Lipid Droplets in Yeast during Stress and Aging

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The baker's yeast *Saccharomyces cerevisiae* is a valuable tool for aging research, as many aging- and disease-associated pathways such as DNA repair mechanisms, lipostasis, proteostasis, oxidative stress responses, regulated cell death, nutrient signaling, autophagy, and regulation of the cell cycle are evolutionarily conserved to a high degree. Lipid droplets (LDs) are evolutionary conserved structures that were mentioned for the first time by Van Leeuwenhoek in 1674, but their reassessment as autonomous organelles with important key roles in lipid and energy metabolism occurred many years later. LDs originate from the endoplasmic reticulum (ER). In the first step, neutral lipids are synthesized at the ER and are redirected into the bilayer, leading to an aggregation of the highly motile lipids. Emerging evidence suggests that LDs also fulfil impotant functions during aging and in protein homeostasis.

LDs autophagy mitochondria protein aggregates lipid peroxides

1. Replicative and Chronologic Lifespan

In general, in yeast cells, two forms of aging mechanisms can be distinguished, namely, replicative and chronological aging. For both, cell death terminates the lifespan, caused by the intrinsic, mitochondrial outer membrane permeabilization (MOMP)-based activation of programmed cell death (PCD)/apoptosis emerging from increased reactive oxygen species (ROS) production and genomic instability, which provokes damage to the cellular proteome, lipidome, and organelles such as mitochondria [1]. Upon nutritional stress (i.e., exhaustion of nutrients), yeast cells stop dividing and enter a stationary phase which allows survival up to several weeks depending on strain type and culture conditions [2]. This survival period in the stationary phase is termed the chronological lifespan[3] and has to be distinguished from the yeast replicative lifespan, which is measured by the number of daughter cells that can be formed from a mother cell before it stops dividing[4]. The average lifespan in the yeast background BY4741 (the most used genetic background, generally considered as the wild type) lasts 25 generations. Aged (mother) cells are larger, and reveal a slowing down of the cell cycle and a declined protein synthesis. Each daughter cell that is formed leaves a bud scar on the mother cell surface that can be observed microscopically by calcofluor-white staining [5]. It is believed that damaged proteins and organelles (e.g., mitochondria) are specifically retained by the mother cells, which explains the "rejuvenation" of daughter cells resulting from asymmetric segregation [1][6]. Representing a mitosis-based lifespan definition, replicative aging in yeast cells mimics the limited mitotic capacity of non-transformed proliferating mammalian cells types, including undifferentiated stem cells as defined first by the *Hayflick limit* [7]. On the other hand, many phenotypical

characteristic described for the chronological aging of yeast cells residing in the stationary phase share similarity with the phenotype of aged, post-mitotic cells in higher eukaryotes mainly comprising the class of terminally differentiated cell types such as cells of the central nervous system[8].

In some respects, several properties of *S. cerevisiae* render this fungal cell system a preferred aging model advantageous to human in vitro cell culture models. For instance, large numbers of cells can be monitored in comparatively short time periods under in vivo conditions in yeast. Of note, contrasting the well-conserved intracellular aging mechanisms common to both, yeast cells fail to display intercellular effects seen in multicellular organisms, such as inflammatory or systemic responses (e.g., regulated by hormones and/or the immune system), as well as other mechanisms involved in cell–cell communication [2]. However, it should not be overseen that, in vitro cell cultures, as for instance derived from mammalian tissues, are devoid of systemic, physiologic "cross-talks and feedback loops" if used as primary cell lines, and co-culturing with other cell types will reflect only part of the systemic complexity directing individual cell fate in vivo, in particular under the aspect of aging. Moreover, interpretation of experimental findings based on immortalized eukaryotic cell lines, self-evidently, is complicated due to the fundamentally altered growth control.

