CELLULAR AGEING: DECIPHERING THE ROLE OF PEROXISOMES IN YEAST

A Thesis

submitted in partial fulfilment of the requirements for the degree of

Doctorate of Philosophy

by

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Dedicated to the society

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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DECLARATION

I do hereby declare that the research findings in this thesis is the result of research work carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of **Dr. Shirisha Nagotu** and **Dr. Priyadarshi Satpati.**

I also declare that the contents of this thesis have not been the basis for award of any other degree, diploma, fellowship or any other similar title of any other University or Institution.

As per general norms of reporting research findings, due acknowledgments have been made, whenever findings of other researchers have been cited in this thesis.

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CERTIFICATE

This is to certify that the work described in the thesis entitled **"Cellular Ageing: Deciphering the role of peroxisomes in yeast**" is an authentic record of the results obtained from the research work carried out by **Rachayeeta Deb** in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India, under my supervision and this work in part or as a whole has not been submitted elsewhere for the award of any other degree.

Dr. Shirisha Nagotu

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"If we knew what it was we were doing, it would not be called research, would it?" – Albert Einstein. That was my situation for almost two years into my PhD, at least in terms of my PhD objectives. I thought it was a shot in the dark but now as I am on the verge of submitting this thesis. I am delighted to see that the risk paid off. My PhD journey at IIT Guwahati has been a truly life-changing experience. While writing this acknowledgment, I feel as if I'm reliving the last six years filled with beautiful memories, priceless moments of joy and tears, and sleepless hours of conducting experiments and publishing papers. All of this would not have been possible without the love, support, and encouragement of many individuals. I'm delighted to express my heartfelt gratitude to everyone who, knowingly or unknowingly, helped and supported me over this memorable trip in my life.

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ABSTRACT

Ageing is a necessary evil experienced by all living organisms. It results in a progressive decline in the normal functioning of cells and eventually culminates with the death of the organism. The irreversible damage due to ageing is attributed to many factors and leads to accumulation of molecular and cellular damage over time. Nine biological processes that universally regulate the ageing process in all studied organisms termed as the hallmarks of ageing are now identified. Research on the cellular causes of ageing will provide a foundation for not only understanding ageing mechanisms but also generating novel anti-ageing treatments to improve human health. Various model organisms have been instrumental in our current understanding of the complex mechanism of ageing.

Budding yeast has emerged as an appropriate model organism for ageing research and is used to study two aspects of ageing namely replicative and chronological ageing. Studies in yeast have enabled our understanding of the age-associated fundamental processes conserved in all eukaryotic species. One such conserved eukaryotic feature is compartmentalization of cellular functions into membrane bound structures called organelles. Various studies have emphasized the function of subcellular organelles in the regulation of yeast ageing. The relationship between ageing and organelle function is bidirectional: ageing impairs the optimum functioning of the organelles and dysfunctional organelles result in accelerated ageing. One such organelle that is ubiquitously present in all eukaryotic cells is the peroxisome. β-oxidation of fatty acids and metabolism of reactive oxygen species are the conserved functions of peroxisomes. The proliferation of this organelle via division of pre-existing organelles and *de novo* formation from the endoplasmic reticulum is extensively studied in various model organisms. Defects in the biogenesis and function of peroxisomes are also associated with ageing and age-related disorders. However, the molecular mechanisms that link peroxisomes with ageing are still under investigation. Our study sought to comprehend the

alterations in peroxisome dynamics during replicative and chronological ageing to understand their role in yeast ageing.

The changes in peroxisome number and morphology upon replicative and chronological ageing were investigated in wild type (WT) cells cultured in peroxisome-inducing oleic acid (OA) and non-inducing glucose (YND) media conditions. Fluorescence microscopy and quantitative analysis revealed an increase in peroxisome number in the early replicatively aged cells in both media conditions. However, the increase was higher in replicatively aged cells of OA as compared to the YND medium. We further analysed the chronological lifespan (CLS) of the cells in calorie restriction (CR) and OA media conditions. The cells displayed increased mean and maximal CLS in OA medium. Microscopic analysis also revealed an increase in the number of peroxisomes in OA-cultured cells when compared to YND on the 0^{th} day of chronological ageing experiment. Interestingly, we also observed a change of phenotype of the fluorescence marker used to tag peroxisomes from punctate to cytosolic with the progression of ageing. Our quantification data revealed that OA grown cells displayed delayed change in the phenotype from punctate to cytosolic. This suggests an intricate link between the morphology/protein import of peroxisomes and chronological ageing.

The increase in peroxisome number in replicatively aged cells prompted us to look at three possibilities for this. Firstly, if this increase is due to increased peroxisome division in old cells. Second, if this is due to retention of non-functional peroxisomes by mother cells and third, if this is in response to dysfunction of mitochondria. To address the above possibilities, we investigated replicative ageing of *pex11*, *pex25* and *pex11pex25* cells which lack proteins involved in peroxisome fission. Our quantitative analysis revealed that aged cells lacking Pex11 showed a significant reduction in peroxisome number as compared to WT even in peroxisome-inducing OA medium, thereby suggesting a role of this protein in the regulation

of peroxisome number in replicative ageing. Further we assessed ROS accumulation and catalase activity in the replicatively aged cells. *pex11* and *pex11pex25* cells in OA media showed an interesting reduction in ROS levels and increase in catalase activity, despite having significantly reduced number of peroxisomes. This suggests that these organelles are not hampered in their function. Microscopy and flow cytometry analysis revealed reduced mitochondrial function in the aged cells. However, the increase in peroxisome number in the replicatively aged cells may not be solely due to mitochondrial dysfunction and needs further investigation. Overall, our findings clearly point towards the role of fission protein Pex11 in the alteration of peroxisome dynamics in replicatively aged cells.

To understand whether peroxisome abundance has any effect on yeast chronological ageing, we analysed the CLS of cells deleted for *PEX11*, *PEX25*, *PEX27* and the double deletion *pex11pex25* in CR and OA media conditions. Interestingly, reduced peroxisome number in the deletion cells had no influence on their CLS in CR medium. However, in OA medium, *pex11*, *pex25*, and *pex11pex25* cells displayed a significant decrease in the mean and maximal CLS as compared to WT cells. This is consistent with the reduced number of peroxisomes in these cells in OA medium. *pex27* cells with similar peroxisome number and phenotype as WT also displayed similar CLS as WT. *pex11pex25* cells with the least mean and maximal CLS in OA medium, also exhibited higher ROS levels and reduced catalase activity. Interestingly, mutant cells with reduced peroxisome number also depicted enhanced FFA accumulation and increased number of lipid droplets.

In conclusion, our study offers some interesting perspectives on the relationship between peroxisomes and yeast ageing and reveals a previously uncharacterized role of the fission proteins in both replicative and chronological ageing of yeast. However, if the effect on ageing observed in our study, can be attributed solely to the altered peroxisome number and function needs further investigation. In line with this, we also report altered mitochondrial

function and lipid droplet number upon ageing. Future work needs to look into organelle communication and their shared functions that may be affected due to the lack of fission proteins.

LIST OF ABBREVIATIONS

Abbreviations

AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis of Variance
Aβ	Amyloid β
$CaCl_2$	Calcium Chloride
CFU	Colony Forming Unit
CLS	Chronological Lifespan
CR	Calorie Restriction
EE	Ergosteryl Esters
EMC	Enriched Mother Cell
ER	Endoplasmic Reticulum
ERCs	Extrachromosomal rDNA Circles
FFA	Free Fatty Acid
H_2-DCFDA	2',7'-Dichlorodihydrofluorescein Diacetate
H_2O_2	Hydrogen Peroxide
HD	Huntington's disease
HGPS	Hutchinson Gilford Progeria Syndrome
HPLC	High-Performance Liquid Chromatography
LB	Luria Bertani
LD	Lipid Droplet
LiAc	Lithium Acetate
MAD	Miniature-chemostat Ageing Device

MEP	Mother Enrichment Programme
OA	Oleic Acid
OMC	Old Mother Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PI	Propidium Iodide
RLS	Replicative Lifespan
ROS	Reactive Oxygen Species
RTG	Retrograde
TAG	Triacylglycerols
TMRE	Tetramethylrhodamine Ethyl Ester
VLCFAs	Very Long Chain Fatty Acids
WT	Wild Type
YMC	Young Mother Cell
YPD	Yeast extract-Peptone-Dextrose
YPDG	Yeast extract-Peptone-Dextrose-Glycerol

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Chapter 1

Introduction
&
Review of Literature

Abstract

Ageing refers to the deterioration of biological processes accompanied by a gradual decrease in the normal functioning of a cell, ultimately resulting in cell death. Nine cellular processes have been identified as the hallmarks of ageing. Classical model systems like mice, worms, flies, yeast, *etc*., have helped in the identification of several conserved genes and metabolic pathways that regulate the ageing process. Over the last few decades, the budding yeast *Saccharomyces cerevisiae* has become a desirable model organism for ageing research because of its short lifespan, thorough knowledge at the genetic and molecular level, and easy genetic modifications. Replicative and chronological ageing are two types of ageing in budding yeast. Various genetic factors associated with organelles that regulate ageing have been identified in yeast. Peroxisome is one such organelle that performs a wide variety of cell and tissue-specific functions. Defects in peroxisome functions have been linked to ageing and severe age-related disorders. However, the underlying molecular mechanisms are still under investigation. This chapter provides a comprehensive overview of the hallmarks of ageing, age-associated alterations in various organelles, the role of peroxisomes in the regulation of yeast ageing and the proteins involved in regulating the number and size of peroxisomes in yeast.

This chapter is a part of

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1.1 Cellular and molecular hallmarks of ageing

Ageing is a global phenomenon characterized by a time-dependent deterioration in the physiological functioning of a cell that leads to cell death (Banerjee et al. 2020). Extensive research over years has identified genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication as the nine hallmarks of ageing, which are conserved in all studied organisms and determine the cellular phenotype upon ageing (Fig. 1.1) (Aunan et al. 2016; López-Otín et al. 2013). These hallmarks are further grouped as primary, antagonistic, and integrative hallmarks. López-Otín and colleagues set out the criteria to be fulfilled by each hallmark: (i) it should appear during normal ageing, (ii) its experimental exacerbation should accelerate the process of ageing, and (iii) its amelioration should slow down the ageing process and thereby lengthen normal lifespan (López-Otín et al. 2013).

The primary hallmarks, genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis, are also described as damage-inducing hallmarks. ***Genomic instability*** refers to various alterations in the DNA that change the information content of the genome (Fig. 1.1). Exogenous and endogenous threats challenge the integrity of DNA (Chatterjee and Walker 2017). Organisms have developed specific mechanisms, such as a complex network of DNA repair mechanisms, maintenance of proper length of functional telomeres, *etc.*, to counteract this accumulation of damaged DNA (Chatterjee and Walker 2017). Excessive DNA damage or insufficient DNA repair is observed upon ageing (Vijg and Montagna 2017). Several human progeroid diseases, including Werner syndrome, Bloom syndrome, Cockayne syndrome, and Seckel syndrome, have been linked to abnormalities in DNA repair processes that accelerate ageing (Burla et al. 2018). Genome instability due to altered nuclear architecture is observed in age-associated disease conditions such as Hutchinson Gilford Progeria syndrome

(HGPS) (Gonzalo and Kreienkamp 2015; Zhang and Cao 2017). Interestingly, this is also a characteristic feature of most types of cancer (Aguilera and Gómez-González 2008; Killcoyne et al. 2021; Negrini et al. 2010).

Telomere attrition refers to the progressive shortening of telomeres with each mitotic cell division and is associated with several age-related disorders (Fig. 1.1) (Chakravarti et al. 2021; Vaiserman and Krasnienkov 2020). Studies have reported that telomere dysfunction accelerates ageing in mice and humans (Calado and Dumitriu 2013; Rossiello et al. 2022). Interestingly, increased telomerase expression can delay ageing in mice (Bernardes de Jesus et al. 2012; Muñoz-Lorente et al. 2019). In line with this, telomere deficiency in humans results in the development of diseases such as pulmonary fibrosis, aplastic anaemia, *etc.* (Arias-Salgado et al. 2019). ***Epigenetic alterations*** change the structure of chromatin without modifying the DNA sequence, which affects gene expression and leads to accelerated ageing (Fig. 1.1) (Gonzalo 2010; Pagiatakis et al. 2021). DNA methylation, ATP-dependent chromatin remodelling by DNA and histone modification and non-coding RNAs are some such alterations (Gonzalo 2010; Pagiatakis et al. 2021). Interestingly, altered epigenetic changes have also been observed in progerias (Arancio et al. 2014). The ***proteostasis*** network includes various mechanisms that regulate proteome homeostasis in the cell. This network involves chaperone-mediated protein folding, which protects the cells from accumulating misfolded proteins and clearance of damaged proteins via proteasomal or lysosomal degradation (Hartl et al. 2011; Meller and Shalgi 2021). The aggregation of dysfunctional (unfolded or misfolded) proteins in the cells due to a gradual decline in the maintenance of the proteostasis network leads to the development of several age-related neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Fig. 1.1) (Haigis and Yankner 2010; Höhn et al. 2020; Taylor and Dillin 2011).

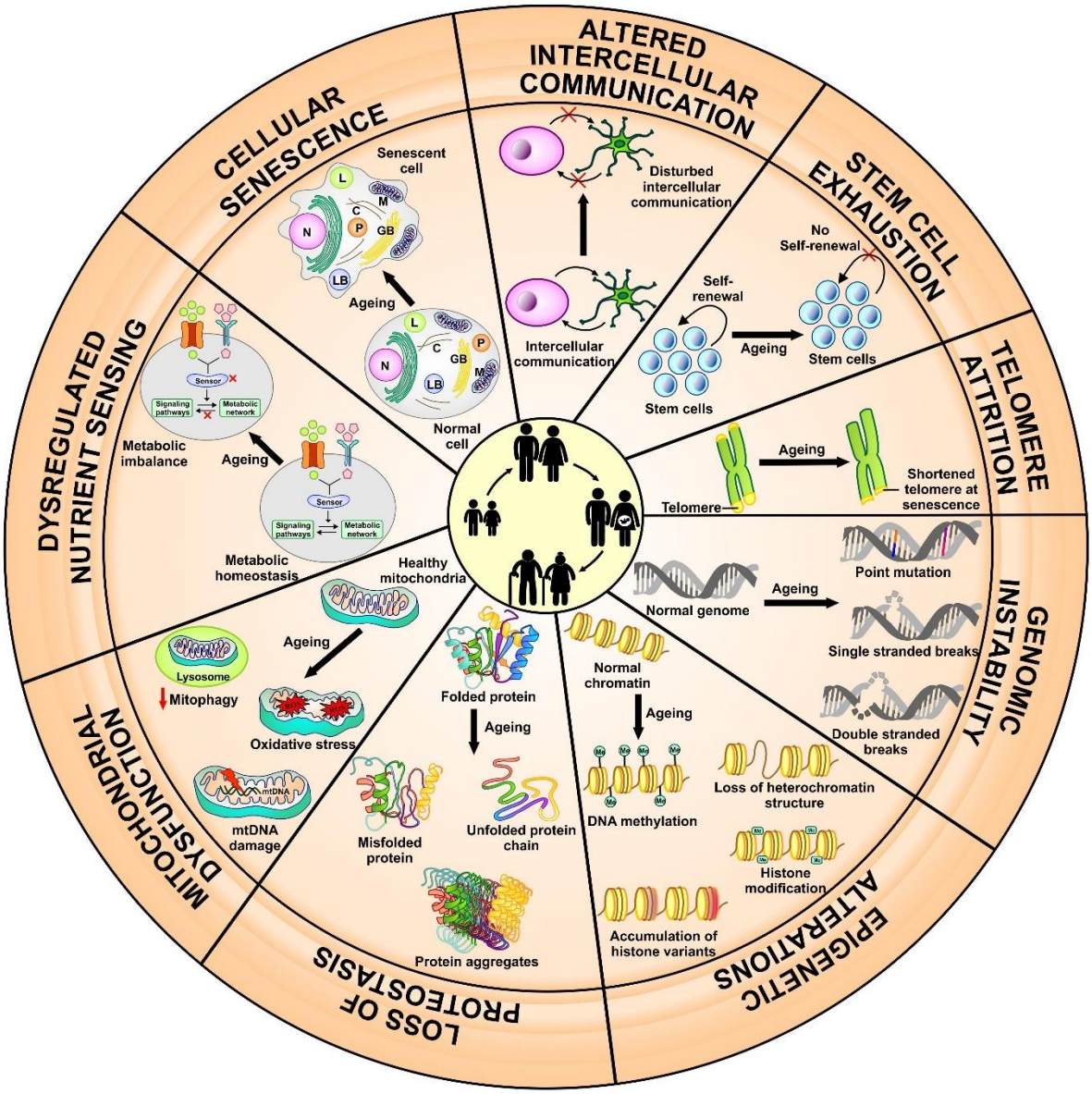

Fig. 1.1: The nine hallmarks of ageing.
The schematic representation depicts the nine hallmarks of ageing. These hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (López-Otín et al. 2013).

Dysregulated nutrient-sensing, mitochondrial dysfunction, and cellular senescence are antagonistic hallmarks, as they produce opposite consequences based on their intensity upon ageing (Fig. 1.1). These factors provide beneficial effects at low or moderate levels and protect the organism from damage, whereas they result in detrimental effects at high or chronic levels (López-Otín et al. 2013). Metabolic homeostasis in the cells is maintained with the help of several signalling pathways which sense the presence of nutrients and growth factors (Bettedi

and Foukas 2017). Alterations in pathways like insulin growth factor (IGF; sense glucose), mechanistic target of rapamycin (mTOR; sense high amino acid concentration), adenosine monophosphate kinase (AMPK; sense low energy) are associated with ***dysregulated nutrient sensing*** (Efeyan et al. 2015; Micó et al. 2017). Altered expression and function of an interesting family of proteins called sirtuins which act as nutrient sensors, upon ageing is also reported (Aiello et al. 2017; Micó et al. 2017). Calorie restriction (CR) and decreased nutrient sensing are now well accepted as consistent and effective methods to extend longevity in different species (Bettedi and Foukas 2017). Along the same lines, treatment of cells with rapamycin that mimics reduced nutrient availability results in lifespan extension (Aiello et al. 2017).

Mitochondrial dysfunction is one of the crucial hallmarks of ageing. Damaged mitochondria lead to an increase in the levels of intracellular reactive oxygen species (ROS) and result in oxidative stress (Guo et al. 2013; Jara et al. 2019). This oxidative stress causes damage to the nucleic acids, proteins, and lipids, further resulting in ageing and cell death (Haas 2019; Payne and Chinnery 2015; Seo et al. 2010). Dysfunctional mitochondria can also affect apoptotic signaling, cellular signaling, inter-organelle crosstalk, *etc* (Mammucari and Rizzuto 2010). Reduced biogenesis and decreased mitophagy (clearance of mitochondria) also contribute to the ageing process (Chen et al. 2020; Gonzalez-Freire et al. 2015). An increase in the accumulation of senescent cells is one of the primary markers of ageing (López-Otín et al. 2013). ***Cellular senescence*** prevents the proliferation of damaged cells in young individuals. Increased damage and reduced clearance resulting in the accumulation of senescent cells contribute to ageing (van Deursen 2014).

Integrative hallmarks such as stem cell exhaustion and altered intercellular communication occur due to accumulated damage by other hallmarks and directly contribute to the clinical phenotype of ageing (Aunan et al. 2016; López-Otín et al. 2013). ***Exhaustion of stem cells*** *i.e.*, reduction in the number and function of stem cells due to a decline in their regenerative

potential, is considered as one of the essential characteristics of ageing (Ren et al. 2017). A decrease in the differentiation of hematopoietic stem cells (HSCs) leads to reduced generation of adaptive immune cells resulting in anaemia and myeloid malignancies in aged individuals (Fig. 1.1) (Li et al. 2014; Sestier 2015; Yamashita et al. 2020). Stem cell exhaustion is commonly observed in several age-related disorders like HGPS, Werner syndrome, and PD (Liu et al. 2012; Liu and Rando 2011; Zhang et al. 2015). Interestingly, stem cell rejuvenation has been shown to reverse the ageing phenotype (Honoki 2017; Ullah and Sun 2018). Beyond the changes within an individual cell, ageing is also controlled by ***alterations in intercellular communication*** (Fig. 1.1). This alteration leads to an increase in inflammatory reactions upon ageing which affects the mechanical and functional properties of cells and tissues (López-Otín et al. 2013). Interestingly long-term administration of anti-inflammatory agents such as aspirin also increased longevity in mice and other models (Liu 2022; Strong et al. 2008). Restoring defective intercellular communication using dietary restriction approaches resulted in the extension of a healthy lifespan (Ma et al. 2020; Okawa et al. 2020).

