the balance towards the peroxisome becoming an overall oxidant producer. Lon has been viewed as a cellular defence protease to prevent the loss of organelle function due to oxidative damage, whether in the mitochondrion or the peroxisome. Therefore, it will be interesting to

explore the aging-related decline of the LonP1 and LonP2 protease isoforms and their impact on these sister organelles.

XI. CONCLUSIONS

1. The peroxisome has an important role in metabolizing fatty acids in the cell at the expense of H₂O₂ generation. Therefore, peroxisomal proteins are at an increased risk of oxidative damage due to their close proximity to oxidant production.
2. LonP2 plays an important role in removing such oxidatively modified proteins, thus preventing their accumulation, and permitting continued normal peroxisomal function.
3. It is unclear whether Lon adaptation to oxidative stress is regulated transcriptionally or translationally, however it may be that the protein aggregates that accumulate during aging may actually sequester Lon, and inhibit proteolytic activity.
4. To our knowledge, little to no evidence currently exists on the role of peroxisomal Lon during aging, highlighting a significant gap in our understanding of the peroxisome quality-control system.
5. During aging, peroxisomes exhibit a gradual decline in metabolic function. This decline may be attributed, in part, to an aging-associated loss of peroxisomal Lon expression or activity. The age-dependent increase in damaged proteins and the presence of inclusion bodies of oxidized, aggregated, and cross-linked proteins within these organelles is indicative of dysfunctional protein homeostasis.
6. Interestingly, the similarities between mitochondria and peroxisomes, including the duality of the two Lon proteases, have led to new evidence indicating potential crosstalk between these redox-regulated organelles.
7. The protease activity of Lon plays a critical role in maintaining function in both mitochondria and peroxisomes.
8. Understanding the protein quality-control mechanisms in both these entities may help to highlight how a detrimental change in one can affect the other.
9. While it is clear that mitochondria are major mediators for cellular aging, further research is needed to incorporate peroxisomes into the cellular aging processes and to understand fully the dynamic relationship between Lon and the peroxisome.

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Fig. 1.

Sources of superoxide, hydrogen peroxide, nitric oxide radical, peroxynitrite, and hydroxyl radical production in the peroxisome. Hydrogen peroxide (H_2O_2) is the main metabolic by-product of fatty acid metabolism in peroxisomes. The primary oxidase to carry out the direct generation of H_2O_2 is acyl CoA oxidase (Foerster *et al.*, 1981). In addition, superoxide ($O_2^{\bullet-}$), a by-product of the breakdown of xanthine, is scavenged by copper-zinc superoxide dismutase (CuZn SOD) and converted into H_2O_2 and O_2 (Foerster *et al.*, 1981). The majority of H_2O_2 produced in the peroxisome is degraded by catalase and glutathione peroxidase. The remainder may undergo Fenton chemistry, resulting in the formation of the hydroxyl radical ($\bullet OH$). In addition, a portion of H_2O_2 is leaked into the cytoplasm, where it is reduced by glutathione peroxidase (Halliwell & Gutteridge, 1989). Breakdown of amino acids may generate the nitric oxide radical ($NO^{\bullet}$) which reacts rapidly with $O_2^{\bullet-}$ to generate peroxynitrite ($ONOO^-$).

Fig. 2.

Peroxisomal and mitochondrial Lon protease. (A) The sequence alignment between the mitochondrial (LonP1) and peroxisomal (LonP2) forms of Lon. Identical sequences are shown in red and similar sequences are shown in grey. (B, C) Conserved domains of the P2 peroxisome-specific (B) and P1 mitochondrion-specific (C) isoforms of Lon. Both LonP1 and LonP2 contain a substrate recognition, 'N' domain to bind oxidized proteins. Both isoforms possess a classical ATPase domain containing Walker (A) and (B) motifs that have a characteristic tertiary structure commonly found in ATP-binding proteins. Lastly, both contain a proteolytic domain that relies upon a catalytically active serine residue for substrate degradation. However, unlike LonP1, which has a mitochondrial targeting sequence on the N-terminus, LonP2 relies upon the peroxisomal targeting sequence, which consists of a conserved three amino acid SRL motif.

Fig. 3.

The Lon protease and aging-related changes. (A, C) During periods of acute oxidative stress, peroxisomes and mitochondria quickly adapt to the oxidative insult by up-regulating LonP1 and LonP2. (A) In young mitochondria, the resulting increase in proteolytic activity of LonP1 ensures rapid degradation of damaged proteins and maintenance of homeostasis. (B) Young peroxisomes adapt to changes in metabolic demand by rapidly increasing the breakdown of odd-chain fatty acids for increased acetyl-CoA production, which consequently fuels mitochondrial energy production. To compensate for the excess generation of hydrogen peroxide, up-regulation of LonP2 counteracts the increased peroxisome-dependent protein damage. (B, D) Senescent cells that are exposed to chronic oxidative stress lose ability to degrade oxidized organelle proteins. (B) In aged mitochondria, the ability to rapidly up-regulate LonP1 declines, leading to the accumulation of protein aggregates, and eventual loss of mitochondrial function. (D) In peroxisomes, a similar phenomenon may occur, in which the decreased ability to elevate LonP2 levels rapidly could also lead to protein aggregation and the gradual loss of peroxisome function.