Both replicative and chronological lifespan in yeast can be extended by caloric restriction, which can be obtained by lowering glucose availability in the culture media (e.g., from 2% to 0.5%) [9]. In the absence of caloric restriction, chronologically aged yeast cells accumulate ethanol produced by glucose fermentation [9]. It is speculated that this counteracts the expression of β-oxidation regulatory enzymes Fox1p, Fox2p, and Fox3p (peroxisomal fatty acid β-oxidation core enzymes) leading to a decline in peroxisomal oxidation of LD-derived non-esterified "free" fatty acids that are synthesized in the ER and are stored in LDs [10][11]. In turn, non-oxidized free fatty acids will accumulate in LDs under normal nutritional conditions (i.e., 2% glucose) which promotes an inhibitory feedback loop on the ER-based synthesis of triacyclglycerols (TAG) [10]. It is hypothesized that lipid dynamic remodeling of this kind can shorten lifespan in chronologically aged yeast cells grown without caloric restriction (i.e., in the presence of 2% glucose) by three different mechanisms: (i) via necrotic cell death ensuing from the peroxisomal failure to oxidize free fatty acids, (ii) apoptosis stimulated by the accumulation of diacylglycerol and free fatty acids in the ER ("*lipoapoptosis*"), or (iii) diacylglycerol initiated protein kinase C-dependent signaling [10].

This accounts for a pivotal role of lipid dynamics in yeast aging, which is further supported by the finding that LD biogenesis in yeast is elevated in the course of replicative and chronological aging as well as under stress conditions [12][13][14]. Of special relevance, Beas et al. reported that overexpression of the *BNA2* gene encoding indoleamine 2,3-dioxygenase (*BNA2* is the yeast homolog of mammalian IDO1) leads to a 40% reduction in LD accumulation during replicative aging, which identifies *BNA2* as an important regulator of LD abundance [2]. Bna2p catalyzes the first step of NAD^+ synthesis converting tryptophan to formyl-kynurenine; hence, this finding reveals a connection between the NAD^+/kynurenine pathway and LD formation in the course of aging. It is proposed that the glycolytic flux in aging yeast cells is directed towards neutral lipid synthesis and LD generation, but Bna2p overexpression diverts the glycolytic flux from pyruvate and acetyl-CoA to the shikimate pathway (responsible for the synthesis of the amino acids phenylalanine, tyrosine, and tryptophan) and as a result lowers LD accumulation in the aged cells. Importantly, this investigation reveals that this kind of Bna2p-mediated "metabolic rewiring" in

aged yeast cells is not directly associated with longevity. Moreover, the findings indicate that LD accumulation does not cause lifespan shortening, but, conversely, exerts protection of aged cells under stress conditions, which might provide a selective growth advantage under variable environmental conditions [14].

2. Lipid Droplets and Stress Adaptation

This concept is supported by another study that substantiates the role of LDs as key players in cellular stress adaption. The yeast cell growth rate declines when phosphatidylcholine biosynthesis is deficient, which changes the cellular phospholipid content and causes ER stress, alterations in ER morphology, and enhanced LD formation. In this case, an excess of phospholipids is converted to TAG by the acyltransferases Lro1p and Dga1p, which is immediately sequestered by LDs. This LD-generating process allows yeast cells to rebalance the pool of freely available phospholipids as an indispensable prerequisite for organelle morphology retrieval and cell growth [15]. Besides this pathway of ER-based regulation of lipid homeostasis yielding LD formation in yeast, ER stress arising from lipid imbalance is also at risk of activating the unfolded protein response (UPR). In most model organisms, it is shown that the UPR protects cells from the detrimental effects of proteotoxicity and is of great importance for the aging process [16]. Therefore, it is not surprising that all interventions that increase the activity of the UPR clearly extend the replicative lifespan of yeast cells [17].