1.2 Budding yeast: An apt model for ageing studies

Ageing is an inevitable biological process; every organism undergoes phenotypic and functional changes associated with ageing. The use of diverse model systems such as *Caenorhabditis elegans*, *Drosophila melanogaster*, mouse models, yeast, zebrafish, primates, *etc.*, has contributed to the present understanding of the pathways that control ageing (Brunet 2020). Despite the differences in the maximum lifespan, the shape of the lifespan or mortality curves is similar and conserved across all the studied model organisms (Crane et al. 2020; Mitchell et al. 2015; Sinclair et al. 1998). Among these numerous models, the budding yeast *Saccharomyces cerevisiae* has emerged as a suitable and most extensively used model for ageing research over the last few years. The yeast cell is 5-10 µm in diameter and belongs to the division of Ascomycota, class Saccharomycetes, family Saccharomycetaceae and genus

Saccharomyces (Sudiyani et al. 2021). The entire genome sequence of *S. cerevisiae* was published in 1996 and has been regularly updated in the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org) (Goffeau et al. 1996). This organism consists of 16 chromosomes (2n=16), 12068 kb DNA, and approximately 6000 genes (Goffeau et al. 1996).

S. cerevisiae offers unique advantages over other model systems owing to its non-pathogenicity, ease of cultivation, and faster growth rate (Sudiyani et al. 2021). Complete knowledge of the yeast genome also allows easy genetic isolation and manipulation (Sudiyani et al. 2021). Numerous studies have shown that yeast and human genes are remarkably conserved, and 31% of yeast's genes are reported to have a human homologue (Goffeau et al. 1996; Montes de Oca et al. 2016). Thus, yeast has long been used to study the fundamental cellular and molecular mechanisms such as protein quality control pathways, vesicular trafficking, mitochondrial metabolism, cell cycle progression, *etc.*, shared by all eukaryotic organisms. Disruption of these mechanisms leads to various age-related disease conditions in humans. Interestingly, although yeast lacks neuron-specific structure and processes, it has laid down the platform to study proteins involved in age-related neurodegenerative disorders and helped to understand the molecular mechanisms that result in cellular ageing (Oliveira et al. 2017).

The pathophysiology of Friedreich's ataxia (FRDA), a rare fatal neurodegenerative disease, was first studied using *S. cerevisiae* as a model. Mutations in the *FXN* gene, responsible for FRDA, were reported to be associated with mitochondrial dysfunction and oxidative stress (Bulteau et al. 2007; Kaplan 1999; Lupoli et al. 2018). Several studies employing yeast models have also elucidated the mechanisms by which disease-causing mutations result in cell death in neurodegenerative diseases like PD, AD, ALS, Huntington's disease (HD), *etc.* (Hofer et al. 2018; McDonald et al. 2020; Menezes et al. 2015). Studies in humanized yeast models for PD revealed that disturbance in protein degradation machinery

and impaired vesicular trafficking are the root causes of cellular toxicity upon α-synuclein expression and aggregation (Franssens et al. 2013; Outeiro and Lindquist 2003; Tenreiro et al. 2017). These models also emphasized the role of mitochondrial dysfunction and mitophagy in α-synuclein toxicity (Büttner et al. 2008; Su et al. 2010; Tenreiro et al. 2017). Although amyloid β (Aβ) and tau proteins (involved in the pathogenesis of AD) do not have orthologs in yeast, their heterologous expression in yeast models has helped in understanding various cellular mechanisms involved in the etiology of this disease such as modelling of amyloid precursor protein (APP) processing, *in vivo* Aβ oligomerization, Aβ-associated toxicity, and tau phosphorylation (Bharadwaj et al. 2010; McDonald et al. 2020; Seynnaeve et al. 2018). Yeast models of HD have highlighted the importance of several pathways associated with this disease and also provided therapeutic understanding by identifying pharmacological agents against HD (Hofer et al. 2018; Mason and Giorgini 2011).

Studies in yeast have identified of pathways linked to ageing such as DNA repair mechanisms, protein folding, lipostasis, mitochondrial homeostasis, stress response, secretion, proteostasis, and regulated cell death (Bilinski et al. 2017; Carmona-Gutierrez et al. 2011; Eisenberg and Büttner 2014; Janssens and Veenhoff 2016; Knorre et al. 2016; Lasserre et al. 2015; Longo and Fabrizio 2002; Postnikoff et al. 2017; Tenreiro and Outeiro 2010). Pro- and anti-ageing mechanisms such as growth in CR condition, TOR-Sch9 nutrient-sensing, Ras-AC-PKA signalling, *etc.* are also conserved from yeast to human (Sudiyani et al. 2021).

1.3 Two paradigms of yeast ageing: Replicative and chronological ageing

Replicative and chronological ageing are the two types of ageing studied in *S. cerevisiae* (Fig. 1.2) (Banerjee et al. 2020). The yeast replicative ageing model mimics the ageing of asymmetrically dividing stem cells of higher eukaryotes, whereas the chronological ageing model is similar to the ageing of non-dividing cells like neurons (Denoth Lippuner et al. 2014).

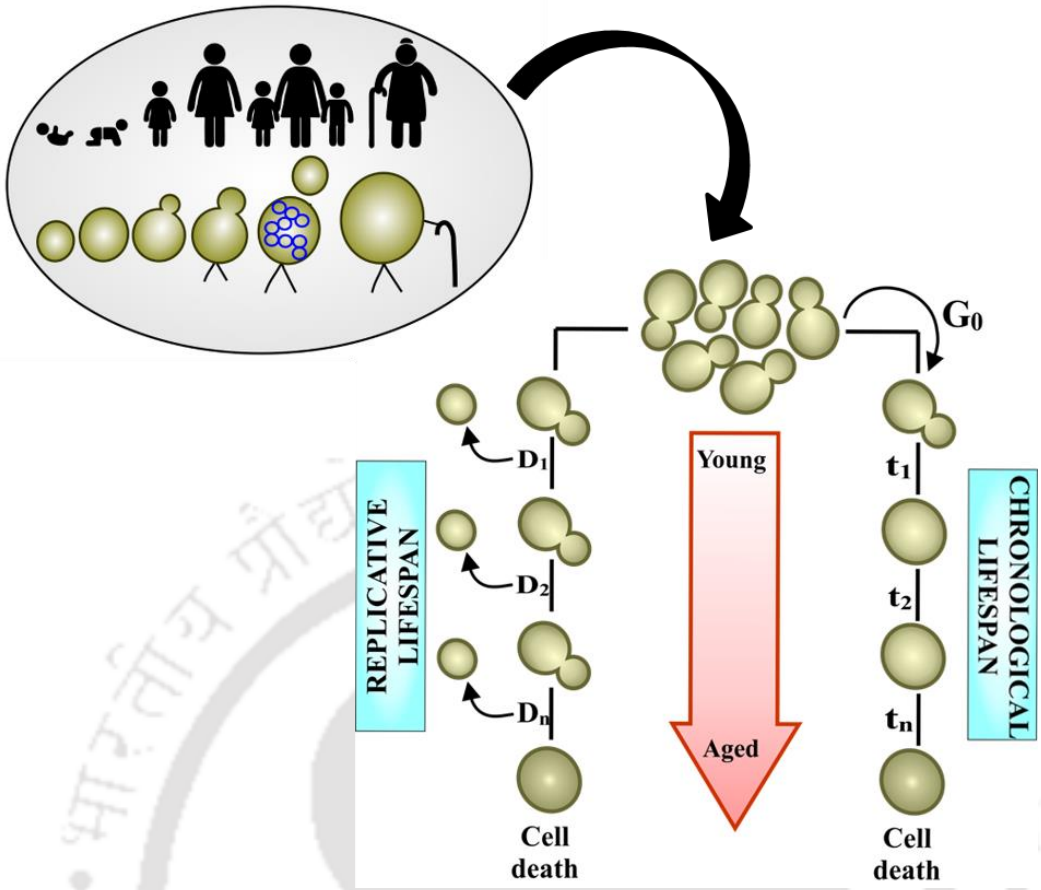

Fig. 1.2: Two models of yeast ageing: replicative and chronological ageing.
The ageing process in budding yeast is comparable to that in higher eukaryotes. Yeast can be used to study two types of ageing, namely replicative and chronological ageing. The replicative ageing model reflects the ageing of asymmetrically dividing stem cells of higher eukaryotes. In contrast, the chronological ageing model is akin to the ageing of non-dividing cells like neurons (Denoth Lippuner et al. 2014). Replicative lifespan (RLS) is the number of daughter cells produced by a mother cell before death (Müller et al. 1980). Chronological lifespan (CLS) is the length of time an individual yeast cell can remain viable in the stationary phase (Fabrizio and Longo 2003).

1.3.1 Replicative ageing

The replicative ageing model discusses the ageing of mitotically active cells. Replicative lifespan (RLS) is defined as the number of times an individual yeast mother cell divides to produce a daughter cell before it reaches senescence (He et al. 2018; Müller et al. 1980). Every yeast cell can undergo a finite number of divisions before it reaches senescence, which is termed as the replicative capacity of the cell (Sinclair et al. 1998). With each cell division, a ring-like scar mainly composed of chitin, called a bud scar, is left on the yeast mother cell wall (Holan et al. 1981; Kollár et al. 1995; Seichertová et al. 1973). Therefore, the number of bud scars present on the mother cell surface is directly proportional to the number

of times the cell has divided and thus provides a measure of the replicative age of the cell (Fig. 1.3) (Barton 1950; Powell et al. 2003; Sinclair et al. 1998).

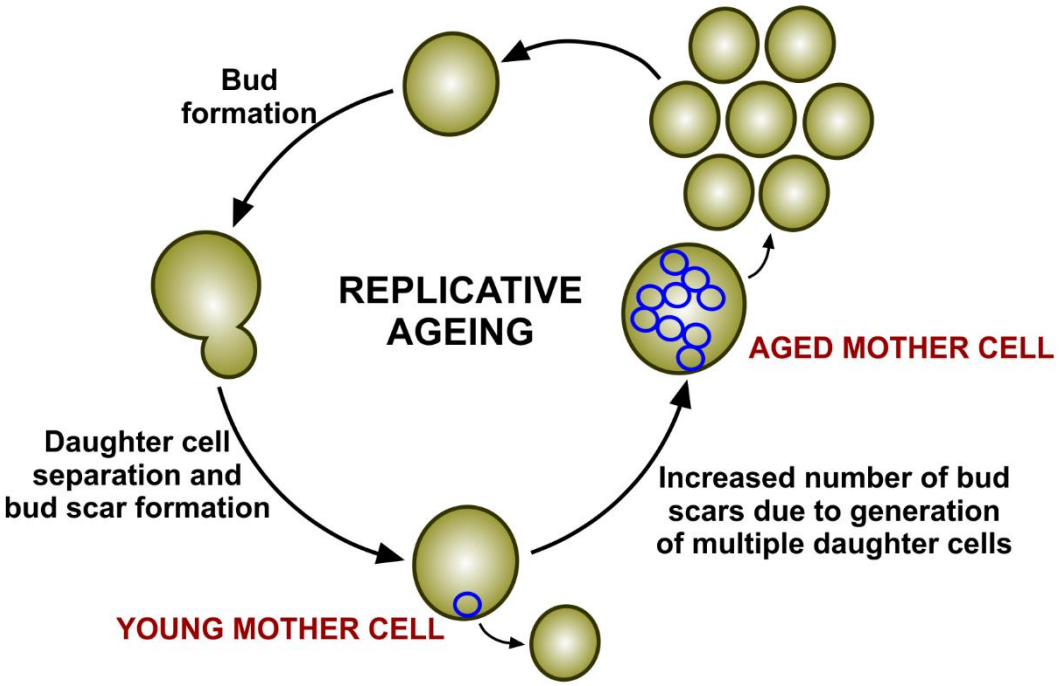

Fig. 1.3: Replicative ageing in budding yeast.
During each cell division in yeast, the birth of a daughter cell is accompanied by the formation of a bud scar on the mother cell wall (Kollár et al. 1995). Thus, the number of bud scars present on the mother cell surface directly correlates with the number of times the cell has divided, indicating the replicative age of the mother cell (Powell et al. 2003).

Various techniques have been developed to isolate the replicatively aged cells and analyse the RLS of a mother cell (Fig. 1.4) (Banerjee et al. 2020; Bhattacharya et al. 2020). Micromanipulation was the earliest and gold standard method used to study RLS (Fig. 1.4a) (Mortimer and Johnston 1959). In this method, the daughter cells produced by a single mother cell cultured on the solid medium are visualized under the microscope and physically removed using a dissection needle. The budding events are scored manually and used for calculating the RLS of the mother cell (Park et al. 2002; Steffen et al. 2009). However, this mechanical method is very laborious and time-consuming. Additionally, the growth media could deteriorate or become contaminated, which will have an impact on the evaluation of RLS. The limitations of the micromanipulation method led to the discovery of high-throughput microfluidic devices (Fig. 1.4b). The device includes a microscopic slide with numerous distinct channels, each with

an inlet and outlet passage that permits the continuous flow of growth medium. Each channel comprises several microbuckets in a row that trap a single mother cell individually. The trapped mother cells are retained in the buckets, and the produced buds are washed out with the help of continuously flowing media. As this method is semi-automated, the budding events are recorded, and RLS is determined by calculating the number of events (Chen et al. 2017a; Kumar et al. 2018; Zhang et al. 2012). To isolate a large number of aged cells and for a high-throughput identification of genes involved in the regulation of RLS, an inducible genetic method termed as mother enrichment program (MEP) has been developed (Fig. 1.4c). This method selectively inhibits the proliferative potential of daughter cells by adding estradiol, whereas the mother cell population is allowed to replicate and maintain normal RLS (Lindstrom and Gottschling 2009). Another approach to study RLS is elutriation, in which the mother and daughter cells are separated based on size (as mother cell size is more than the daughter), and then centrifugal force and flow rate are used to pool the mother cells with continuous elimination of daughter cells (Fig. 1.4d) (Laun et al. 2005; Woldringh et al. 1995). Replicatively aged mother cells can also be isolated using biotin-streptavidin based magnetic sorting (Fig. 1.4e) (Hendrickson et al. 2018). This approach of separating the aged mother cells came from the observation that the cell wall of the daughter cell is synthesized *de novo* at the budding site and is not inherited from the mother cell (Smeal et al. 1996). Thus, biotin labelled cell surface is retained by the mother cells through multiple rounds of cell division. In this method, the primary amines of proteins on the yeast cell surface are tagged with biotin, and the biotinylated cells are allowed to grow for desired number of generations. After that, the aged mother cells are coated with streptavidin magnetic beads and separated with the help of a magnetic column. Biotin tagged mother cells are also isolated from non-biotinylated daughter cells using techniques like purification, flow cytometry, or an improved miniature-chemostat ageing device (MAD) (Hendrickson et al. 2018; Janssens et al. 2015; Sarnoski et al. 2017). The

number of generations, *i.e.* the replicative age of the isolated mother cells, can be determined by staining them with calcofluor white which stains the chitin present in the bud scar (Sorokin et al. 2014).

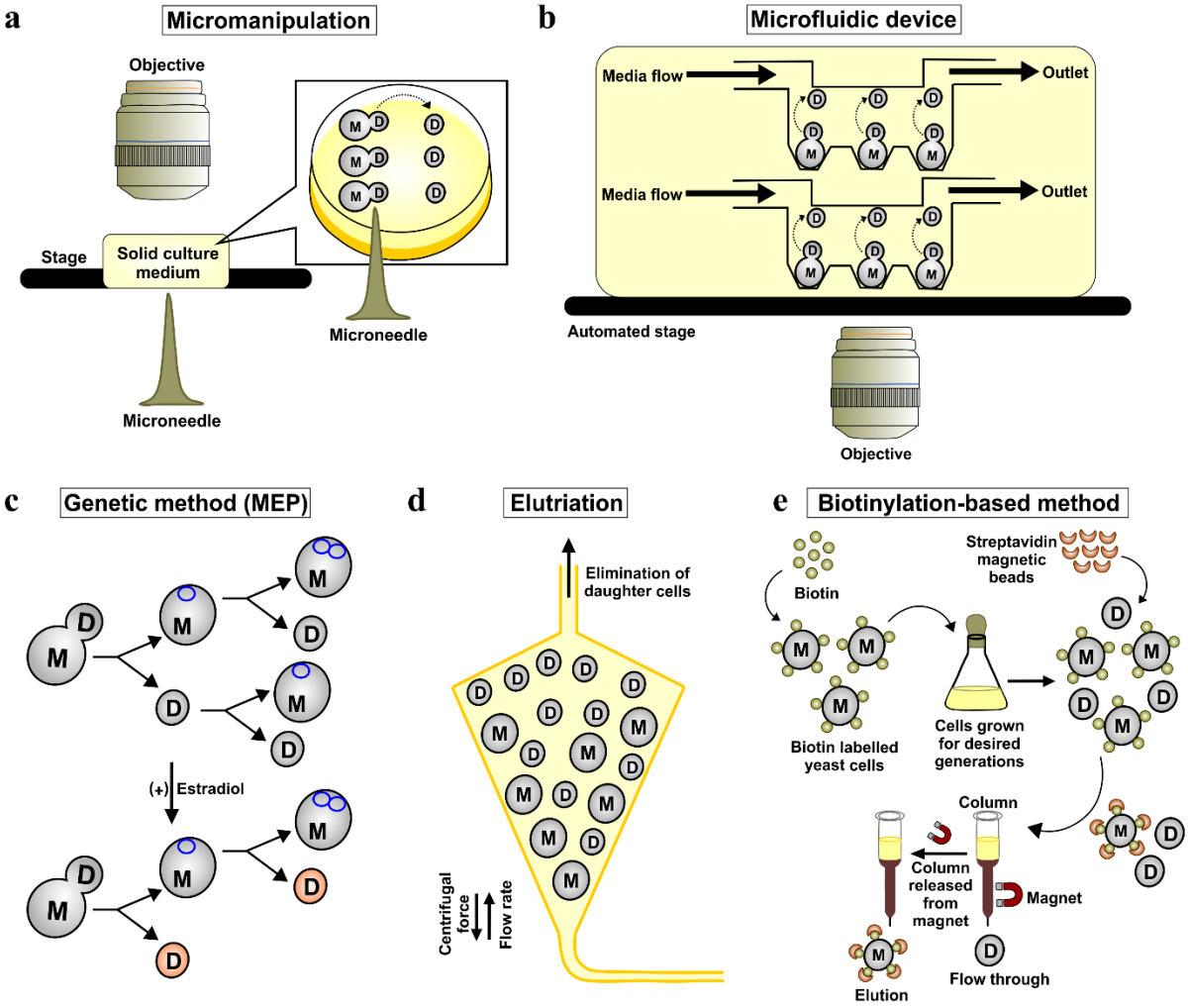

Fig. 1.4: Methods developed to study replicative ageing in budding yeast.
(a) Micromanipulation involves manual removal of daughter cells produced by mother cells cultured on a solid medium with the help of a microneedle and a microscope (Park et al. 2002). **(b)** A number of microfluidics-based automated devices have been developed which enable investigation of numerous age-related parameters in single or group of cells, when used in combination with techniques like microscopy (Chen et al. 2017a; Jo and Qin 2016). **(c)** Mother enrichment programme (MEP) is an inducible genetic approach that pools mother cell populations by selectively inhibiting daughter cell proliferation upon addition of estradiol (Lindstrom and Gottschling 2009). **(d)** Elutriation is another process which separates mother and daughter cells based on their size, and pools mother cells using centrifugal force and flow rate, with continuous elimination of daughter cells (Laun et al. 2005; Woldringh et al. 1995). **(e)** Biotinylation-based methods label mother cells with biotin and separate them from the non-biotinylated daughter cells using purification techniques like affinity-based columns, flow cytometry, or micro chemostat ageing device (MAD) (Hendrickson et al. 2018; Smeal et al. 1996). M – Mother cells, D – Daughter cells

1.3.2 Chronological ageing

Chronological lifespan (CLS) is the measure of time a yeast cell can maintain viability in a non-dividing quiescent (G_0-arrested) state (Fig. 1.5) (Fabrizio and Longo 2003). CLS is analysed when the cells enter the post-diauxic state and they exit cell cycle (Longo et al. 1996). In this stage, the cells are depleted of nutrients (glucose) in the medium and shift the mode of metabolism to respiration (Fig. 1.5) (Werner-Washburne et al. 1993). Chronological ageing model describes the ageing of post-mitotic cells.

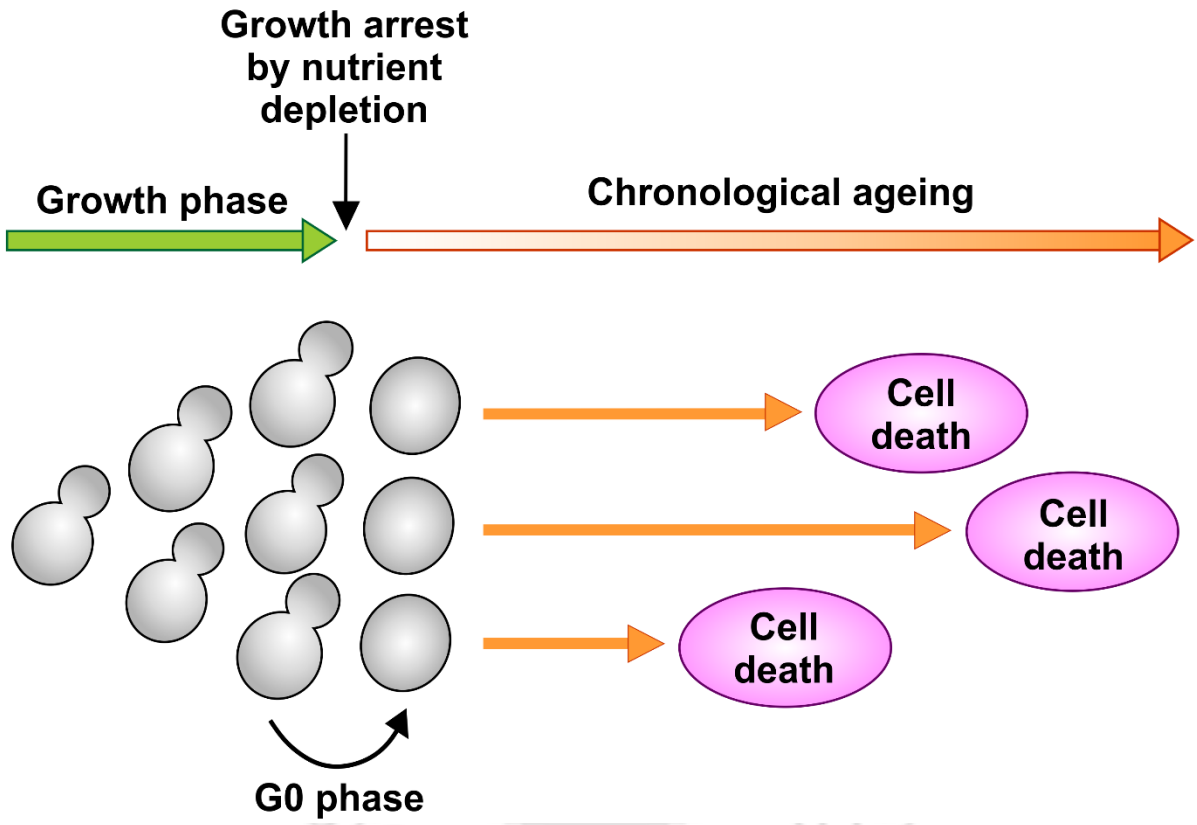

Fig. 1.5: The chronological ageing in budding yeast.
The chronological lifespan (CLS) is defined as the ability of cells to remain viability in stationary phase. In this stage, the nutrients in the medium become depleted and cell growth is arrested (Longo and Fabrizio 2012).

CLS of yeast cells can be measured by several methods like colony forming unit (CFU) assay, dye-based, genetic and high-throughput methods (Fig. 1.6) (Banerjee et al. 2020). CFU assay is one of the widely used method to study CLS (Fig. 1.6a) (Fabrizio and Longo 2007). In this assay, a sample of cells is collected from the culture on different days of ageing and

plated on solid agar plates. The number of colonies are counted and CLS curves are plotted (Fabrizio and Longo 2007).

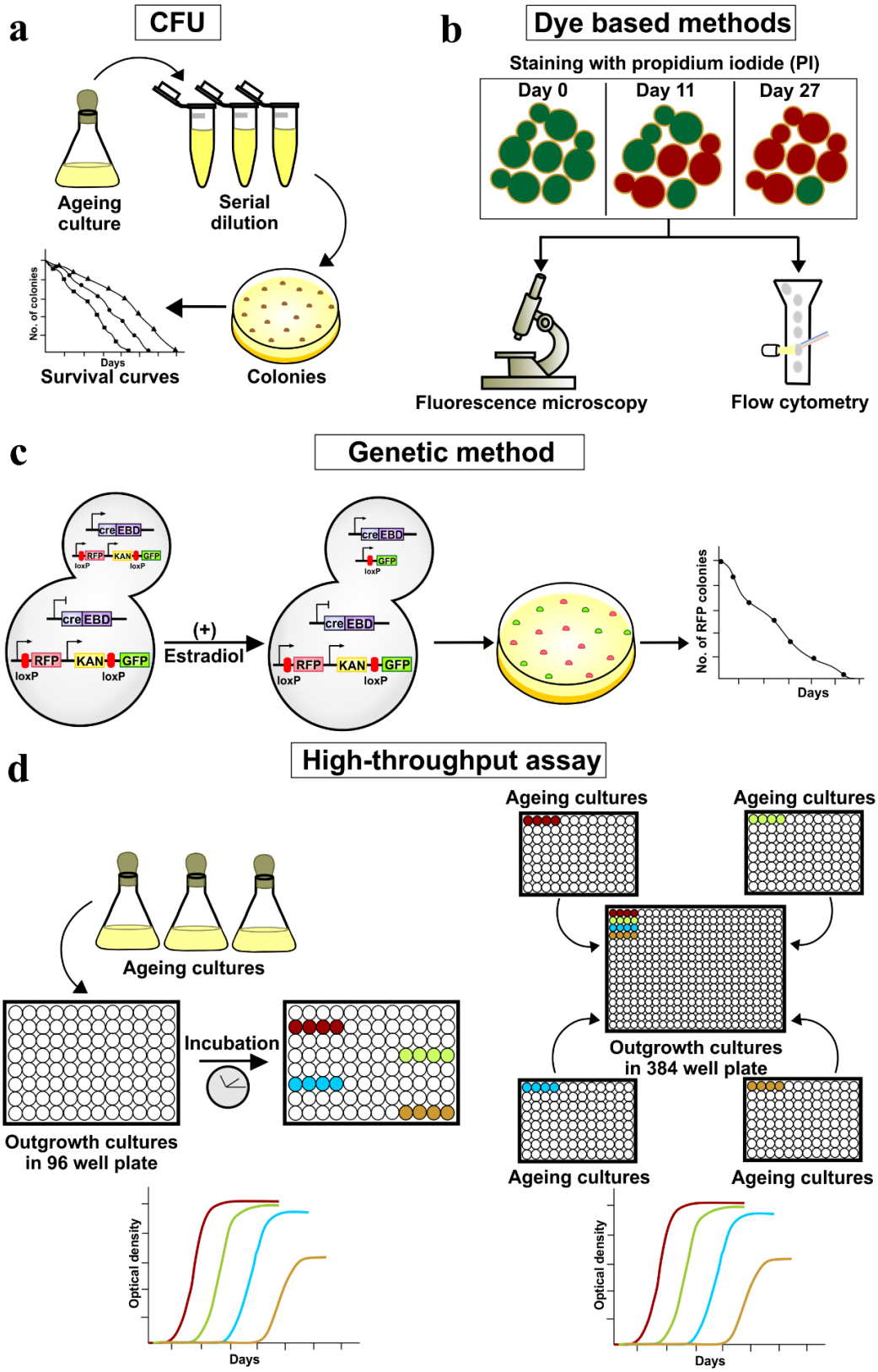

Fig. 1.6: Methods developed to study chronological ageing in budding yeast. (a) The colony-forming unit (CFU) method involves periodic removal of an aliquot of ageing culture and plating them on agar plates, followed

by counting the number of reproductively competent cells that can form colonies (Fabrizio and Longo 2007). **(b)** Chronological ageing of the yeast cells can be assessed by estimating their cell viability using dyes like propidium iodide (PI) which is further analysed with the help of techniques like fluorescence microscopy or flow cytometry (Ocampo and Barrientos 2011). **(c)** Genetic method uses a daughter cell-specific promoter and crelox-based production of fluorescent proteins in order to separate mother and daughter cells in the stationary phase culture. After plating, colonies derived from the mother and daughter cells express RFP and GFP, respectively. Only the colonies that express RFP are counted to produce the lifespan curves (McCleary and Rine 2017). **(d)** In order to evaluate the CLS of numerous strains or strains maintained under diverse conditions, high-throughput approaches have been developed. These methods allow genome-wide analysis to find mutants or compounds that affect CLS and rely on the evaluation of doubling time on subsequent days by inoculating a sample of the ageing cultures into fresh medium (Jung et al. 2015).

Dye-based labelling of live and dead cells (Propidium iodide or PI) followed by fluorescence microscopy and flow cytometry has also been developed to assess the chronological age of yeast cells (Fig. 1.6b) (Ocampo and Barrientos 2011). In order to improve the genome-wide screening of various factors regulating CLS, genetic method based on daughter cell-specific promoter and cre-lox dependent expression of fluorescent proteins has been developed for distinguishing the mother and daughter cells in stationary phase (McCleary and Rine 2017). Upon plating, the mother and daughter cells form colonies expressing RFP and GFP respectively and the RFP expressing colonies are counted to generate CLS curves (Fig. 1.6c). Recent development of high-throughput methods has advanced the process of large-scale determination of CLS in a wide variety of strains under different growth conditions and has also enabled the genome-wide investigation of compounds or genes which can alter CLS (Fig. 1.6d) (Jung et al. 2015).

1.4 Yeast cell organelles: structural and functional alterations upon ageing

Cells undergo several structural and functional changes upon ageing. Various studies in yeast have reported the role of subcellular organelles in regulating cellular ageing (Banerjee et al. 2020; Denoth Lippuner et al. 2014). This section gives an overview of the effects of ageing on the structure and function of organelles like nucleus, endoplasmic reticulum (ER), mitochondria, vacuole and lipid droplets (LDs). Several age-associated alterations in organelles such as change in size, morphology and dynamics, altered protein import and degradation,

disrupted inheritance, effect on genetic stability and metabolic activity *etc.* have been reported upon replicative and chronological ageing.

Enlarged and fragmented nucleolus was observed in both replicatively and chronologically aged yeast cells (Kaeberlein et al. 1999; Lewinska et al. 2014; Sinclair and Guarente 1997). Alterations in mitochondrial morphology are another important indicator of ageing. *S. cerevisiae* cells lacking Dnm1 (a protein involved in mitochondrial fission) exhibited increased mean RLS suggesting the role of this process in yeast ageing (Scheckhuber et al. 2007). Deletion of *DNM1* and *FIS1* resulted in the loss of mitochondrial fission and prolonged RLS in yeast mother cells (Scheckhuber et al. 2007). On the other hand, impaired mitochondrial fusion in cells lacking Mgm1 resulted in reduced CLS and RLS (Scheckhuber et al. 2011). However, deletion of both *DNM1* and *MGM1* also resulted in reduced RLS (Bernhardt et al. 2015; Sesaki et al. 2003). The number and size of vacuoles were reported to be altered with replicative ageing (Li and Kane 2009). Enlarged vacuole with wrinkled membrane was observed in replicatively aged yeast cells (Lee et al. 2012). Studies have reported the requirement of proteins involved in vacuolar fusion (Erg6, Ypt7 and Nyv1) for the extension of RLS (Tang et al. 2008). Vacuolar fragmentation was also reported upon early replicative ageing (after 4 doublings) (Ghavidel et al. 2018). Reduction in the accumulation of LD extended RLS in *S. cerevisiae* (Beas et al. 2020).

Cellular ageing is also influenced by changes in protein composition within an organelle. Changes in the composition of nuclear pore complex (NPC) resulted in an imbalanced nucleo-cytoplasmic exchange and accelerated replicative ageing in yeast (Meinema et al. 2022). Studies have also reported a number of structural changes in chromatin with age, including reduced levels of total histones and altered histone modifications (Feser and Tyler 2011). Replicatively aged mother cells exhibited reduced levels of total and DNA-bound histones compared to young daughter cells, and elevating the histone expression caused

a significant increase in yeast RLS (Feser et al. 2010). In addition, reduced and enhanced CLS were observed in budding yeast when the amounts of histone proteins were decreased and increased, respectively (Mei et al. 2019). Histone methylation and acetylation also plays an important roles in chromatin organization and thereby in the regulation of ageing process. It was observed that defects in histone methylation led to a decrease in both RLS and CLS (Cruz et al. 2018; Mei et al. 2019). Altered histone deacetylation due to a mutation in the histone deacetylases Hst3, Hst4 and Sir2, also resulted in reduced RLS (Dang et al. 2009; Feser et al. 2010). However, prevention of histone deacetylation in yeast cells lacking histone acetyltransferase Sas2 resulted in extended RLS (Dang et al. 2009). Screening of histone mutant library also led to the identification of novel histone modifications which regulate yeast RLS (Sen et al. 2015). A role of Sir2-dependent chromatin silencing in replicative ageing was also reported (Li et al. 2017). Extrachromosomal rDNA circles (ERCs), which are retained in the mother cells during cell division, are one of the distinguishing characteristics of replicatively aged mother cells (Sinclair and Guarente 1997). Reduced RLS of yeast was observed upon artificial introduction of ERCs into the daughter cells (Sinclair and Guarente 1997). Altered RLS upon inhibition or enhanced formation of ERC by Sir2 and Fob1 respectively have also been reported (Kaeberlein 2010). It was observed that *SIR2* overexpression or *FOB1* deletion resulted in enhanced RLS, whereas deletion of *SIR2* decreased RLS in budding yeast (Defossez et al. 1999; Kaeberlein et al. 1999; Steinkraus et al. 2008). However, extended CLS was observed upon deletion of *SIR2* under CR conditions (Fabrizio et al. 2005). *N*-glycosylation is one of the most prevalent protein modifications in the ER (Lehle et al. 2006). Defects in one of the subunits in the oligosaccharyltransferase complex (which catalyses *N*-glycosylation of proteins in ER) resulted in shortened CLS (Hauptmann and Lehle 2008). Reduced CLS was also observed in cells lacking the vacuolar sorting proteins

Vps25 and Vps27, which are important in sorting ubiquitinylated proteins into endosomes (Fabrizio et al. 2010).

Asymmetric inheritance of cellular components from the mother to daughter cells during cell division is a significant characteristic of budding yeast. Dysfunctional organelles are retained by the mother cells whereas the fitter organelles are transported to the daughters (Henderson and Gottschling 2008). Organelle inheritance is a tightly controlled process, and any interference with balanced inheritance accelerates the ageing process. It was observed that cells devoid of proteins such as Mfb1 (required for the retention of mitochondria in the mother cell tip) and Ypt11 (required for mitochondrial attachment at bud cell tip) exhibited reduced and enhanced RLS, respectively (Pernice et al. 2016; Rafelski et al. 2012). Compromised inheritance of fitter mitochondria in the daughter cells lacking Mmr1 (protein involved in mitochondrial inheritance to buds) led to oxidative stress and thereby shortened RLS (McFaline-Figueroa et al. 2011). In the similar lines, overexpression and deletion of *VAC17* and *VAC8* (required for vacuolar inheritance) resulted in an increase and decrease in yeast RLS, respectively (Hill et al. 2016; Tang et al. 2008). It was also observed that yeast mother cells exhibited reduced expression of Vac17 during replicative ageing (Weisman 2006).

Degradation of organelles also contributes to organelle homeostasis and is altered upon cellular ageing. ER-associated protein homeostasis is mediated by proteins called chaperones which prevent the aggregation of substrate protein (Nishikawa et al. 2005). It was observed that cells lacking ER chaperones Hsp70 and Lhs1 exhibit decreased CLS and RLS (Perić et al. 2016). ER stress combined with altered natural stress responses such as unfolded protein response (UPR), nutrient levels *etc.* resulted in shortened RLS (Chadwick and Lajoie 2019). Reduced RLS was also observed in cells lacking the protein Ydj1, which aids in the identification of cytosolic misfolded proteins for ubiquitin-dependent degradation (Saarikangas et al. 2017). Overexpression of another ER chaperone *KAR2* (involved in protein folding in

ER) also resulted in extended RLS in *S. cerevisiae* (Cui et al. 2015). A high-throughput study by Garay and colleagues identified an association between the ER protein Arv1 and autophagy which alters the CLS in budding yeast (Garay et al. 2014). Reduced CLS was observed in cells lacking Arv1 (Tinkelenberg et al. 2000). However, deletion of *ARV1* in the autophagy mutants resulted in enhanced CLS (Garay et al. 2014). The mitochondrial quality control mechanism in yeast ensures the removal of dysfunctional mitochondria through an autophagy pathway known as mitophagy (Fukuda and Kanki 2018; Lemasters 2005). Atg32 is a mitochondrial protein which acts as a receptor for selective removal of mitochondria and thus initiate mitophagy (Hirota et al. 2012). Absence of Atg32 resulted in accelerated chronological ageing in yeast owing to the accumulation of dysfunctional mitochondria and thereby increased oxidative damage in the cell (Richard et al. 2013). A genome-wide screen identified several proteins linked to mitophagy (Atg33, Uth1, Fzo1, Aup1) and cells lacking these proteins resulted in shortened CLS, thereby highlighting the role of mitophagy in regulating chronological ageing (Breitenbach et al. 2014; Fabrizio et al. 2010). Extended CLS was also observed upon initiation of lipophagy in *S. cerevisiae* cells (Seo et al. 2017).

Eukaryotic cells contain a variety of signal transduction pathways that regulate growth and metabolism depending on the cellular environment. Majority of these pathways are regulated by TORC1 kinase complex (comprising of Tor1/Tor2), which in turn regulates the downstream kinase Sch9 (Deprez et al. 2018). It was reported that deletion of *TOR1* and *SCH9* resulted in decreased signalling and thereby led to the extension of both CLS and RLS, as similar to CR conditions (Wei et al. 2009). A reduction in the accumulation of damaged DNA was observed in *sch9* cells during chronological ageing (Burhans and Weinberger 2007). A crucial pathway which regulates cell cycle progression and lifespan in yeast is the nutrient signaling Ras–cAMP–PKA pathway (Sun et al. 1994). This pathway involves two *RAS* genes, *RAS1* and *RAS2,* which were first genes reported to influence the yeast lifespan (Kataoka et al.

1984). Cells deleted for *RAS1* exhibited extended RLS, whereas *RAS2* deletion resulted in reduced RLS and extended CLS (Fabrizio et al. 2003; Fabrizio and Longo 2003; Sun et al. 1994).

Oxidative homeostasis achieved by the presence of oxidases and antioxidants is another cellular process conserved in yeast. ER, mitochondria and peroxisomes contribute to ROS homeostasis in yeast cells (Yoboue et al. 2018). Mitochondria acts as one of the major sources of ROS in the cell and generates wide variety of ROS molecules such as reactive hydroxide radicals, negatively charged superoxide and neutrally charged hydrogen peroxide (Zorov et al. 2014). Studies have reported reduced CLS upon oxidative damage resulting from mitochondrial dysfunction in the cells (Pan 2011; Ruetenik and Barrientos 2015). However, the presence of enzymes like superoxide dismutases (SOD) and catalase in the cells helps in detoxifying the produced ROS (Ighodaro and Akinloye 2018). Deficiency of mitochondrial Sod2 resulted in the decrease of both CLS and RLS in yeast (Ogata et al. 2016; Steinkraus et al. 2008). Another organelle essential in maintaining the redox balance in a cell is the peroxisome. Peroxisome houses both oxidases that produce ROS and the antioxidant catalase that scavenges ROS.