The UPR provides cellular maintenance by specific handling of accumulated misfolded protein as well as facing lipid bilayer stress in the ER. Besides ER expansion, UPR signaling comprises the activity of a number of UPR-related gene products which direct the response either towards re-established homeostasis or, if not adequately facing a prolonged stress condition, participate in apoptosis onset (for a review see [18]). Essential to a successful outcome is the proper elimination of the ER stressor. A misfolded protein that initially accumulates inside the ER is translocated to the cytosol, where it is polyubiquitinylated by ubiquitin-conjugating enzymes residing at the cytosolic ER surface, the polyubiquitination serving as tag for proteasomal degradation [19]. However, lipid bilayer stress may also stimulate UPR in the ER (UPR^{ER}) [20][21] which converges with the UPR triggered by the misfolded protein at the central UPR effector Ire1p (inositol-requiring enzyme 1) [22]. Interestingly, in mouse hepatocytes, ER stress stimulates Ire-1 and downstream targets such as DGAT2 (diacylglycerol-acyltransferase 2) [23], with DGAT2 (as well as DGAT1) being essential to LD biosynthesis [24]. Referring to this and findings demonstrating ROS-triggered LD biogenesis and antioxidant properties of LDs in *Drosophila* [25], Walther et al. suggested that the Ire1p/DGAT2-stimulated LD formation could antagonize phospholipid oxidation via LD-mediated ROS scavenging [26]. This also underlines the importance of LDs for the aging process as the accumulation of ROS is one of the most prominent features at the terminal lifespan [27].

Moreover, linking LD formation to UPR-dependent responses in yeast, it was shown that ER-derived LDs can be associated with polyubiquitinylated proteins and also can be enriched in Kar2p, an ER chaperone involved in protein folding [15]. This led to the conclusion that un-/misfolded proteins accumulating in the ER are cleared from this compartment via LD formation, the released LDs being degraded terminally in the yeast vacuole by a process resembling microautophagy, termed *microlipophagy*. It has to be emphasized that this process differs from starvation-induced macroautophagy, since it does not involve the ATG-dependent initiation of

(macro)autophagosomes, but instead requires ESCRT components (endosomal sorting complexes required for transport) and the ER-stress response factor Esm1 (ER stress-induced microlipophagy protein 1) [15][28]. Both stimulation of autophagy and ESCRT components extend the chronological lifespan of yeast cells [29]. A further study also clearly links LDs with the removal of aggregates consisting of misfolded proteins. Moldavski et al. showed that so-called inclusion bodies (IBs) are functionally and spatially linked to LDs [30]. Upon stress induction, unfolded or misfolded proteins, which cannot be cleared by the quality control machinery (e.g., due to quality control system overload or failure) aggregate and form inclusion bodies. In an extensive screening approach, Moldavski and co-workers identified thirteen proteins that are crucial for an efficient and rapid IB clearance. Interestingly one of these proteins, namely, Iml2p, strongly associates with LDs via interaction with the LD-resident proteins Pet10p and Pdr16p. It should be noted that Pet10p is the yeast perilipin, which is the only perilipin discovered so far in *S. cerevisiae* [31]. This interaction especially happens during cell stress, when Iml2 is exclusively located in inclusion bodies. Under such stress conditions, a physical tethering between LDs and IBs can be monitored, the physical binding of LDs to IBs allowing aggregate clearance. Iml2 is essential to this clearance process, which is considered to be mediated by a soluble sterol derivate effusing from LDs via interaction with Iml2 [30]. These findings highlight the role of LD-dependent protein aggregate clearance during aging, which is still poorly studied considering the substantial influence of cellular aging on both protein misfolding and protein toxicity [32]. Besides Pet10p and Pdr16p, another LD-resident protein, Ubx2p, could be involved in protein homeostasis [33][34]. This UBX-domain-containing protein resides in the ER but relocates to LDs upon their formation. UBX2 deletion leads to abnormal cellular numbers of LDs of reduced size and TAG content [35]. At the same time, this protein is also involved in protein homeostasis, in that Ubx2p recruits Cdc48p and both interact to support ER-associated protein degradation [36].

3. Lipid Droplets: Guardians of Mitochondrial Integrity

In line with these findings, the researchers' research also indicates a linkage between LD formation and the removal of un-/misfolded, potentially harmful proteins in yeast and mammalian cells. Moreover, the researchers demonstrated that several, proteins including yeast Mmi1p and Erg6p, as well as mammalian BAX, BCL-X_L, and TCTP, can be transferred from mitochondria to LDs via a V-shaped domain consisting of two alpha helices [13]. The V-domain shows a higher binding affinity to the LD membrane than to the outer membrane of mitochondria, which explains the directed transfer [37][13]. Among different possible contexts, this directed protein shuttling is of special relevance to the control of PCD/apoptosis onset mediated by the pro-apoptotic bcl-2 family members BAX and BAK. It has to be clearly stated that apoptosis and aging are deeply interconnected in yeast as well as in mammalian cells [38][39][40], and LDs seem to be involved in both processes. In most cells, apoptosis is increased with the dysregulation of the apoptotic program, enhancing the risk of cancer and cellular senescence [38]. Induced by a plethora of potential intrinsic cell death stimuli, BAK and BAX translocate to the mitochondrial outer membrane where they form the mitochondrial-apoptosis-induced channel (MAC), resulting in MOMP. As a consequence, the release of cytochrome C from the mitochondrial intermembrane space to the cytosol promotes apoptosome formation, caspase 9 activation, and the terminal progression of intrinsic apoptotic signaling [41][42]. Particularly under cellular stress conditions, the anti-apoptotic mammalian bcl-2 family member BCL-X_L, as well as TCTP, also

translocate to mitochondria but suppress MOMP by antagonizing BAX/BAK oligomerization [43]. In a similar way, Mmi1p, the yeast homolog of TCTP, also participates in the apoptotic machinery, with the deletion of Mmi1p leading to an extended replicative lifespan [44][45]. From this, it can be speculated that under a given stress condition both pro-and anti-apoptotic proteins locate to the outer mitochondrial membrane, continuously challenging MOMP onset. Such potentially harmful mitochondria may be specifically removed by mitophagy, a selective mode of macroautophagy [46].

Emphasizing its specificity for mitochondria, mitophagy in yeast depends on the activity of Uth1p which localizes to the outer mitochondrial membrane and is required for mitophagy, but not for starvation-induced bulk macroautophagy [47]. As previously stated, mitophagy is crucial to cellular maintenance under stress conditions by eliminating dysfunctional mitochondria, which is complicated by the fact that stress-induced macroautophagy/mitophagy may confer cell protection in one stress context, but conversely can contribute to cell death (i.e., autophagic cell death) under different stress conditions [48][49]. Besides BAX/BAK-mediated MAC, excessive ROS generation can lead to the formation of another mitochondrial permeability transition pore (mPT). The mPT pore complex is composed of VDAC (voltage-dependent anion channel) in the outer membrane, cyclophilin D in the matrix, and ANT (adenine-nucleotide translocator) in the inner membrane, and opening of the mPT, leading to mitochondrial swelling in many cases followed by necrotic cell death [41]. However, mPT opening may also initiate BAX/BAK-mediated MAC/MOMP; to a large degree, the outcome of this depends on cellular ATP availability comprising cell death by either necrosis or apoptosis, which also may involve enhanced autophagy/mitophagy [50]. Reminiscent of this, for yeast mutants lacking Mdm38p, a K^+/H^+ exchange-regulator residing in the inner mitochondrial membrane has been reported, which develops a drop of the mitochondrial membrane potential that is accompanied by mitochondrial swelling, deterioration in mitochondrial morphology, and vacuolar changes indicative of mitophagy [51].

LDs and mitochondrial homeostasis. It has to be emphasized that mitophagy does not necessarily need to be associated with conditions of enhanced stress, but represents an important physiological regulator of mitochondrial homeostasis. In postmitotic mammalian cells, mitophagy is crucial to the control of mitochondria numbers under normal physiologic conditions, as well as the removal of dysfunctional mitochondria in starving cells [52]. In this context, the age-dependent decline in autophagic activity seen in mammalian cells [53] deserves particular attention since it may weaken the cellular clearance from dysfunctional mitochondria. Hence, it is conceivable that additional mechanisms may support cellular maintenance in aged cells by protecting them from the onset of premature cell death via apoptosis caused by “stressed” mitochondria. The above-mentioned V-domain-based shuttling of Mmi1p, BAX, and other MOMP agonists to LDs could fulfill this task considering that LDs closely locating to mitochondria are capable of sequestering pro-apoptotic proteins, and as a result antagonize the onset of MOMP-dependent apoptosis [13]. Terminally, such potentially harmful BAX-enriched LDs will be degraded in the yeast vacuole. Indeed, in yeast cells, the researchers demonstrated the V-domain/LD based protection from apoptosis, but, conversely, human HepG2 hepatoma cells treated with the apoptosis inducer staurosporine revealed a substantially elevated susceptibility for apoptosis upon the V-domain-mediated translocation of BAX and Bcl-X_L from mitochondria to LDs [13]. Explaining this, the researchers observed the translocation of pro-apoptotic Bcl-X_S to the mitochondria in staurosporine-treated HepG2 cells. Opposing anti-apoptotic Bcl-X_L (i.e., the long