1.5 Peroxisomes: A versatile organelle

Peroxisomes are single membrane-bound organelles with a size ranging from 0.1 to 1 μm in diameter (Smith and Aitchison 2013). These organelles were identified by Rhodin in 1954 during his work on mouse kidney proximal tubule cells and were originally called microbodies (Rhodin 1954). Later, De Duve and colleagues were the first to isolate and biochemically characterize these organelles from rat liver using differential and density gradient centrifugation techniques. These biochemical studies revealed the presence of hydrogen peroxide **(**H_2O_2)-producing oxidases like D-amino acid oxidase, urate oxidase, L-hydroxy acid oxidase and an H_2O_2-degrading enzyme catalase in the organelle matrix (De Duve

and Baudhuin 1966). These findings prompted De Duve to name this newly identified organelle "peroxisome" as they contain hydrogen peroxide producing and degrading enzymes. Later, a specific cytochemical staining procedure to analyse peroxidase activity of catalase using alkaline 3,3-diaminobenzidine (DAB) reaction was developed (Fahimi 1969). This method later facilitated identification of peroxisomes in most eukaryotic cells.

The most conserved functions of peroxisomes across organisms are ROS metabolism and β-oxidation of fatty acids (Islinger et al. 2018). The first discovered function of this organelle was its significant role in cellular ROS production and scavenging (De Duve and Baudhuin 1966). Peroxisomes harbor various H_2O_2-producing flavin-containing oxidases that can be organism and tissue-specific and are hence one of the major sites of intracellular H_2O_2 production (Baker and Graham 2002; De Duve and Baudhuin 1966; del Río 2013; Fahimi and Sies 2012). Some of the important enzymes which generate H_2O_2 in peroxisomes of different organisms are: (i) glycolate oxidase (GOX) [in plants], (ii) urate oxidase, (iii) aspartate oxidase, (iv) pipecolic acid oxidase, (v) hydroxyacidoxidase, (vi) polyamine oxidase [in mammals], (vii) xanthine oxidase, (viii) nitric oxide synthase, (ix) acyl CoA oxidases (ACOX1) [in yeast, mammals and plants] (del Río and López-Huertas 2016; Fransen et al. 2012; Schrader and Fahimi 2006). Apart from ROS production, peroxisomes can also scavenge the accumulated ROS as they contain antioxidant systems like catalase (CAT), SOD, glutathione S-transferase (GST), *etc.* (Gill and Tuteja 2010; Sandalio et al. 2013). In mammals and plants, catalase enzymes are confined to peroxisomes. However, *S. cerevisiae* has genes encoding two catalase enzymes – cytosolic catalase T (*CTT*) and peroxisomal catalase A (*CTA*) (Cohen et al. 1988; Hartig and Ruis 1986).

In addition to ROS metabolism, β-oxidation of fatty acids is another crucial function of peroxisomes conserved in all organisms (Poirier et al. 2006) (Fig. 1.7). The role of peroxisomes in fatty acid oxidation was first discovered in 1976 in rat liver cells (Lazarow and De Duve

1976). Fatty acids and their derivatives play multiple structural and functional roles in living organisms and also help in energy production and metabolism. β-oxidation is the major degradative pathway for fatty acid esters. It takes place in both mitochondria and peroxisomes in animal cells and solely in peroxisomes in plants and most yeast and fungal species (Poirier et al. 2006).

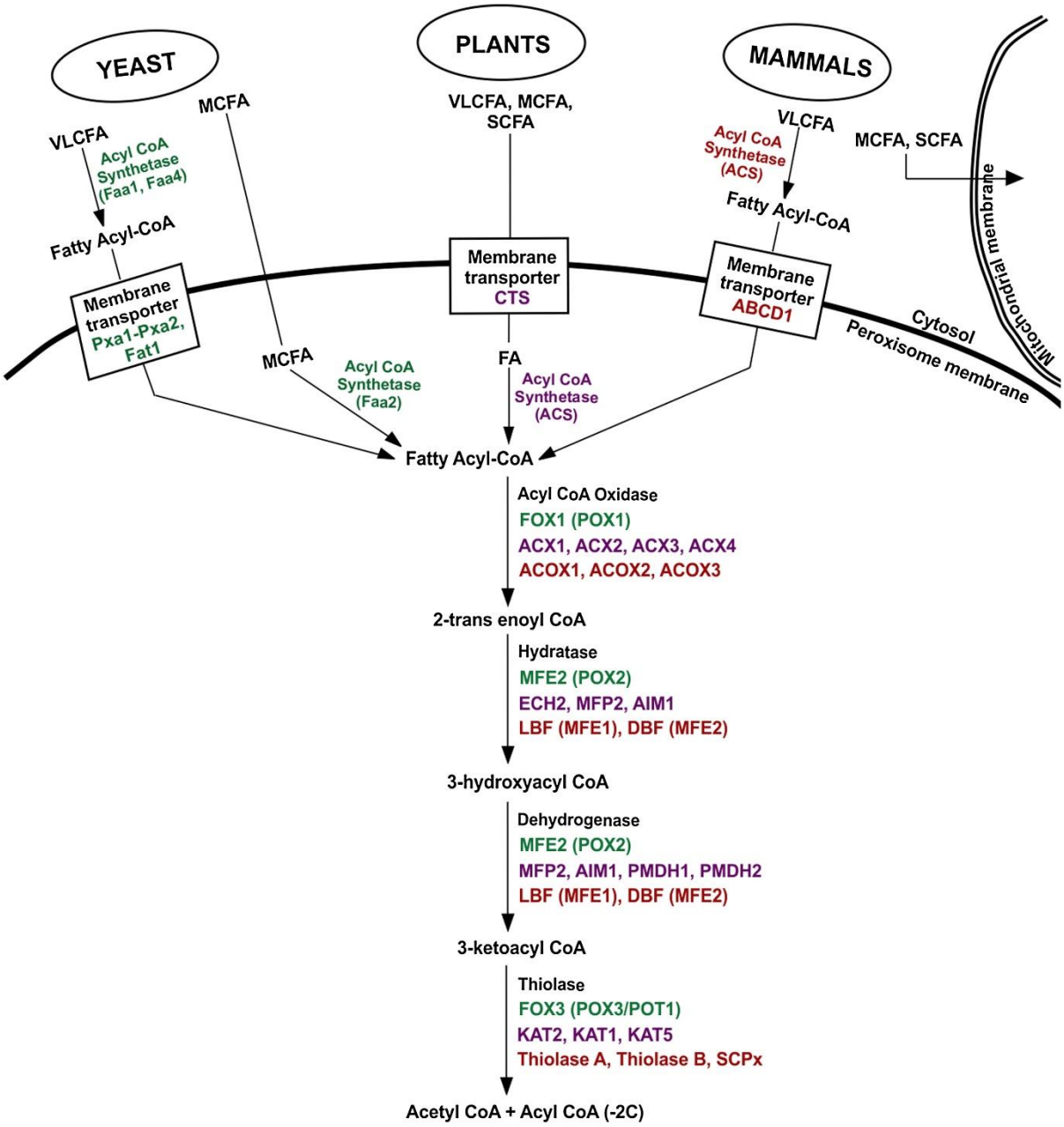

Fig. 1.7: Schematic depicting β-oxidation of fatty acids in various model organisms.
β-oxidation occurs solely in peroxisomes in most fungi and plants, while in mammals, it takes place both in peroxisomes and mitochondria. In yeast (*S. cerevisiae*), very long chain fatty acids (VLCFAs) are activated to acyl CoA in cytosol by acyl CoA synthetases Faa1 and Faa4 and are transported into peroxisomes via the membrane transport protein complex Pxa1-Pxa2 and Fat1. Unlike VLCFAs, MCFAs are activated to their acyl

CoAs inside peroxisomes by acyl CoA synthetase, Faa2 (Faergeman et al. 2001; Færgeman et al. 1997; Hettema et al. 1996; Hiltunen et al. 2003). In plants, fatty acids are transported into peroxisomes through ABC transporter comatose (CTS) and then activated to fatty acyl CoAs by acyl CoA synthetase (Baker et al. 2010). In mammals, conversion of VLCFAs to acyl CoAs by synthetase occurs in cytosol followed by their transport to peroxisomes with the help of ABCD1 transporter (Demarquoy and Le Borgne 2015). The fatty acyl CoAs then enter the core β-oxidation reactions which consists of four steps: a) oxidation by acyl CoA oxidase, b) hydration of double bond by hydratase, c) dehydrogenation of hydroxyl group to ketone by dehydrogenase and d) thiolytic cleavage of ketoacyl CoA by thiolase to give the final products, acetyl CoA and acyl CoA shortened by two carbon atoms. The figure represents the corresponding enzymes of all the above-mentioned enzymatic reactions in yeast, plants and mammals. Text in green is for yeast (*S. cerevisiae*), purple for plants and red for mammal specific enzymes that take part in this process.

β-oxidation in peroxisomes is mainly involved in the oxidation of very long chain fatty acids (VLCFAs) (> C22) and branched fatty acids and is primarily related to biosynthesis pathways (Demarquoy and Le Borgne 2015). This pathway starts with the activation of fatty acids to their acyl CoA derivatives. In *S. cerevisiae*, VLCFAs are activated in cytosol by acyl CoA synthetases, Faa1 and Faa4, and then transported into peroxisomes via Pxa1-Pxa2 and Fat1, whereas activation of middle chain fatty acids (MCFAs) occurs in peroxisomes by Faa2 (Black and DiRusso 2007; Faergeman et al. 2001; Færgeman et al. 1997; Hettema et al. 1996; Hiltunen et al. 2003). In mammals the fatty acyl CoA is transported into peroxisomes through ABCD1 transporter after activation in the cytosol (Demarquoy and Le Borgne 2015). Acyl CoAs in plants are activated in peroxisomes after transport via ABC transporter comatose (CTS) (Fig. 1.7) (Baker et al. 2010). The pathway then proceeds through four sequential catalytic reactions of dehydrogenation, hydration, dehydrogenation and thiolytic cleavage to finally produce acetyl CoA and acyl CoA shortened by two carbon atoms. The enzymes involved in these reactions, namely acyl CoA oxidase, hydratase, dehydrogenase and thiolase, though conserved in most fungi, plants and mammals differ in organism specific number of homologues. All these enzymes are enlisted in Fig. 1.7. *S. cerevisiae* can utilize both saturated long chain fatty acids like myristic and palmitic acid as well as unsaturated fatty acids like oleic acid, linoleic acid, *etc.* as substrates for β-oxidation (van Roermund et al. 2003).

Apart from the above-mentioned conserved functions, peroxisomes carry out diverse functions in various conventional model systems (Fig. 1.8). In yeast and filamentous fungi,

peroxisomes regulate the biosynthesis and metabolism of essential compounds and are also essential for maintaining virulence of a number of phytopathogenic fungi (Chen et al. 2017b; van der Klei and Veenhuis 2013). They also play an important role in the growth and survival of some fungi and help in controlling cellular ageing (Lefevre et al. 2013). Plant peroxisomes are involved in the biosynthesis of essential compounds, photorespiration and photomorphogenesis, pollen development and function and also in the regulation of glyoxylate cycle. ROS generated by peroxisomes in plants also provides protection against herbivores and pathogens. Other functions of plant peroxisomes include purine and polyamine catabolism and induction of senescence (del Río and López-Huertas 2016; Reumann and Bartel 2016).

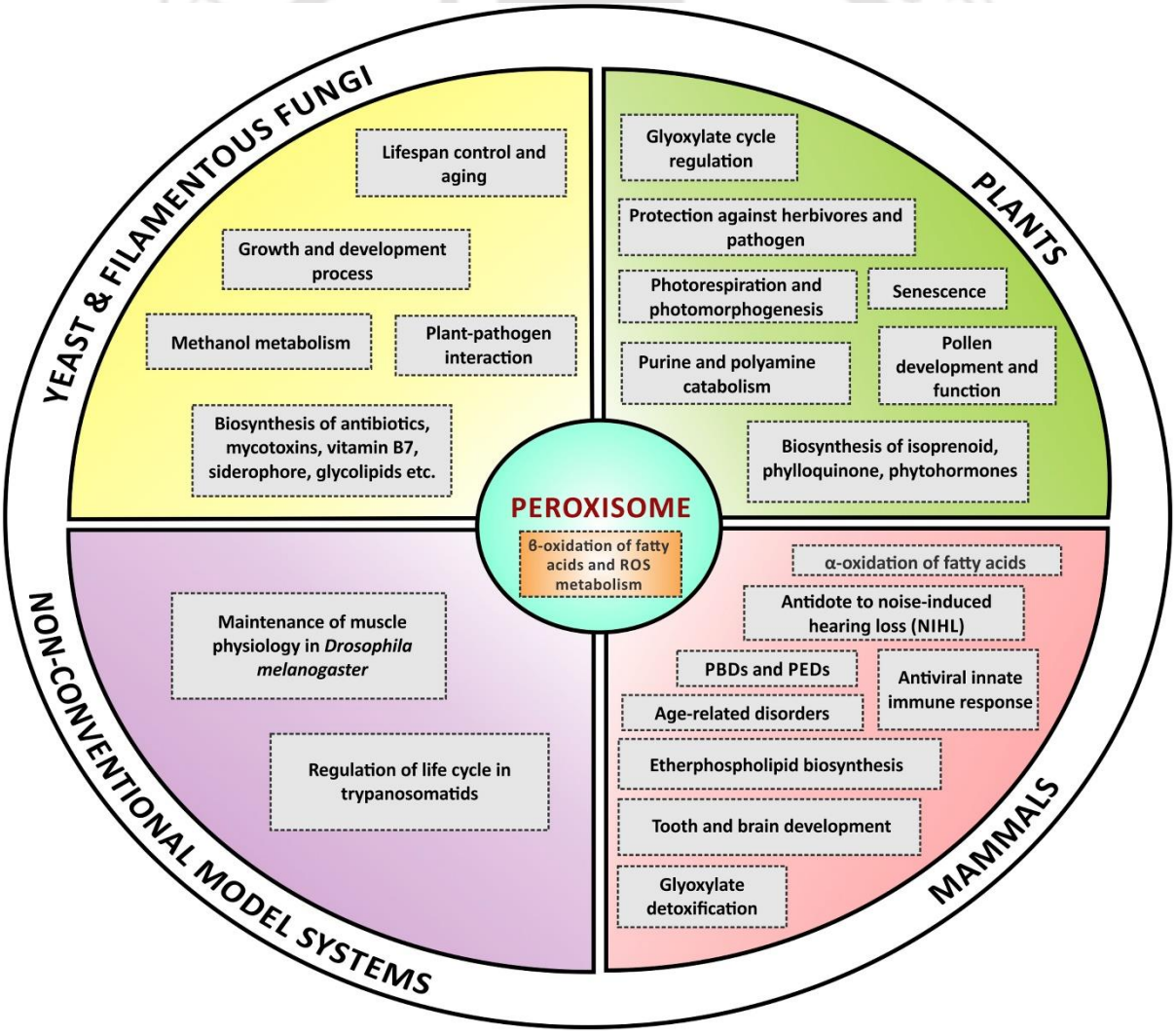

Fig. 1.8: Schematic depicting the various functions of peroxisomes in different model organisms.
Peroxisomes are single membrane-bound ubiquitous organelles in most eukaryotic cells. They can alter their size, number per cell and protein content in response to different environmental stimuli and depending on the type of

cell in which they occur. This remarkable plasticity is responsible for a role of peroxisomes in various metabolic functions in different organisms. Fungi, plants and mammals are the most commonly used model systems for peroxisome research. However, researchers are also unravelling several new and important functions using model systems, like Trypanosomatids, Drosophila, *etc*. Peroxisomes are required for the maintenance of muscle physiology in Drosophila and specialized peroxisomes termed glycosomes are essential for life cycle regulation in trypanosoma which cause sleeping sickness in humans. Glycosomes harbor the first steps of glycolysis and are essential for the life of the parasite in the blood stream (Faust et al. 2014; Szöor et al. 2010).

Mammalian peroxisomes are immensely important for normal health and development. Peroxisomes help in tooth and brain development and peroxisome proliferation acts as an antidote to noise-induced hearing loss (NIHL) (Delmaghani et al. 2015; Poll-The et al. 2004; Stelzig et al. 2013). Antiviral innate immune response is also induced by peroxisomes in collaboration with mitochondria and ER (Dixit et al. 2010; Magalhães et al. 2016; Odendall and Kagan 2013). Other metabolic functions of peroxisomes in mammals include fatty acid α-oxidation, etherphospholipid biosynthesis, glyoxylate detoxification (Waterham et al. 2016). Peroxisome malfunctioning can also lead to lethal disorders (Fig. 1.8). These disorders are classified into two groups: Peroxisome Biogenesis Bisorders (PBDs) and single Peroxisome Enzyme Deficiencies (PEDs). Zellweger syndrome (ZS), Rhizomelic Chondrodysplasia Punctata (RCDP) Type 1, Neonatal adrenoleukodystrophy (NALD) and Infantile Refsum Disease (IRD) are together grouped as PBDs. Most frequently occurring disorder among PEDs is the X-linked adrenoleukodystrophy (X-ALD) (Wanders 2014). A role for peroxisomal ROS in age-related disorders like Type II diabetes, cancer, AD and PD is also under investigation (Fransen et al. 2013). Several studies have also reported alterations in the peroxisomal functions in the brain of patients with neurodegenerative disorders (Jo and Cho 2019; Uzor et al. 2020). Peroxisomal dysfunction has been proposed to play an important role in the progression of these diseases (Table 1.1).

Table 1.1 Important peroxisome functions altered in various neurodegenerative diseases.
Disruption of peroxisome metabolic functions results in the development of various neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis and glaucoma. The table summarizes the peroxisome functions altered in these diseases

Neurodegenerative Disease	Regions/ Cells of Brain Most Affected	Hallmark Feature	Altered Peroxisome Functions	References
Alzheimer's Disease (AD)	Cerebral cortex (frontal and temporal)	Accumulation of extracellular Aβ peptide and intracellular neurofibrillary tangles of tau proteins	Decreased activity of antioxidant enzymes leading to oxidative stress	(Marcus et al. 1998)
			Reduced levels of plasmalogens (PtdEtn, PtdCho, PtdIns)	(Igarashi et al. 2011; Pettegrew et al. 2001; Tajima et al. 2013)
			Accumulation of VLCFAs	(Kou et al. 2011)
			Increase in peroxisome volume density	(Kou et al. 2011; Santos et al. 2005)
Parkinson's Disease (PD)	Substantia nigra pars compacta (SNpc)	Accumulation of α-synuclein (Lewy bodies) resulting in the loss of dopaminergic neurons	Oxidative stress due to reduced catalase levels	(Yakunin et al. 2014)
			Reduced plasmalogen levels	(Fabelo et al. 2011)
Multiple Sclerosis (MS)	Oligodendrocytes	Demyelination of axons leading to the progressive loss of their functions	Elevated levels of VLCFAs	(Gray et al. 2014)
			Reduced levels of ethanolamine plasmalogens	(Senanayake et al. 2015)
Amyotrophic Lateral Sclerosis (ALS)	Motor neurons controlling voluntary muscles	Demyelination of motor neurons causing muscle weakness	D-amino acid oxidase (D-AAO) inactivity leading to elevated levels of D-serine	(Sasabe et al. 2007; Sasabe et al. 2014)
			Elevated concentration of cholesterol	(Abdel-Khalik et al. 2017)

Glaucoma	Retinal ganglion cells (RCGs)	High intraocular pressure (IOP) in the optic nerve and abnormalities in trabecular meshwork (TM) resulting in blindness	Oxidative stress	(Ferreira et al. 2004; Kumar and Agarwal 2007)

1.6 Peroxisomes and ageing in yeast

Our understanding of peroxisome biology has significantly expanded over the past few years. At the molecular level, the mechanisms of organelle biogenesis, proliferation, autophagy *etc.* have been uncovered. The involvement of dysfunctional peroxisomes in cellular ageing is a relatively new field of peroxisome research. Recent studies have emphasised the relationship between impaired peroxisome function and yeast replicative and chronological ageing (Kumar et al. 2018; Lefevre et al. 2013; Scheckhuber 2020). One such function is the scavenging of intracellular ROS by antioxidant enzyme catalase (Wang et al. 2015). It was observed that *S. cerevisiae* cells lacking peroxisomal catalase Cta1 showed reduced CLS whereas inactivation of both the isoforms (Cta1 and cytosolic catalase Ctt1) resulted in extended CLS (Mesquita et al. 2010; Petriv and Rachubinski 2004). However, the CLS of catalase-deficient *Ogataea polymorpha* cells grown in media like glucose/glycerol (which do not require peroxisomes) was similar to the wild type (WT) cells. On the contrary, enhanced CLS was observed in these cells when cultured in methanol or methylamine that require peroxisomes for metabolism (Kawałek et al. 2013; Kumar et al. 2012). Accumulation of intracellular ROS and thus decreased cell viability was observed in *O. polymorpha* cells lacking peroxisomal Lon protease Pln (Aksam et al. 2007). Absence of the antioxidant enzyme peroxiredoxin (Pmp20) also led to enhanced oxidative stress, leaky peroxisomal membranes and increased susceptibility to necrotic cell death in *O. polymorph*a (Aksam et al. 2008).

In yeast, β-oxidation pathway solely occurs in peroxisomes (Poirier et al. 2006). Lefevre and colleagues reported that deletion of *POT1* (thiolase), the third enzyme of the β-oxidation pathway, completely blocked the pathway and resulted in shortened CLS in *S. cerevisiae* (Lefevre et al. 2013). Another study reported that cells lacking Fox1, Fox2 and Fox3 (fatty-acyl coenzyme A oxidases involved in the β-oxidation pathway) also resulted in the reduction of CLS (Arlia-Ciommo et al. 2018; Goldberg et al. 2009a).

Table 1.2: Effect on CLS and RLS in *S. cerevisiae* cells deleted for peroxisomal proteins.
This table lists all the peroxisomal proteins whose absence leads to defects in peroxisome structure and function and are also associated with altered chronological and replicative ageing in *S. cerevisiae*.

Peroxisomal processes studied	Cells lacking the peroxisomal protein	Effect on ageing	Growth conditions	References
Peroxisome fission	Pex3	CLS ↓ RLS ↓	Glucose (0.5%) Glucose (2%)	(Lefevre et al. 2013) (Kumar et al. 2018)
	Fis1	CLS ↑	Glucose (0.5%)	(Lefevre et al. 2015)
	Pex11	CLS ↓	Oleic acid (0.1%)	(Scheckhuber 2020)
Peroxisome protein import	Pex5	CLS ↓	Glucose (0.5%)	(Lefevre et al. 2013)
	Pex6	CLS ↓	Glucose (0.5%)	(Lefevre et al. 2013)
	Pex7	CLS ↓	Glucose (0.5%)	(Lefevre et al. 2013)
Peroxisome inheritance	Inp1	CLS ↓ RLS ↓	Oleic acid (0.1%) Glucose (2%)	(Scheckhuber 2020) (Kumar et al. 2018)
	Inp2	RLS ↑	Glucose (2%)	(Kumar et al. 2018)
Peroxisome functions	Fox1, Fox2, Fox3	CLS ↓	Glucose (0.2%)	(Arlia-Ciommo et al. 2018)
	Pot1	CLS ↓	Glucose (0.5%)	(Lefevre et al. 2013)
	Cta1	CLS ↑	Glucose (0.5%)	(Mesquita et al. 2010)

An involvement of peroxisome biogenesis proteins in regulating cellular ageing has also been reported (Table 1.2) (Lefevre et al. 2015; Lefevre et al. 2013; Scheckhuber 2020). Deletion of *PEX3* resulted in defective peroxisome biogenesis and led to a reduction in the CLS in *S. cerevisiae* (Lefevre et al. 2013). Similar reduction in RLS was also observed in *pex3* cells (Kumar et al. 2018). Reduced CLS was observed in cells with partly or fully mislocalized peroxisomal matrix proteins (*pex5*, *pex6* and *pex7*) (Lefevre et al. 2013). Studies have reported

that cells lacking and overexpressing Pex11 (protein involved in peroxisome fission) exhibited reduced and enhanced CLS, respectively (Chalermwat et al. 2020; Scheckhuber 2020). However, inhibition of peroxisome fission in *vps1fis1* cells resulted in increased CLS (Lefevre et al. 2015). Reduced RLS and CLS were reported in cells lacking Inp1, required for the retention of peroxisomes in the mother cell (Kumar et al. 2018; Scheckhuber 2020). On the contrary, cells lacking Inp2 (required for the inheritance of peroxisomes to daughter cells) displayed enhanced RLS (Kumar et al. 2018). All these studies point towards a connection between the abundance of functional peroxisomes and cellular ageing.

1.7 Peroxisome biogenesis: Role of Pex11 family in the regulation of peroxisome number

An interesting feature of yeast peroxisomes is that they respond dynamically to the changes in the cellular environment (Smith and Aitchison 2013). Depending on different environmental stimuli and growth conditions, peroxisomes alter their size, number and protein content. As peroxisomes are sole site for fatty acid oxidation in yeast, peroxisome proliferation is induced in medium containing fatty acids as the carbon source (Veenhuis et al. 2000). Glucose-grown *S. cerevisiae* cells contain one or very few small peroxisomes. But the shift of these cells in media containing fatty acids like oleic acid as sole carbon source results in a remarkable increase in the number and size of peroxisomes (Erdmann and Blobel 1995; Veenhuis et al. 1987). This ability to induce the proliferation of peroxisomes has made yeast an attractive system for the study of peroxisome biogenesis.

Peroxisome biogenesis occurs by two separate pathways: fission of existing peroxisomes and *de novo* budding from the ER (Hettema and Motley 2009; Nagotu et al. 2010). Peroxisomes have long been considered to be autonomous organelles which multiply by growth and division (Lazarow and Fujiki 1985). According to this model, peroxisomes proliferate by fission of pre-existing peroxisomes (Lazarow and Fujiki 1985). Peroxisomes grow and mature by importing

matrix and membrane proteins and this is followed by the elongation and fission of the peroxisome membrane. This is mediated by the proliferation machinery that includes Pex11 family, Dnm1/Vps1, Fis1 and Mdv1/Caf4 (Farré et al. 2019; Kuravi et al. 2006; Motley et al. 2008; Opaliński et al. 2011). Various steps involved in the growth and division of peroxisomes are as follows: (i) membrane elongation, (ii) constriction of the elongated membrane, (iii) fission to form new nascent peroxisomes, and (iv) maturation (Fig. 1.9) (Deori et al. 2018; Motley and Hettema 2007; Motley et al. 2008).

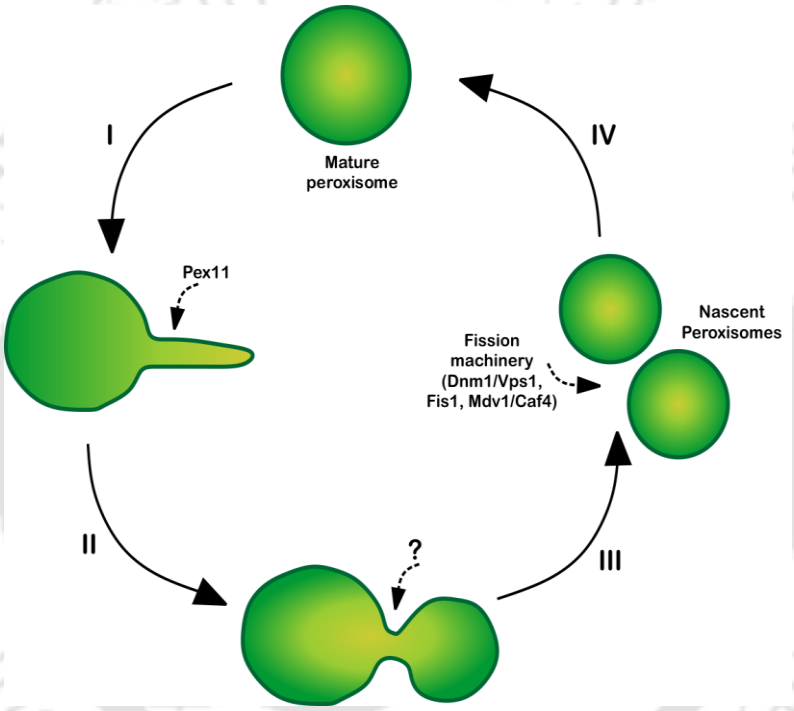

Fig. 1.9: Model representing different stages of peroxisome division in yeast: (I) Elongation or tubulation of peroxisome membrane, (II) Constriction, (III) Fission and (IV) Maturation. The proteins which are mainly involved in the elongation of peroxisome membrane are the Pex11 family, which comprises of Pex11, Pex25 and Pex27 (Huber et al. 2012). The role of Pex11 in peroxisome fission is well established. However, much is not known about the role of Pex25 and Pex27 in this process. The elongated membrane is then constricted in an unknown manner. The final step of fission is mediated by the fission machinery, comprising of the dynamin-like proteins (Dnm1, Vps1), tail-anchored protein (Fis1), and adaptor proteins (Mdv1, Caf4) (Kuravi et al. 2006; Motley et al. 2008). Fission leads to the formation of nascent peroxisomes, which ultimately become mature peroxisomes after the import of membrane and matrix proteins.

The peroxins which play a vital role in peroxisome proliferation and thereby regulate the number of peroxisomes in a cell belong to the Pex11 family (Akşit and van der Klei 2018; Koch and Brocard 2011). These proteins are extremely conserved across different yeast species (Kiel et al. 2006). In *S. cerevisiae*, the Pex11 family consists of three proteins namely Pex11,

Pex25 and Pex27 (Huber et al. 2012; Jansen et al. 2021). Despite the fact that all the three proteins are involved in controlling peroxisome number in *S. cerevisiae*, each protein appears to have a unique function in this process. Pex11 facilitates the proliferation of pre-existing peroxisomes and thus helps in the maintenance of peroxisome number in the cell. Pex25 appears to contribute to the *de novo* biogenesis of peroxisomes from ER, whereas Pex27 may have a negative effect on peroxisome function (Huber et al. 2012).

Pex11 (YOL147C) is an integral membrane protein which was first reported to be involved in peroxisome proliferation (Baerends et al. 2000; Erdmann and Blobel 1995; Marshall et al. 1995). Studies in various yeast models have reported that deletion of *PEX11* results in reduced number of enlarged peroxisomes, whereas *PEX11* overexpression led to an increase in peroxisome number (Erdmann and Blobel 1995; Joshi et al. 2012; Krikken et al. 2009). Loss of Pex11 was associated with altered morphology of peroxisomes in *Yarrowia lipolytica* and *O. polymorpha* (Cepińska et al. 2011; Chang et al. 2015). Pex11 has also been linked to the segregation of peroxisomes during cell division in *O. polymorpha* (Krikken et al. 2009). Pex11 and Pex34 were found to be crucial in the development of peroxisome-mitochondria contact sites in *S. cerevisiae*, whereas Pex11 has been connected to peroxisome-ER contact sites in *O. polymorpha* (Shai et al. 2018; Ušaj et al. 2015; Wu et al. 2020). Moreover, Pex11 was also reported to be involved in fatty acid oxidation in *S. cerevisiae* and maintenance of lipid homeostasis in *Y. lipolytica* (Dulermo et al. 2015; van Roermund et al. 2000).

Pex25 (YPL112C) and Pex27 (YOR193W) were first characterized in *S. cerevisiae* as peripheral membrane proteins (Smith et al. 2002; Tam et al. 2003). These two proteins exhibit extensive sequence similarity to each other and also to Pex11 (Smith et al. 2002; Tam et al. 2003). However, the literature available till date is not sufficient to understand the exact role of Pex25 and Pex27 in peroxisome biogenesis and regulation of peroxisome number. Cells deleted for *PEX25* displayed distinct growth defect on oleate medium (Smith et al. 2002). Cells

lacking both *PEX11* and *PEX25* also displayed growth defect on oleate medium similar to *pex11* cells and exhibited enlarged peroxisomes (Tam et al. 2003). However, the peroxisome number and morphology in *pex11* cells was restored upon overexpression of *PEX25* and the cells displayed the presence of numerous clustered peroxisomes (Tam et al. 2003). Interestingly, the concomitant absence of Pex27 in *pex11* cells (*pex11pex27*) restored the ability of these cells to utilize and grow on oleate medium, thereby suggesting a negative effect of Pex27 on peroxisome function (Huber et al. 2012). *S. cerevisiae* cells deleted for all the three genes (*PEX11*, *PEX25* and *PEX27*) resulted in fewer number of enlarged peroxisomes, thereby confirming the role of these proteins in the regulation of peroxisome size and number (Rottensteiner et al. 2003). Huber and colleagues also reported a role of Pex25 in *de novo* formation of peroxisomes from ER in cells devoid of functional peroxisomes (*pex3*) (Huber et al. 2012). According to another study, only Pex25, but not Pex11 and Pex27, localised to the pre-peroxisomal vesicles (PPVs) in *pex3* cells, which indicates a possible role of Pex25 in PPV maturation from ER (Akşit and van der Klei 2018; Wróblewska et al. 2017). In addition, Pex25 was reported to be involved in actin assembly by the recruitment of GTPase Rho1 to the peroxisome membrane (Marelli et al. 2004). Differential distribution of Pex11 and Pex25 was also observed upon peroxisome inheritance in *O. polymorpha* cells (Cepińska et al. 2011). A genome-wide screen in *S. cerevisiae* revealed a potential relevance of *PEX25* to yeast chronological lifespan and classified *pex25* cells as long lived mutants (Fabrizio et al. 2010).

All the three members of the Pex11 family (Pex11, Pex25 and Pex27) were reported to self-interact with each other (Tam et al. 2003). An interaction between Pex25 and Pex27 was also detected (Tam et al. 2003). It was observed that regulation of peroxisome number by the peroxin Pex34 required its interaction with Pex11, Pex25 and Pex27 (Tower et al. 2011). Interactions of Pex25 with Pex14 (required for peroxisome protein import) and Inp1 was also reported (Fagarasanu et al. 2005). An interesting study by Agarwal and colleagues revealed a

role for Pex25 in strengthening the interactions between Pex19 and different peroxisomal membrane proteins (PMPs), thereby contributing to the process of peroxisome biogenesis (Agarwal et al. 2017).

1.8 Perspectives on future work

Extensive research in yeast over the past few decades has tremendously improved our understanding of cellular ageing. Yeast has proven to be a useful model for the initial discovery and characterization of some of the most promising longevity variables and prospective anti-ageing medications. For example, rapamycin and resveratrol, the two compounds that were first reported to delay ageing in yeast, have moved to clinical trials for disorders associated with ageing (Kaeberlein 2010). However, a significant issue is to understand the interplay of ageing-associated damage in various organelles and defining the underlying molecular pathways.

Peroxisomes are one such vital organelle required for the optimum functioning of a cell. Alterations in several peroxisome functions have been observed in age-related disorders. Impairment in the processes of peroxisome inheritance, protein import, and β-oxidation of fatty acids were also reported to result in cellular ageing in yeast.

Several questions that still need to be addressed for a better understanding of the molecular mechanisms underpinning the involvement of peroxisome dysfunction in yeast ageing are as follows:

- Does peroxisome abundance influence yeast chronological and replicative ageing?
- What is the effect of ageing on peroxisome morphology and function?
- Does ageing effect peroxisome communication with other cellular organelles, thereby effecting their function?

1.9 Motivation of the research work

A characteristic hallmark of eukaryotic cells is the presence of dynamic membrane-bound compartments called organelles. These sub-cellular structures perform various essential functions and regulate the optimum functioning of a cell. One such versatile organelle is the peroxisome which plays important roles in crucial cellular functions such as ROS metabolism and β-oxidation of fatty acids, making it indispensable for human health and development. These organelles have an interesting ability to adapt their number, size and metabolic functions in response to the physiological needs of the cell. Interestingly, altered shape, number and functions of these organelles is associated with developmental and neurodegenerative disorders. Peroxisome division/proliferation is one such mechanism that maintains organelle homeostasis under different conditions.

Interestingly an association of altered peroxisome protein import, inheritance and biogenesis with ageing has been reported in yeast. In line with the connection between peroxisomes and age-associated disorders, a role for optimum number of peroxisomes can be envisaged for proper functioning of cells. Yeast has been an instrumental model organism in identifying conserved cellular processes in ageing and age-associated disorders. Pex11 protein family has been extensively studied for their role in regulating peroxisome number in various organisms. Yeast has three proteins that belong to this family: Pex11, Pex25 and Pex27. However, not much is known as to how peroxisome proliferation regulated by these proteins influences both replicative and chronological ageing of yeast cells. Our literature review suggests an interesting link between functional peroxisomes and cellular ageing. However, the molecular details of this connection are not completely understood. Therefore, it is important to decipher these details for a better understanding of the complex process of ageing. This may in future also provide new insights to develop therapeutic strategies for the treatment of conditions linked to cellular ageing.

1.10 Objectives of the thesis

Based on the available literature and the potential for future research in understanding the role of altered peroxisome dynamics in yeast ageing, the following objectives were formulated:

i. To understand the peroxisome dynamics in the replicative and chronological ageing of WT *S. cerevisiae* cells.
ii. To understand peroxisome dynamics in the replicatively aged yeast cells deleted for Pex11 family proteins.
iii. To understand the effect of altered peroxisome number on chronological ageing of yeast cells deleted for Pex11 family proteins.

1.11 Organization of the Ph.D thesis

Chapter 1 provides an introduction about ageing and the models of yeast ageing. This chapter also gives a brief overview about several age-associated alterations in organelles. The proteins which regulate the number and size of peroxisomes in yeast cells is also discussed in details in this chapter.

Chapter 2 includes a detailed description of the materials and methods used to perform the experiments. The name of the manufacturers for the chemicals and other components used is mentioned and all the steps involved in each of the methods is also outlined exclusively.

In ***Chapter 3,*** the replicative and chronological ageing in WT *S. cerevisiae* cells cultured under different media conditions was analysed. In addition, the morphology and number of peroxisomes was also analysed in young and replicatively/chronologically aged cells.

Chapter 4 describes a correlation between peroxisome number and early replicative age in yeast cells. Here we reported how peroxisome dynamics change upon replicative ageing using yeast as a model. The alterations in peroxisome number upon replicative ageing and the factors responsible for this were investigated. Further, peroxisome function was also analysed in young and replicatively aged cells.

Chapter 5 reports the effect of altered number of peroxisomes on the chronological lifespan of yeast cells. Various cellular parameters associated with peroxisomes altered upon chronological ageing were also investigated. The effect of these alterations on peroxisome function in young and chronologically aged yeast cells was further analysed.

Chapter 6 comprises the conclusion from the work carried out and highlights the significant findings from this study. This chapter also includes the future scope towards understanding the mechanisms that link peroxisome dynamics to replicative and chronological ageing in yeast.

Chapter 2

Materials and methods

2. 1 MATERIALS

2.1.1 Strains and plasmids

S. cerevisiae strains used in this study are listed in Table 2.1. All the deletion strains used in this study were obtained from the gene deletion library (Dharmacon) and were confirmed by polymerase chain reaction (PCR). Plasmids used in this study are listed in Table 2.2.

2.1.2 Primers

The primers used in the study are listed in Table 2.3. Clone manager 9 software was used to design the primers.

2.1.3 Culture media

The bacterial and yeast culture media components used in this study were of molecular biology grade and purchased either from HiMedia (HiMedia Laboratories Pvt. Ltd., India) or SRL (Sisco Research Laboratories Pvt. Ltd., India). The growth media for cell culture were sterilized by autoclaving at 121 °C and 15 psi for 20 mins. Heat sensitive components like antibiotics were filter sterilized using 0.22 µ membrane filter. The composition of various media used in the study are described below.

2.1.3.1 Bacterial media

Luria Bertani (LB)

5 g/L Yeast extract, 10 g/L Tryptone and 10 g/L NaCl.

For the preparation of LB agar plates, 20 g/L agar was added to the media before autoclaving. LB media was supplemented with 100 µg/ml ampicillin, whenever required. Stock solution of 100 mg/ml was prepared for ampicillin in sterile distilled water, filter sterilized and stored at -20 °C. Antibiotic was added to the autoclaved LB agar media (moderately warm) before pouring plates.

2.1.3.2 Yeast media

Yeast extract-Peptone-Dextrose (YPD) medium

1% Yeast extract, 1% Peptone, 1% Glucose

For preparation of YPD agar plates, 2% agar was added to the media.

Minimal medium (YND)

0.17% yeast nitrogen base (YNB) without amino acids and ammonium sulphate supplemented with 1% glucose and 0.5% ammonium sulphate as nitrogen source. pH was adjusted to 6.0 using KOH (Erdmann et al. 1989). Whenever required, leucine (3 mg/ml), lysine (10 mg/ml), histidine (10 mg/ml) and uracil (3 mg/ml) were added to the media. For growth on plates, 2% agar was added to the media.

Calorie restriction (CR) medium

0.17% YNB without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 0.5% glucose. Whenever required, leucine (3 mg/ml), lysine (10 mg/ml), histidine (10 mg/ml) and uracil (3 mg/ml) were added to the media. pH was adjusted to 6.0 using KOH (Erdmann et al. 1989).