isoform), Bcl-X_S (the short isoform) is a pro-apoptotic splice variant of Bcl-X, the Bcl-X_L/Bcl-X_S ratio being defined by the cell type and cell differentiation, which are dependent (e.g., non-transformed versus tumor cells) by numerous determinants including transcription factors and cytokine signaling [54]. Importantly, the researchers found Bcl-X_S to be devoid of a V-domain [13], which may explain the enhanced onset of apoptosis in staurosporine-treated HepG2 cells. Taken together, this emphasizes the dependence of V-domain/LD-based MOMP inhibition on additional regulatory elements, in particular in mammalian cells, rendering the mechanisms cell-type-specific. Ongoing research demonstrates that the V-domain-based mitochondria to LD shuttling is not restricted to the MOMP/apoptotic settings as presented above, but seem to play a more general role in cellular stress responses, as indicated by the marked protein accumulation by LDs seen during replicative aging and in the initiation of proteotoxic stress [30]. In good correspondence with this, Garcia et al. reported a substantial remodeling of the LD proteome in the presence of ER stress [28].

LDs and DNA repair. Moreover, certain yeast haploid *rad*Δ (radiation damage) deletion strains also show altered lipid storage patterns and a reduced lifespan [55]. *RAD* genes are involved in DNA repair (e.g., nucleotide/base excision repair) which is evolutionary highly conserved. In yeast, repair of double-strand breaks via homologous recombination is accomplished by the MRX complex composed of the RAD gene products Mre11p, Rad50p, and Xrs2p [55]. Deletion of one of these three genes leads to higher levels of TAGs and steryl esters, as well as characteristic changes in lipid-metabolism-associated gene expression. The down-regulated expression of lipolysis-associated genes (e.g., *TGL3*) at an augmented expression of genes involved in lipid synthesis (*LPP1*, *SLC1*), together with high TAG levels, may readily explain the observed increase in LD numbers in *rad*Δ mutants. This is accompanied by chronological lifespan shortening and pronounced mitochondrial fragmentation indicative of premature aging. However, as normally aged cells also displayed higher LD numbers, it is not clear whether the increased LD abundance simply reflects the premature aging process of *rad*Δ mutants or, vice versa, LD accumulation is causal to chronological lifespan shortening [55]. Concerning the considerations made above regarding a cytoprotective role of LD accumulation in stress adaptation, it would be interesting to study the extent to which the severity of the phenotype is altered in *rad*Δ mutants devoid of LDs.

These findings account for a functional triad between LD abundance, mitochondrial integrity, and lifespan in yeast, which is addressed by stress conditions as well as the general aging process. Following the common view of mitochondrial dysfunction as a hallmark of aging [56], the causal relationship between mitochondrial fragmentation and chronological lifespan shortening, as seen in yeast exposed to high glucose levels [57], represents a reliable means of monitoring the aging process already at early stages [6]. Extending this, and in line with the functional triad envisaged above, LD accumulation in the same way may be considered a complementary biomarker for both premature and normal aging, as suggested by Kanagavijayan et al. [55]. With respect to this, determinants of LD synthesis such as the cellular levels of TAG and sterols are of prevalent meaning to the whole context. In yeast, two enzymes are regarded as the main actors in TAG production, Lro1p (lecithin cholesterol acyl transferase related open reading frame) and Dga1p (diacylglycerol acyltransferase 1) [58]. For sterols, the acyl-CoA:sterol acyltransferase Are1p and its paralog Are2p are the main sterol esterification tools in yeast [59]. Together these enzymes regulate the TAG:sterol balance to a ratio of 1:1 in yeast LDs [60]. The researchers showed that the

simultaneous overexpression of all Lro1p and Dga1p enzymes, as well as Are1p and Are2p (single overexpression of each enzyme), yields an extension of both the chronological and replicative lifespan of *S. cerevisiae* [12].