Oleic acid (OA) medium

0.17% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 0.1% yeast extract, 0.05% Tween 80, 0.1% glucose and 0.1% oleic acid. Whenever required, leucine (3 mg/ml), lysine (10 mg/ml), histidine (10 mg/ml) and uracil (3 mg/ml) were added to the media and the pH was adjusted to 6.0 using KOH.

Yeast extract-Peptone-Dextrose-Glycerol (YPDG) medium

1% Yeast extract, 2% Peptone, 0.1% Glucose, 2% Glycerol

For preparation of YPDG agar plates, 2% agar was added to the media.

2.1.4 Buffers and solutions

Phosphate Buffered Saline (PBS)

137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4. The pH was adjusted to 7.2 with HCl. A 10X stock solution was prepared which was further diluted to 1X working solution.

Tris-Acetic acid-EDTA (TAE) buffer

40 mM Tris base, 0.5 M EDTA. The final pH of the buffer was adjusted to 8 with glacial acetic acid. A 10X solution was prepared as a stock which was then diluted to 1X before use.

Tris-EDTA (TE) buffer

10 mM Tris-Cl (pH 8.0), 1 mM EDTA

A 10X solution was prepared as stock which was further diluted to 1X and autoclaved before use.

Yeast transformation buffer

1 M Lithium acetate (LiAc), 50% Polyethylene glycol (PEG 3350), 100 mM Tris-Cl (pH 7.5) with 10 mM EDTA.

Cobalt nitrate reagent (working solution for catalase assay)

70 mM Cobalt (II) nitrate hexahydrate ($Co(NO3)_2 \cdot 6H_2O$), 20 mM Sodium hexametaphosphate (Graham salt), 1 M Sodium bicarbonate. All these components were added in the order mentioned and the volume was made up with water. The solution was then mixed well after preparation.

Hydrogen peroxide (H_2O_2) solution

100 mM H_2O_2 dissolved in 50 mM potassium phosphate (KPi) buffer (pH 7.0).

2. 2 METHODS

2.2.1 Microbiological techniques

2.2.1.1 Bacterial strains and growth conditions

Escherichia coli DH5α cells were used for cloning purposes. The cells were cultured at 37 °C in LB medium and the medium was supplemented with ampicillin (100 µg/ml) whenever required. The strains were stored in glycerol (850 µl cell culture and 150 µl 87% glycerol) at -80 °C. Cells stored as glycerol stocks were revived by streaking them into agar plates containing antibiotic marker.

For plasmid isolation, bacterial cells containing selection marker (ampicillin) were inoculated in LB medium with appropriate antibiotic and allowed to grow overnight at 37 °C and 180 rpm. The overnight cultured cells were then used for the isolation of the plasmids.

2.2.1.2 Bacterial transformation by calcium chloride ($CaCl_2$) method

E. coli DH5α cells were grown overnight to saturation in 5 ml of LB medium at 37 °C and 180 rpm. The overnight cultured cells were then diluted to 20 ml fresh LB medium at $OD_{600\ nm}$ = 0.1 and allowed to grow. After the cells reached an OD_{600} of 0.3-0.5, 2 ml cells were pelleted by centrifugation at 14,000 rpm for 30 secs. The cell pellet was then suspended in 1ml of 0.1 M ice-cold $CaCl_2$ and incubated for 45 mins on ice followed by centrifugation at 14,000 rpm for 30 secs. The cell pellet was carefully re-suspended in 100 µl of 0.1 M ice-cold $CaCl_2$ by pipetting while keeping the cells on ice. Plasmid DNA was then added to the cells and the cell-DNA mixture was incubated on ice for 30 mins followed by a brief heat shock at 42 °C for 105 secs. The cells were then immediately transferred to ice for 1 min. 1 ml of LB medium was added to the cells and they were incubated at 37 °C and 180 rpm for 1 hr. Cells (1 %, 10% and 89%) were then plated on selective plates with the appropriate antibiotic (ampicillin). Colonies were visible on the plates after incubation at 37 °C for 16-24 hrs.

2.2.1.3 Yeast strains and culture conditions

S. cerevisiae cells were grown at 30 °C and 200 rpm overnight and shifted to fresh medium. The cells were pre-cultured twice and allowed to reach an exponential growth phase for all the routine experiments. The yeast strains were stored in glycerol (600 µl cell culture and 400 µl 87% glycerol) at -80 °C. Cells stored as glycerol stocks were revived by streaking them into agar plates containing amino acids or antibiotic marker. WT BY4742 and the cells with gene deletions were streaked into YPD plates. Colonies from the plates were inoculated in liquid media and cultured overnight. The cells grown till stationary phase were pre-cultured twice and then shifted to fresh media for further experiments.

Yeast culture conditions for determination of replicative age

Mother cells for determining replicative age were obtained as described in Jin et al. 2021 with some modifications (Fig. 2.1). Briefly, yeast cell cultures were grown overnight in YND media. The cultures were then diluted twice at OD_{600nm} = 0.1 in fresh media and grown till they reached OD_{600nm} = 1. Cells (4 OD_{600nm}) were washed twice at 3000 g for 5 mins at room temperature with sterile 1X PBS and cell pellets were re-suspended in 0.5 ml of 1X sterile PBS. 0.5 mg of EZ-Link Sulfo-NHS-LC-LC-Biotin (Invitrogen) was dissolved in 0.5 ml of 1X sterile PBS and combined with the re-suspended cell pellet for a final reaction volume of 1 ml. The biotin labelling reaction was carried out at room temperature for 30 min with shaking, followed by washing twice in 1X sterile PBS. A fraction of biotinylated cells was collected as log phase (0 hrs) sample and the remaining cells were transferred in fresh 200 ml YND and OA media and cultured under normal conditions (30 °C, 200 rpm) for 24 hrs. Magnetic streptavidin beads (Pierce, Invitrogen) were washed twice and re-suspended in ice-cold 1X sterile PBS. At different time points (8, 16, 24 hrs), biotinylated cells were collected, washed twice with sterile 1X PBS and cell pellets were dissolved in 10 ml of PBS. These cells were then incubated with the magnetic streptavidin beads at 4 °C with shaking for 2 hrs. The beaded

mother cells were collected with the help of a magnetic separation stand (Promega). The supernatant (containing the daughter cells) was carefully discarded. Biotin labelling in the cells was confirmed by adding fluorescein-conjugated avidin (Av-FITC) to the biotinylated cells and examining them under fluorescence microscope. The mother cell pellet was further processed for microscopic analysis and other assays. The mother cells collected after 24 hrs were re-inoculated in fresh medium (YPD, YND and OA) and allowed to grow for another 24 hrs (Lam et al. 2011). These cells were then separated with the help of magnetic streptavidin beads to enrich the mother cells and processed for further experiments.

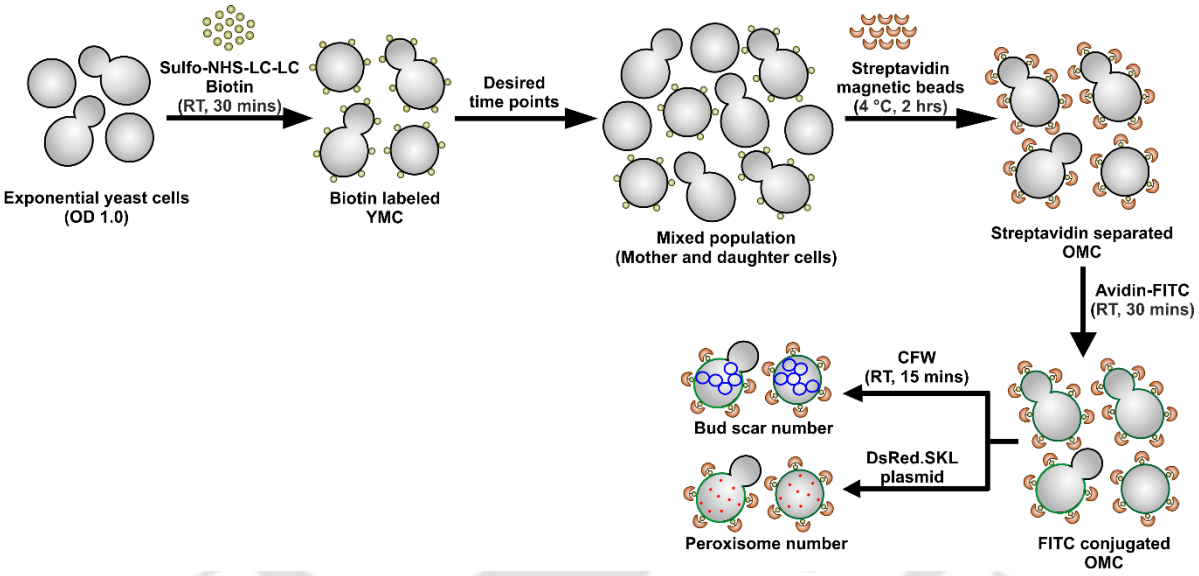

Fig. 2.1 Schematic representation of the biotin-streptavidin affinity-based method of isolating replicatively aged mother cells.
Exponentially grown cells labelled with biotin were shifted to fresh YND or OA medium to allow further cell division. Samples were then collected at desired time points and aged mother cells were separated with the help of streptavidin magnetic beads. Biotin labelling is confirmed by adding Av-FITC to these cells. Further the number of bud scars (stained with CFW) and peroxisomes (marked with DsRed-SKL plasmid) was analysed in these cells.

Yeast growth conditions for CLS assessment

Yeast strains were grown overnight in CR medium and were diluted to $OD_{600\ nm}$ of 0.1 in fresh CR medium twice. After the last pre-culture, cells were diluted in 100 ml CR and OA media to $OD_{600\ nm}$ = 0.1. As maximum cell density is reached after 48 hrs of growth in minimal media, thus 3rd day after inoculation was considered as day 0 of chronological lifespan. Survival was determined at days 0, 3, 7, 11, 15, 19, 23 and 27 either by counting CFUs or by

viability determination using PI staining (Fig. 2.2). Survival at 0th day was considered as 100% of survival.

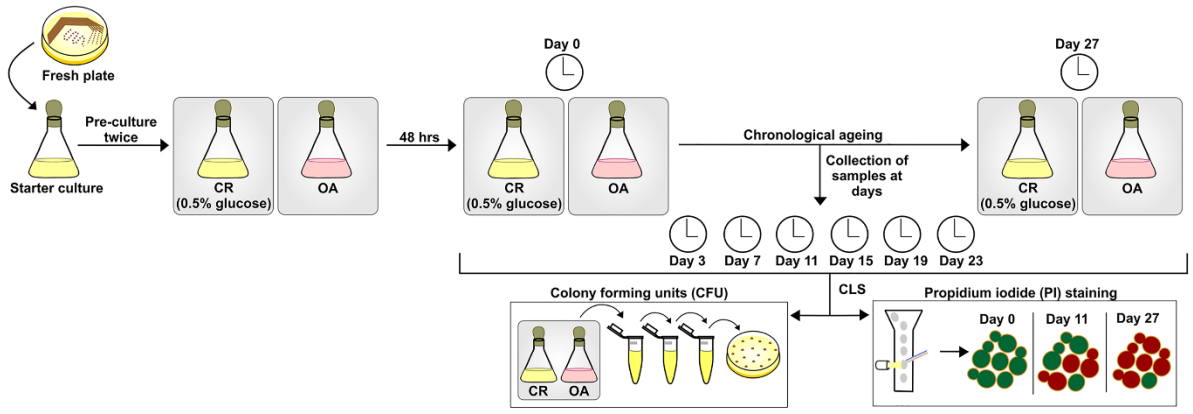

Fig. 2.2 Schematic representation of the CLS assessment method used in this study.
Yeast cells were inoculated from a fresh plate and cultured overnight in CR media (0.5% glucose). Cells were further pre-cultured twice in the same media and finally shifted to 100 ml of CR (and OA) media. After the cells reached maximum cell density (48 hrs), the 3rd day was considered as day 0 of chronological ageing. CLS was then determined every 4 days by calculating percentage cell viability using CFU assay and PI staining followed by flow cytometry.

2.2.1.4 Yeast transformation by lithium acetate (LiAc) method

Yeast transformation was performed using the lithium acetate (LiAc) method (Gietz and Akio 1988). A loop-full of yeast cells from a freshly streaked plate were put in a sterile Eppendorf tube with the help of a sterile loop. The cells are then suspended in 500 µl of 40% PEG (3350) in LiAc/TE by pipetting or vortexing for few secs. 5-6 µl of denatured herring sperm DNA and 2-3 µl of plasmid DNA or 5 µl of PCR product was then added to the cells. The cell mixture was then incubated for 30 mins at 30 °C with shaking. This was followed by the addition of 40 µl DMSO and a heat shock at 42 °C for 5 mins. The cells were then harvested by centrifugation at 5000 rpm for 2 mins. The cell pellet was finally suspended in 100 µl of 1 M sorbitol and the complete suspension was plated on selective plates. Colonies were visible on the plates after incubation at 30 °C for 2-3 days.

2.2.2 Molecular biology techniques

2.2.2.1 Yeast genomic DNA (gDNA) isolation

Yeast cells from a freshly streaked plate were collected and mixed with 30 µl of 0.2% sodium dodecyl sulphate (SDS). The cell-SDS mixture was then vortexed for 15 secs and incubated at 80 °C for 5 mins. The cells were then centrifuged at 13,000 g for 1 min. The supernatant containing the gDNA was isolated to a new tube and stored at -20 °C.

2.2.2.2 Polymerase chain reaction (PCR)

10 ng of DNA was amplified using specific primers (Table 2.3) and Taq DNA polymerase in a 50 µl reaction volume. Taq DNA polymerase required an initial denaturation step of 95 °C for 5 mins before the start of thermal cycle. The PCR was run for 30 cycles according to the following protocol: denaturation at 95 °C for 1 min, annealing at 56 °C to 68 °C for 1 min followed by an extension step at 72 °C for 1.30 mins. After completing all these steps, a final extension step was performed at 72 °C for 10 mins. All the amplified products were then run on agarose gel for visualization.

2.2.2.3 Gel extraction and PCR purification reaction clean-up

The DNA from excised gel fragments and amplified PCR product was extracted and purified respectively by using the Macherey-Nagel (MN) Nucleospin® gel and PCR clean-up kit. All procedures were followed as per the manufacturer's instructions. (https://www.mn-net.com/media/pdf/02/1a/74/Instruction-NucleoSpin-Gel-and-PCR-Clean-up.pdf)

2.2.2.4 Plasmid isolation

Plasmids from bacterial cells were isolated using the MN Nucleospin® plasmid isolation kit. *E. coli* DH5α cells containing the plasmids were revived from glycerol stocks by streaking them on a fresh LB-agar plate containing the appropriate antibiotic (ampicillin). Single colony from the agar plate was then inoculated in 5 ml LB-ampicillin liquid medium

and allowed to grow overnight at 37 °C and 180 rpm. The overnight cultured cells were centrifuged at 11,000 g for 30 secs and plasmid DNA was isolated according to the protocol provided by the manufacturer.

(https://www.mn-net.com/media/pdf/45/51/02/Instruction-NucleoSpin-Plasmid.pdf)

2.2.2.5 Restriction digestion and ligation

The desired regions from the plasmids were obtained by digesting them with appropriate set of restriction enzymes. The digested products were then ligated by using T4 DNA ligase. The ligation mixture consisting of the digested products, ligase and reaction buffer was incubated at 16 °C for 16 hrs and then transformed to bacterial cells.

2.2.2.6 Construction of pRS6-DsRed-SKL plasmid

pRS6-DsRed-SKL plasmid, which was used for visualizing peroxisomes, was constructed as follows: a fragment of 726 bp (comprising of DsRed-SKL) was obtained by restriction digestion of the plasmid pUG34-DsRed-SKL (Kuravi et al. 2006) with enzymes *Bam*HI and *Xho*I. Restriction digestion of the plasmid pRS6-GFP-SKL (Kuravi et al. 2006) with the same enzymes resulted in fragments of sizes 784 bp (containing the GFP-SKL region) and 5453 bp. The 726 bp DNA fragment of pUG34-DsRed-SKL and the 5453 bp fragment of pRS6-GFP-SKL was extracted with the help of gel extraction kit. This was followed by the ligation of both the extracted DNA fragments to construct pRS6-DsRed-SKL with URA3 selection marker. The constructed plasmid was confirmed by restriction digestion by enzymes *Pst*I and *Stu*I. Both the enzymes have one cut site each at the DsRed-SKL region and the vector backbone. Digestion with *Stu*I will give fragments of sizes 4478 bp and 1701 bp, whereas digestion with *Pst*I will give fragments of sizes 3928 bp and 2251 bp (Fig. 2.3).

2.2.2.7 Construction of *pex11pex25* strain

The *pex11pex25* double deletion strain was constructed by replacing the genomic region of *PEX25* with the NAT region of plasmid pFA6a-natNT2 (Janke et al. 2004b) by

homologous recombination (Fig. 2.4). *pex11* strain was transformed with the amplified PCR product obtained using primers pr-RDE019 and pr-RDE020, and plasmid pFA6a-natNT2 as the template. Nourseothricin-resistant colonies were selected and proper integration was further confirmed by PCR using primers pr-RDE021 and pr-NMD028 and genomic DNA as a template (Fig. 2.4).

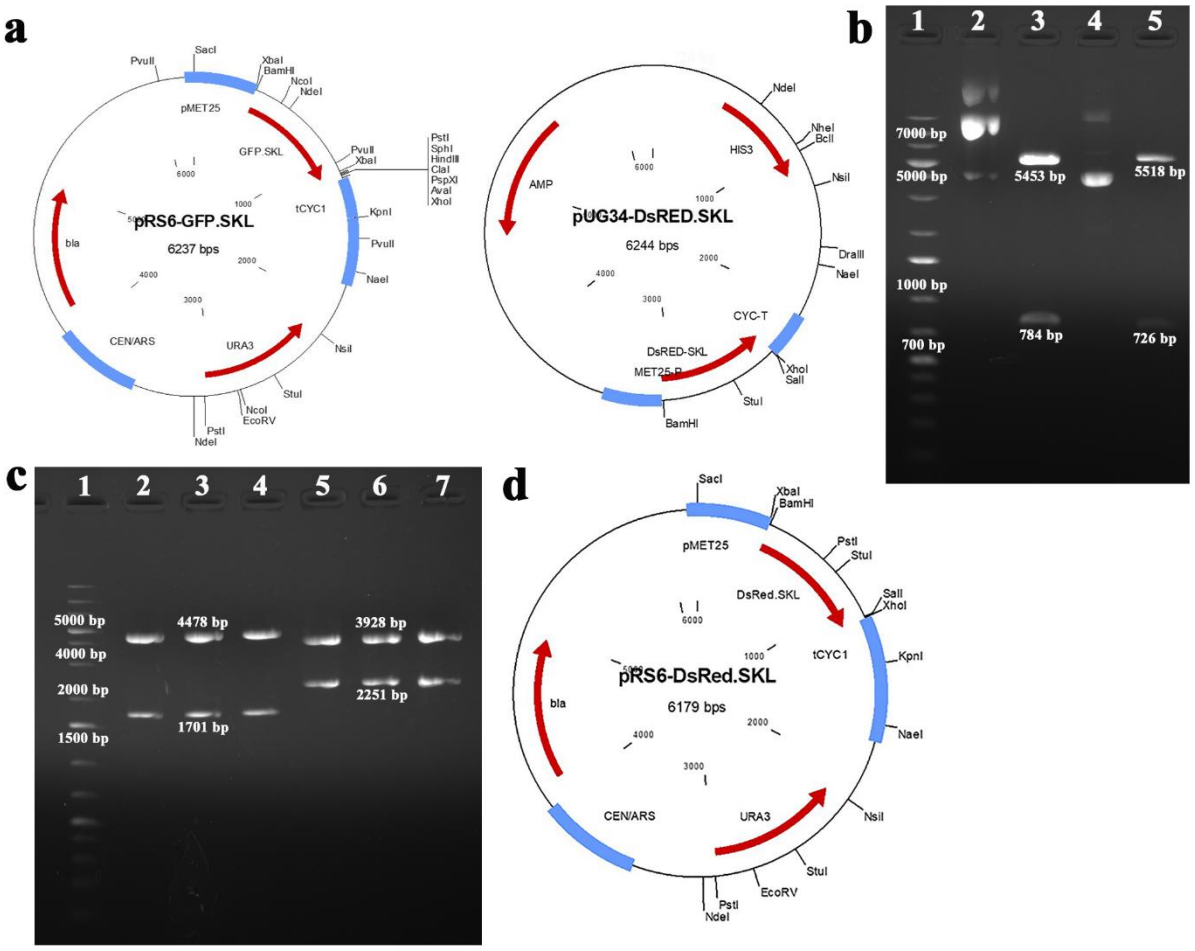

Figure 2.3: Construction of pRS6-DsRed-SKL plasmid.
(a) Plasmid map of pRS6-GFP-SKL and pUG34-DsRed-SKL plasmids. **(b)** Lane 1 shows the DNA ladder. Lane 2 and 4 of the agarose gel image shows the undigested pRS6-GFP-SKL and pUG34-DsRed-SKL plasmids, respectively. Lane 3 and 5 shows the bands obtained upon digesting pRS6-GFP-SKL and pUG34-DsRed-SKL plasmids with *Bam*HI and *Xho*I. **(c)** Screening and confirmation of the colonies containing the ligated product by digestion with *Stu*I and *Pst*I. Lane 2 to 4 represents plasmid digested by *Stu*I whereas lane 5 to 7 represents digestion with *Pst*I. **(d)** Plasmid map of the final ligated product pRS6-DsRed-SKL.

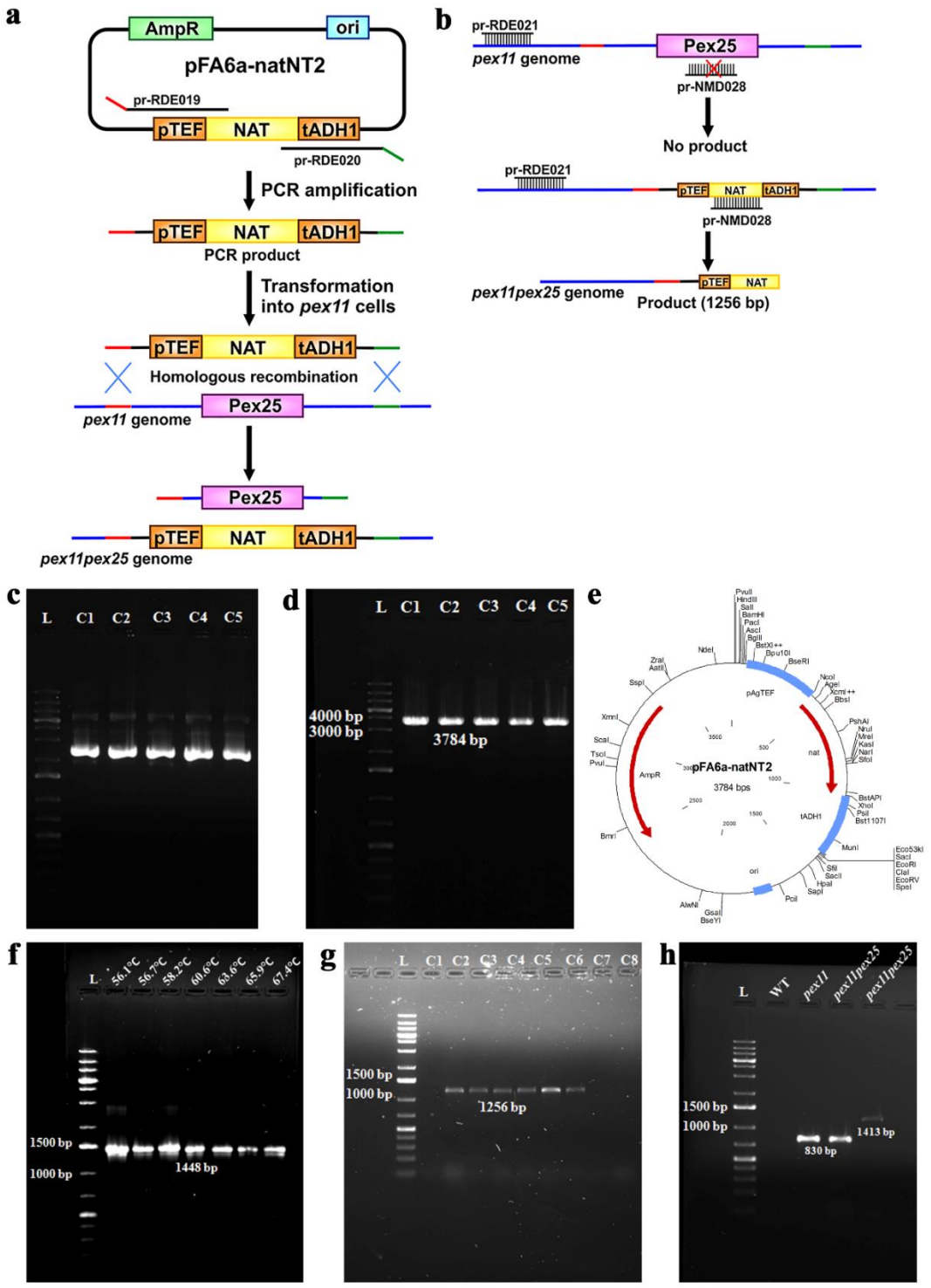

Figure 2.4: Construction and confirmation of *pex11pex25* strain.
(a) and **(b)** depict schematic representation of the strategy for construction and confirmation of *pex11pex25* strain respectively. **(c)** Agarose gel image showing the pFA6a-natNT2 plasmid isolated from different colonies. **(d)** Confirmation of the isolated pFA6a-natNT2 plasmids of size 3784 bp by *Bam*HI digestion. **(e)** Plasmid map of pFA6a-natNT2 plasmid. **(f)** Agarose gel image depicting the amplified deletion cassette which shows a single band of size 1448 bp. **(g)** Screening and confirmation of the transformed colonies. C1 contains genomic DNA from *pex11* cells and as expected does not show any band. All other colonies are with Pex25 deletion and thus yield a band of size 1256 bp. **(h)** Confirmation of *pex11pex25* strain with different set of primers. The first lane contains WT genomic DNA and thus shows no band. The second and third lanes show single bands of 830 bp

obtained by colony PCR using *pex11* and *pex11pex25* genomic DNA respectively and primers pr-SGH001 and pr-RDE012. The fourth lane shows a band of size 1413 bp obtained using *pex11pex25* genomic DNA and pr-SGH001 and pr-NMD028 primers.

2.2.3 Colony forming unit (CFU) assay

CFU assay was performed as described in Lefevre et al. 2013 (Lefevre et al. 2013). Briefly, an aliquot of cells was taken from each culture at specific time points. Serial dilutions of the cells were prepared in sterile demineralized water and 500 cells were spread on YPD plates. Subsequently, plates were incubated at 30 °C for 48 hrs and the number of viable cells per plate was counted. The number of viable cells is defined as the number of colonies growing on the plate and is termed as CFUs. The percentage of CFU is calculated as (number of viable colonies/ total number of colonies plated) x 100%. The assay was initiated from the 0^{th} day and continued till there were no visible colonies on the assay plates. The CFU percentage on the 0^{th} day was taken as a reference and set to 100%. CFUs at each time point were plotted against time to produce the chronological survival curve (Lefevre et al. 2013).

2.2.4 Staining procedures

2.2.4.1 Mother cell and bud scar staining

Cells were stained at room temperature with 10 µl FITC-Avidin (Invitrogen) for 15 mins to identify the biotinylated mother cells (Janssens et al. 2015). Subsequently, 5 µl of calcofluor white stain (Sigma Aldrich) was added for 15 mins (Azbarova et al. 2017) to visualize the bud scars at room temperature. After staining, the cells were washed twice with 1X PBS before fluorescence microscopy.

2.2.4.2 Staining of lipid droplets (LDs)

BODIPY 493/503 (Molecular Probes) was used to label the LDs as described in Bascom et al. 2003 (Bascom et al. 2003). Cells cultured in OA media were collected at different time intervals and fixed with 37% formaldehyde for 10 mins. The fixed cells were then re-suspended in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and permeabilized for 6 mins by

treating them with 0.2% Triton X-100. BODIPY was then added to the cells at a final concentration of 10 µM for 15 mins and the LDs were visualized with the help of fluorescence microscopy (Bascom et al. 2003).

2.2.5 Assessment of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was assessed in the mother cells cultured in YND using membrane potential-dependent cationic dyes tetramethylrhodamine ethyl ester (TMRE) and MitoTracker Orange. For TMRE staining, cells were washed with 1X PBS and incubated with 40 nM TMRE for 30 mins at 30 °C (Dhar et al. 2019). MitoTracker Orange was added to a final concentration of 0.1 µg/ml and incubated for 15 mins at 30 °C (Pozniakovsky et al. 2005). Subsequently cells were analysed by fluorescence microscopy and flow cytometry.

2.2.6 Fluorescence microscopy

Labelled cellular structures were visualized and images were acquired with an Olympus IX83 microscope equipped with a DP80 CCD camera and 100X/1.4 oil immersion objective. pE-300 white CoolLED light source was used to illuminate the samples. LDs (BODIPY 493/503) were visualized with the help of U-FBNA filter having excitation range of 470-495 nm, dichroic beam-splitter of 505 nm and emission range of 510-550 nm. Bud scars (calcofluor white) and peroxisomes (pRS6-DsRed-SKL) were visualized using 378/474/575 nm brightline triple-bandpass filter (Semrock) along with 409/493/596 nm dichroic beam-splitter (Semrock). Acquisition and analysis of images was performed using CellSens software V2.3 and final images were assembled using Adobe Photoshop CC. For quantitative analysis, the cells were fixed with 4% formaldehyde in PBS on ice for 15 mins. The z-stacks were spaced 0.3 µm and obtained from top to bottom of yeast cells. For quantitative analysis of the number of peroxisomes and bud scars in the replicatively aged mother cells, 200 random non-budding mother cells were counted from two independent experiments (100 in each experiment). In

chronological ageing, the number of peroxisomes and LDs were quantified in 50 random single cells from each strain from each experiment (n=2).

2.2.7 Flow cytometry

PI (Molecular Probes) staining was used to discriminate viable and dead cells (Ocampo and Barrientos 2011). Levels of intracellular ROS was measured in viable cells using 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) (Mesquita et al. 2010). Cells were collected at definite time points and incubated with a final concentration of 10 µM H_2-DCFDA for 90 mins and 2 µM PI for 30 mins at 30 °C. Cells were then washed twice with 1X PBS and analysed by flow cytometry. BD FACS Calibur Flow Cytometer (BD Biosciences) with a 488 nm excitation laser was used to analyze 50,000 replicatively aged mother cells or 100,000 chronologically aged cells per experiment at low flowrate. FCS Express 5 software was used for data acquisition and analysis. Heat killed cells (95 °C, 5 mins) and *ctt1* and *cta1* cells were taken as controls for viability staining and ROS staining, respectively.

2.2.8 Spot assay

For the determination of growth on glycerol containing medium, the mother cells of all strains cultured in YND media were pooled using magnetic separation as described above in section 2.2.1.3. Cells were centrifuged and re-suspended in sterile water to OD_{600nm} = 1. Serial dilutions of each strain (four 10-fold dilutions) were plated onto solid YPD and YPDG plates (Zubko and Zubko 2014). YPD plates were incubated for 24 hrs and YPDG plates for 48 hrs at 30 °C to visualize cell growth.

2.2.9 Catalase activity assay

Soluble protein extracts were prepared as previously described (Chen et al. 2017c) with some modifications. Cells were pelleted at 5000 g and dissolved in 0.33 M sodium phosphate buffer. The dissolved cell pellet was treated with zymolyase (G Biosciences) at 30 °C for 5 mins and centrifuged at 10,000 g for 5 mins. The pellets were re-suspended in the same buffer

and mixed with acid-washed glass beads. Cell suspensions were then vortexed (12×30 secs cycles) on ice. Cell debris was removed by centrifugation at 4 °C and the total protein concentration in the soluble protein extracts was measured by Bradford assay with bovine serum albumin (BSA) as a standard (Bradford 1976). Further, the catalase activity of these extracts was measured at 440 nm as described previously (Hadwan 2018). 0.1 ml of 100 mM H_2O_2 dissolved in 50 mM KPi buffer (pH 7.0) was used for the assay. 30 µg of cell lysates containing catalase was incubated with 100 mM H_2O_2 for 2 mins before incubating the aliquots with cobalt nitrate reagent, which measures the residual H_2O_2 (Fig. 2.5).

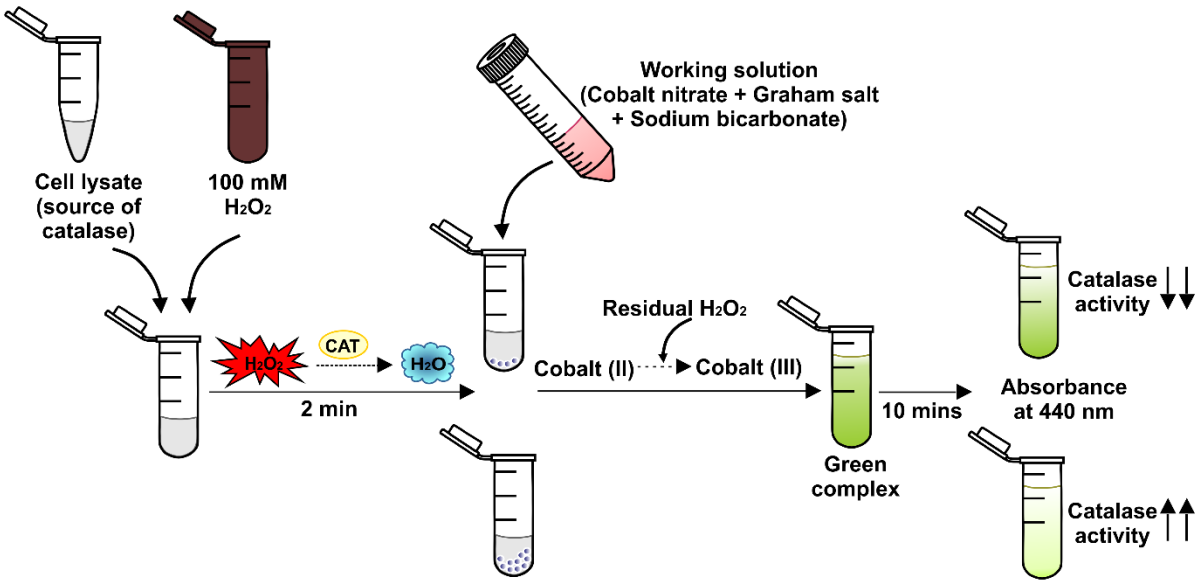

Figure 2.5: Schematic representation of the catalase activity assay.
Cell lysates containing catalase was incubated with 100 mM H_2O_2 for 2 mins. The catalase reduces H_2O_2 to water and thus bubbles are formed. Then working solution containing cobalt nitrate is added which gets oxidized by the residual H_2O_2 and thus forms a green coloured complex solution. Catalase activity is measured by taking absorbance at 440 nm.

2.2.10 Measurement of glucose and ethanol concentration

For measuring glucose and ethanol concentrations in CR conditions, 1 ml of chronologically aged yeast cells collected at different days were centrifuged and the supernatants were frozen at -80 °C. 20 µl of the supernatants were then analysed by high-performance liquid chromatography (HPLC) (SHIMADZU, Japan) equipped with RID detector and auto sampler. Aminex 87H (BioRad) column maintained at 60 °C was used as

stationary phase and 5 mM H_2SO_4 was used as mobile phase with a flowrate of 0.6 ml/min (Chang et al. 2018).

2.2.11 Measurement of free fatty acids (FFA)

Total lipids were extracted from the cells as described in Handee et al. 2016 with slight modifications (Handee et al. 2016). 3 OD_{600nm} unit cells cultured in OA media were collected on 0^{th} and 7^{th} day of the chronological ageing experiment. Cell pellets were obtained upon centrifugation and were washed with 1 ml PBS (pH 7.4) and subsequently dissolved in 300 µl of chloroform: methanol (1: 2 v/v). Glass beads were added to the cell suspension and vortexed for 15 mins, followed by 5 mins incubation on ice. The lysed cells were centrifuged at 13,000 g for 10 mins. The lower chloroform phase was collected, and the samples were vacuum dried for an hour to obtain lipids. The lipids were then dissolved in 50 µl fatty acid assay (FAA) buffer and FFAs were quantified using Free Fatty Acid Quantification Kit (Sigma Aldrich) (Wendt et al. 2019), according to the protocol provided by the manufacturer. (https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/128/465/mak044bul.pdf)

2.2.12 Statistical Analysis

All quantitative experiments reported in the manuscript were performed at least two times. Statistical analysis was performed using Student's t-test (with Welch's correction), one-way or two-way analysis of variance (ANOVA) (with multiple comparisons) using GraphPad Prism 8.2.4. The error bar indicates mean ± SEM. Values were considered significant (*) if $p < 0.05$, very significant (**) if $p < 0.01$ very significant (**), extremely significant (***) if $p < 0.001$ and very extremely significant (****) if $p < 0.0001$.

Table 2.1 *S. cerevisiae* strains used in this study

Strain	Description	Source
BY4742 WT	MATα *his3*Δ *leu2*Δ *lys*Δ *ura3*Δ	Dharmacon Inc
BY4742 WT DsRed-SKL	BY4742 WT.*URA3*::P_{MET25} *DsRed-SKL*	This study
BY4742 *pex11*	*pex11*Δ::kanMX4	Dharmacon Inc
BY4742 *pex11* DsRed-SKL	BY4742 *pex11*Δ.*URA*3::P_{MET25} *DsRed-SKL*	This study
BY4742 *pex25*	*pex25*Δ::kanMX4	Dharmacon Inc
BY4742 *pex25* DsRed-SKL	BY4742 *pex25*Δ.*URA*3::P_{MET25} *DsRed-SKL*	This study
BY4742 *pex27*	*pex27*Δ::kanMX4	Dharmacon Inc
BY4742 *pex27* DsRed-SKL	BY4742 *pex27*Δ.*URA*3::P_{MET25} *DsRed-SKL*	This study
BY4742 *pex11pex25*	*pex11*Δ::kanMX4 *pex25*Δ::natNT2	This study
BY4742 *pex11pex25* DsRed-SKL	BY4742 *pex11Δpex25Δ.URA3*::P_{MET25} *DsRed-SKL*	This study
BY4742 *ctt1*	*ctt1*Δ::kanMX4	Dharmacon Inc
BY4742 *cta1*	*cta1*Δ::kanMX4	Dharmacon Inc

Table 2.2 Plasmids used in this study

Plasmid	Description	Source
pFA6a-natNT2	amp^R; *Sc-NAT*	(Janke et al. 2004)
pRS6-GFP-SKL	P_{MET25} *GFP-SKL*; amp^R; *Sc-URA*3	(Kuravi et al. 2006)
pUG34-DsRed-SKL	P_{MET25} *DsRed-SKL*; amp^R; *Sc-HIS*3	(Kuravi et al. 2006)
pRS6-DsRed-SKL	P_{MET25} *DsRed-SKL*; amp^R; *Sc-URA*3	This study

Table 2.3 Primers used in this study

Primer	Description
pr-SGH001	5'-GAGCCACCGGATCGGTCTTT-3'
pr-RDE012	5'-CTGCAGCGAGGAGCCGTAAT-3'
pr-RDE013	5'-TGGTGAATTTGCTCGGTACG-3'
pr-RDE016	5'-GTCTCTCCTCAGAGAGGCATTG-3'
pr-RDE019	5’-CAACTTCCCTGTAAGTCTTCACCTATAGAAACTG GTCGTAAAACAATGCGTACGCTGCAGGTCGAC-3’
pr-RDE020	5’-TCGCCACATATATATGTACATATCTATATGTATA CATATTTTTATATATCAATCGATGAATTCGAGCTCG-3’

pr-RDE021	5'-TCCAGGTCGGTGATAGTA-3'
pr-NMD028	5'-AATTCGCCTCGAGATTAGGG-3'

Table 2.4 List of commercially used chemicals

Chemical	Name	Manufacturer
Enzymes	Taq DNA polymerase	New England Biolabs (NEB)
	T4 DNA ligase	ThermoFisher Scientific
	*Bam*HI	ThermoFisher Scientific
	*Xho*I	ThermoFisher Scientific
	*Stu*I	ThermoFisher Scientific
	*Pst*I	ThermoFisher Scientific
Kits	Plasmid isolation Miniprep kit	Macherey-Nagel
	PCR purification kit	Macherey-Nagel
	Gel extraction kit	Macherey-Nagel
	Protein concentration estimation kit	BioRad

Chapter 3

Altered peroxisome dynamics upon ageing in *Saccharomyces cerevisiae*

Abstract

Ageing is a degenerative process leading to gradual loss in the normal functioning of a cell. Oxidative stress due to accumulation of reactive oxygen species plays a major role in several age-related neurodegenerative disorders and thus act as one of the primary causes of ageing. Peroxisomes are single membrane-bound organelles which produce and scavenge reactive oxygen species and thus contributes to the redox balance within the cell. Their role in cellular ageing is under investigation in various model systems. Budding yeast has emerged as an attractive model organism for studying the hallmarks of cellular ageing. In yeast, there are two models of ageing termed as replicative and chronological ageing. In this study, we aimed to understand the alterations in peroxisome dynamics upon early replicative and chronological ageing in yeast cells under both peroxisome-inducing (oleic acid) and non-inducing (glucose or calorie restriction) conditions. The number of bud scars and percentage of aged cells increased with time in both the media conditions. However, cells grown in peroxisome-inducing conditions displayed higher number of bud scars. Interestingly, replicatively aged mother cells depicted increased peroxisome number in both the media conditions. The cells displayed extended chronological lifespan in oleic acid media when compared to calorie restriction media. Altered peroxisome number and morphology was also observed in the chronologically aged cells when cultured in both the media conditions.