This stimulation of LD synthesis resulted in less mitochondrial fragmentation and reduced production of ROS, which normally increase during aging. Contrarily, a mutant strain devoid of LDs (*lro1*Δ, *dga1*Δ, *are1*Δ, *are2*Δ) suffers from a significantly shortened chronological lifespan and experiences a burst of ROS production already within one day of cultivation, suggesting severe mitochondrial defects [12]. According to the assumptions made above, mitochondrial functionality is an essential target for age-related cellular decline, and it seems plausible that "fitter" mitochondria with maintained integrity will be beneficial to a prolonged lifespan.

Furthermore, mitochondria have been identified recently to assist the cytosolic proteasome in protein degradation, especially during stress conditions. Underlying this is a process termed MAGIC (mitochondria as guardian in cytosol), which mediates the import of misfolded proteins into mitochondria where protein degradation is performed by the matrix-resident protease Pim1p [61]. Yeast Pim1p is an ATP-dependent mitochondrial Lon protease required for the degradation of misfolded mitochondrial proteins, which is essential to mitochondrial function and maintenance [62]. With aging, the activity of Pim1p ceases, and *pim1*Δ yeast mutants lacking Pim1p are marked by a shortened replicative lifespan and show reduced proteasomal activity connected with an increased accumulation of oxidized and aggregated proteins in the cytosol [62]. In line with this, the researchers also observed a significant shortening of both the replicative and chronological lifespan in *pim1*Δ cells [12]. In addition, the mitochondria of *pim1*Δ cells showed an abnormal morphology accompanied by enhanced ROS production, enlarged LDs, and a delay in the cell cycle. This premature aging phenotype of *pim1*Δ cells could be reversed partially by overexpressing Lro1p [12]. This suggests an important role of LDs in the detoxification/sequestration of the non-degraded, oxidized protein, which underlines the beneficial role of LDs in cell integrity by assisting cellular clearance from protein aggregates.

It is noteworthy that the advantageous effects of LDs cannot be seen solely as a function of LD abundance, but also as a function of LD size and morphology. This is indicated by the observation that *pim1*Δ cells treated with oleate and olive oil showed a reduced lifespan, revealing a drop in the LD number, with the LDs themselves becoming massively enlarged. In contrast, overexpression of Lro1p/Dga1p on the *pim1*Δ restored the strains' normal replicative lifespan but led to numerous but smaller LDs [12]. Furthermore, cells of the mutant strain *sei1*Δ (SEI, yeast seipin controls LD size, number, and morphology) show a reduced replicative lifespan but no significant differences to wild-type cells in overall neutral lipid levels. Different from the wild type, the LDs of *sei1*Δ cells are smaller and show LD clustering. Hence, LD size and distribution also obviously play an important role in the effect of LDs on lifespan in yeast [12]. In this context, it is worth mentioning that, in yeast cells, life-prolonging interventions such as caloric restriction [63], rapamycin treatment (blockage of the TOR kinase; for details see the following chapters) [64], and sirtuin inhibition [65] induce the formation of LDs [66][67][68]. In fact, in the experiments the researchers observed a modest 1.15–1.20-fold increase in the LD content upon treatment of BY4741 cells with 10 µM resveratrol (unpublished data).

Similar research was performed in the filamentous ascomycete *Podospora anserina* [10]. Here, deletion of the gene *PaATG24*, encoding a sorting nexin, resulted in impaired autophagy, a reduced vacuolar size, lowered growth rate, and lifespan shortening. Addition of oleic acid stimulates LD production and gives rise to an extended lifespan in wild-type as well as *PaATG24*Δ cells, revealing a restored autophagic flux and normal vacuolar phenotype. Interestingly, oleic acid treatment also diminishes ROS production in *Podospora* as result of a bypass of complex I and II of the mitochondrial electron transport chain [69].

Taken together, the research on LDs in yeast provides substantial evidence that LDs, apart from their well-defined role in lipid metabolism, can also serve as hitherto underrated “detoxification organelles”, which in orchestration with other processes involved in cellular maintenance, in particular the autophagic flux, serve as lifespan determinants.

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