This chapter is a part of

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Deb R, Nagotu S (2022) The nexus between peroxisome abundance and chronological ageing in *Saccharomyces cerevisiae*. Biogerontology. https://doi.org/10.1007/s10522-022-09992-9

3.1 Introduction

Ageing is an unavoidable degenerative process which results in gradual loss of cellular productivity over time and is associated with several physiological changes (Banerjee et al. 2020). Budding yeast *S. cerevisiae* is an attractive eukaryotic model organism for studies related to ageing, owing to its simplicity, the ease in performing genetic and molecular manipulations, and its relatively short lifespan (He et al. 2018). Interestingly, yeast can be used to study two types of ageing processes, namely replicative ageing and chronological ageing (Fig. 1.2) (Banerjee et al. 2020). CLS is the measure of time a population of non-dividing yeast cells can remain viable in the stationary phase (Fabrizio and Longo 2003), whereas RLS is the number of daughter cells produced by an individual mother cell before its death (Müller et al. 1980). Yeast RLS and CLS studies are relevant as conserved mechanisms in humans are associated with ageing of asymmetrically dividing mammalian cells like fibroblasts and lymphocytes and non-dividing cells such as neurons, respectively (Denoth Lippuner et al. 2014).

A combination of genetic and environmental factors and various disease conditions act as contributors to age-associated cellular changes (Karol 2009; López-Otín et al. 2013; Schmidt 2019). Oxidative stress that occurs due to ROS imbalance within the cell is one of the major causes of ageing and age-related neurodegeneration and thus increases the rate of morbidity and mortality (Anik et al. 2022; Liguori et al. 2018). Peroxisomes are single membrane-bound ubiquitous organelles essential in maintaining redox balance in the cell. These organelles produce and scavenge intracellular ROS as they contain oxidases and the antioxidant enzyme catalase. Enhanced accumulation of ROS upon ageing has been reported in various model organisms including yeast highlighting the role of these organelles in this process (Ayer et al. 2014). Increased number of peroxisomes in aged fibroblasts was reported suggesting their role in the accumulation of ROS (Legakis et al. 2002). Recently, several studies in yeast have also

pointed towards a role for peroxisomes in both CLS and RLS (Kumar et al. 2018; Lefevre et al. 2013; Scheckhuber 2020).

Earlier studies have analysed RLS and CLS of *S. cerevisiae* WT cells in various growth media. Variation in carbon source was reported to influence yeast lifespan (Kumar et al. 2018; Lefevre et al. 2013; Scheckhuber 2020; Smith et al. 2007; Van Zandycke et al. 2002). Reduced RLS and CLS was reported when ethanol was used as a carbon source (Orlandi et al. 2013; Van Zandycke et al. 2002). Studies have also reported extended RLS and CLS in CR conditions (Arlia-Ciommo et al. 2018; Goldberg et al. 2009b; Kaeberlein et al. 2004). CLS of *S. cerevisiae* WT cells was also analysed in growth media containing carbon sources such as glucose, glycerol, oleate, dextrose, sucrose, maltose, trehalose and raffinose (Burtner et al. 2009; Fabrizio and Longo 2007; Lefevre et al. 2013; Scheckhuber 2020; Smith et al. 2007). Extended CLS (mean and maximal lifespan) of WT cells was observed in cells cultured in peroxisome-inducing oleate media (Lefevre et al. 2013; Scheckhuber 2020). However, the effect of ageing on peroxisome dynamics has not been studied in detail.

Hence, this study aimed at understanding how peroxisome dynamics change upon replicative and chronological ageing in different growth conditions using yeast as a model organism. Our data suggests increased peroxisome number as a marker for early replicative ageing. Cells cultured in peroxisome-inducing conditions exhibited enhanced CLS. An overall change in peroxisome morphology and number was observed upon ageing.

3.2 Results

In yeast, a bud scar is left on the mother cell wall with each cell division. Therefore the number of bud scars is directly proportional to the replicative age of mother cells (Powell et al. 2003). The replicatively aged WT yeast mother cells were obtained using biotin-streptavidin-dependent magnetic sorting, as mentioned in section 2.2.1.3 (Jin et al. 2021). Exponentially grown cells labelled with biotin (0 hr) were shifted to fresh YND or OA medium to allow

further cell division. Aged mother cells were then collected at different time points (8, 16, 24 hrs) (Fig. 3.1) (Wang et al. 2014). In yeast, abnormal morphology of the mitochondrial network and increased production of ROS were reported already after 4 cell divisions (Lam et al. 2011; Sorokin et al. 2014). Pronounced changes were reported to occur in yeast mother cells during their first ten budding events (Knorre et al. 2018). Taking these studies into account and to analyse if similar alterations are also observed with respect to peroxisomes in early replicative age, we categorised mother cells collected at 8, 16 and 24 hrs into two groups – cells with zero to one bud scar as young mother cell (YMC) and with four or more than four bud scars as old mother cell (OMC).

Further, in order to recover definitively old cells, mother cells separated at 24 hrs were re-inoculated into fresh medium (YND or OA) and collected by magnetic separation for analysis. These cells were termed as enriched mother cells (EMC) (Fig. 3.1). All the aged cells were then incubated with calcofluor white that stains bud scars. Post-staining, microscopic analysis was performed, and several images were captured.

3.2.1 Number of bud scars and the percentage of aged WT cells increase with time in YND and OA grown conditions

An increase in the number of bud scars with time was observed in WT cells grown in both YND and OA media (Fig. 3.1a and 3.1b). At 0 hr, the predominant population of WT cells (67.5%) was observed to be YMC with 0 or 1 bud scar (Fig. 3.1a and 3.1b). No OMC (≥ 4 bud scars) were observed at this time point. Interestingly at 8 hrs more percentage of WT cells had ≥ 4 bud scars when cultured in YND (40.5%) as compared to OA (12%). At 16 and 24 hrs, the cells mostly had 4-9 bud scars in both the media conditions (Fig. 3.1b).

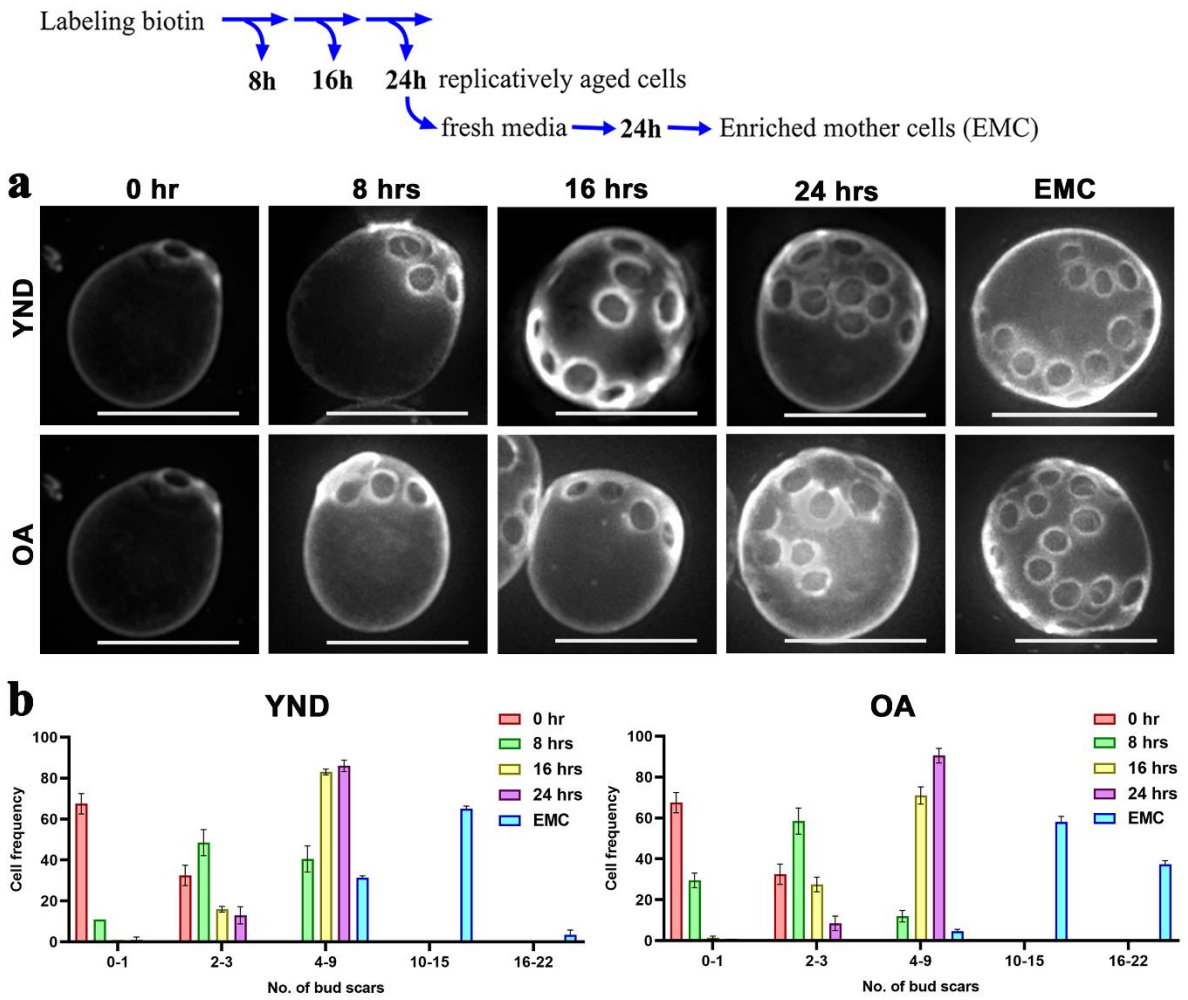

Fig. 3.1: The number of bud scars increase with time in WT cells.
(a) Representative images depicting bud scars in biotinylated mother cells of WT in YND and OA media at different time points are shown. Bud scars stained with calcofluor white were visualized in the mother cells. Scale bar represents 5 µm. **(b)** Variation in the number of bud scars in the WT cells at different time points are represented as bar graphs in YND and OA media. Cells with 0 to 1 bud scar are termed as YMC and those with ≥ bud scars are referred to as replicatively aged OMC.

As mentioned earlier, mother cells collected at 24 hrs were enriched by allowing them to grow for another 24 hrs in fresh media conditions. Following separation these cells were termed as EMC. Our enrichment method was successful as 100% of EMC of WT contained 4 or more than 4 bud scars in both the media conditions (Fig. 3.1b and 3.2a). An increase in the number of bud scars in WT EMC was observed in OA as compared to YND (Fig. 3.2a). In OA medium, more percentage of WT EMC (37.30%) had 16-21 bud scars as compared to YND (3.56%) (Fig. 3.2a). The maximum number of bud scars in WT EMC was 18 and 20 in YND

and OA media respectively. In these cells, the average number of bud scars was 9.92 ± 0.22 and 14.52 ± 0.26 in YND and OA, respectively (Fig. 3.2b).

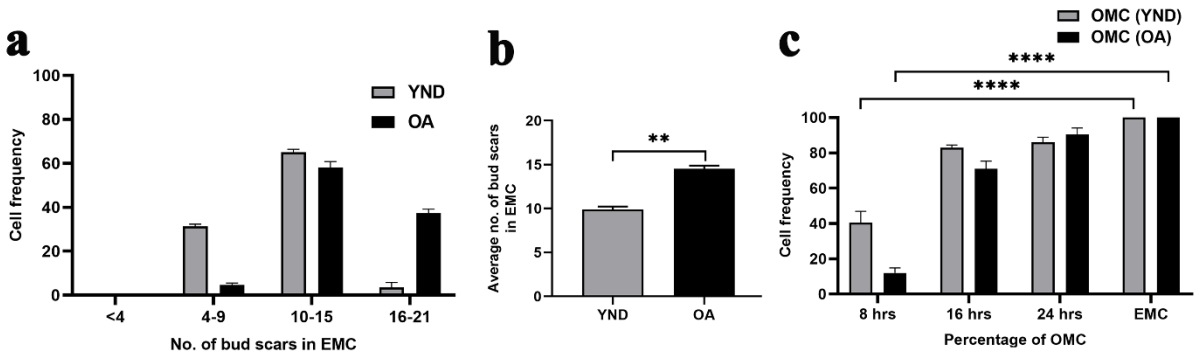

Fig. 3.2: The percentage of OMC increase with time in WT cells.
(a) Variation in the bud scar number in EMC of WT are shown in YND and OA media. **(b)** Average number of bud scars in WT EMC in both the media conditions are represented as bar graph **(c)** Percentage of WT OMC at different time points are represented in YND and OA media. Error bars indicate absolute deviations from the mean value. Significance was determined using Student's t-test (with Welch's correction) or two-way ANOVA (with multiple comparisons) from two independent experiments (n = 2). Values of P < .05 were considered significant (*), P < .01 very significant (**), and P < .001 extremely significant (***), P<0.0001 very extremely significant (****).

The percentage of OMC also increased with time in both the media conditions (Fig. 3.2c). The percentage of OMC in 8, 16 and 24 hrs increased to 40.5%, 83% and 86% in YND medium and 12%, 71% and 90.5% in OA medium (Fig. 3.2c).

3.2.2 Increased number of peroxisomes is observed in replicatively aged WT cells

The number of peroxisomes was analysed in the WT cells obtained at various time intervals in both the media conditions using DsRed-SKL as a marker (Fig. 3.3a). No significant change was observed in the peroxisome number in YMC of WT at 24 hrs in YND compared to YMC at 0 hr (Fig. 3.3b). However, the number of peroxisomes in YMC at 24 hrs increased by 2-fold in OA medium compared to YMC at 0 hr owing to the induction of peroxisomes on OA (Fig. 3.3b). On the other hand, the number of peroxisomes in OMC of WT increased with time in both the media conditions (Fig. 3.3b). The average peroxisome number in OMC of WT at 24 hrs in YND and OA medium increased by approximately 2 and 3-fold compared to the YMC at 0 hr (Fig. 3.3b). A significant increase in the average number was also observed in the

WT OMC at 24 hrs when compared to 8 hrs (Fig. 3.3b). This experiment clearly demonstrates increase in peroxisome number in OMC in both the media conditions in WT cells.

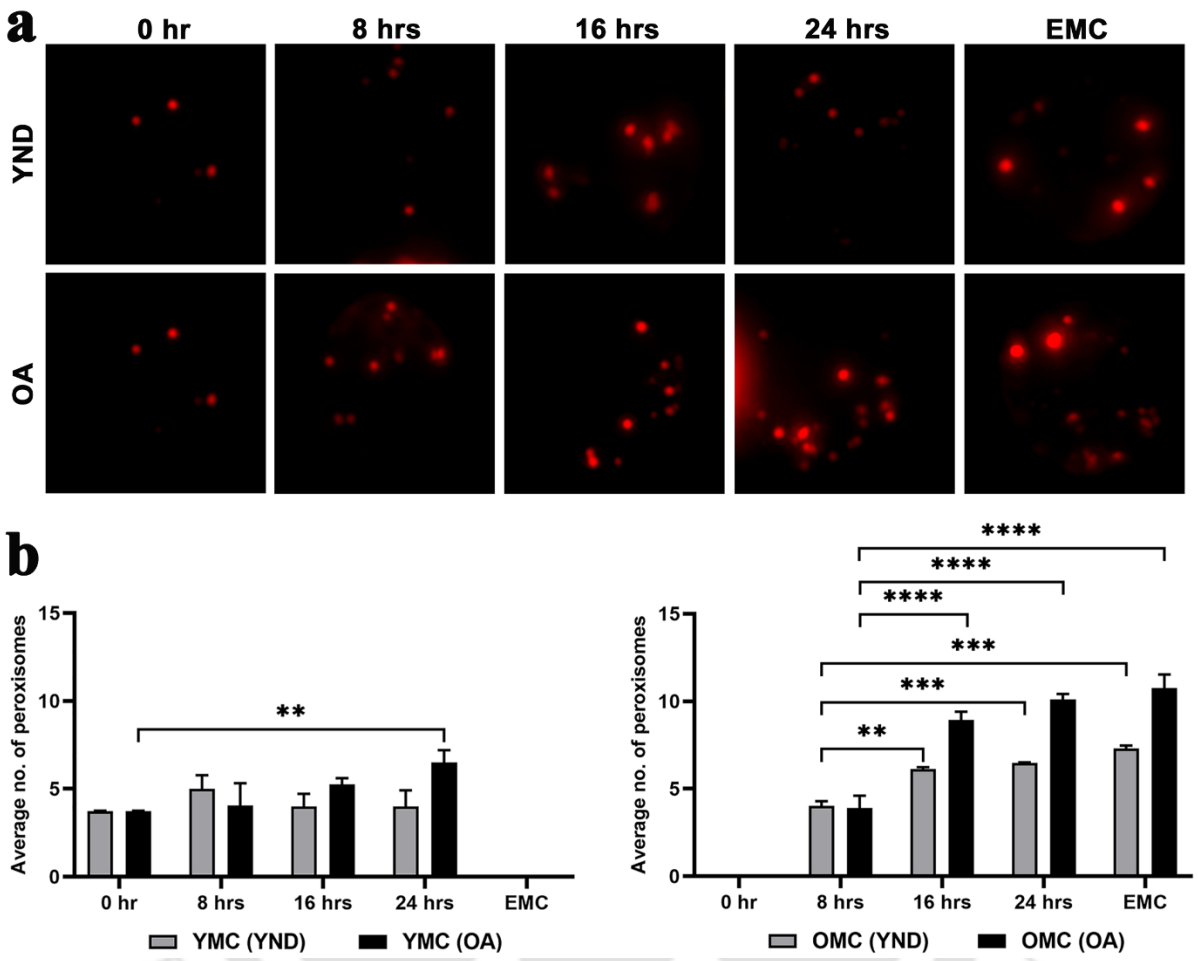

Fig. 3.3: The number of peroxisomes increase with time in WT cells.
(a) Representative images depicting the morphology of peroxisomes in biotinylated WT mother cells in YND and OA media at different time points are shown. Peroxisomes marked with DsRed-SKL were visualized in the mother cells. **(b)** The average number of peroxisomes in the YMC and OMC of WT at different time points are represented in YND and OA media. Error bars indicate absolute deviations from the mean value. Significance was determined using two-way ANOVA (with multiple comparisons) from two independent experiments (n = 2). Values of $P < .05$ were considered significant (*), $P < .01$ very significant (**), and $P < .001$ extremely significant (***), $P<0.0001$ very extremely significant (****).

Irrespective of a significant increase in the bud scar number in the EMC, no significant increase in peroxisome number was observed in the EMC of WT when compared to their respective OMC at either 16 or 24 hrs in both the media conditions (Fig. 3.3b and 3.4a). This suggests that increase in peroxisome number can be a marker of early replicative age. Our study for the first time comprehensively demonstrates the changes in peroxisome number with increase in the number of bud scars in WT yeast cells in both peroxisome-inducing and non-

inducing growth conditions (Fig. 3.4b). A positive correlation was observed between the number of peroxisomes and bud scars in both the media conditions (Fig. 3.4b).

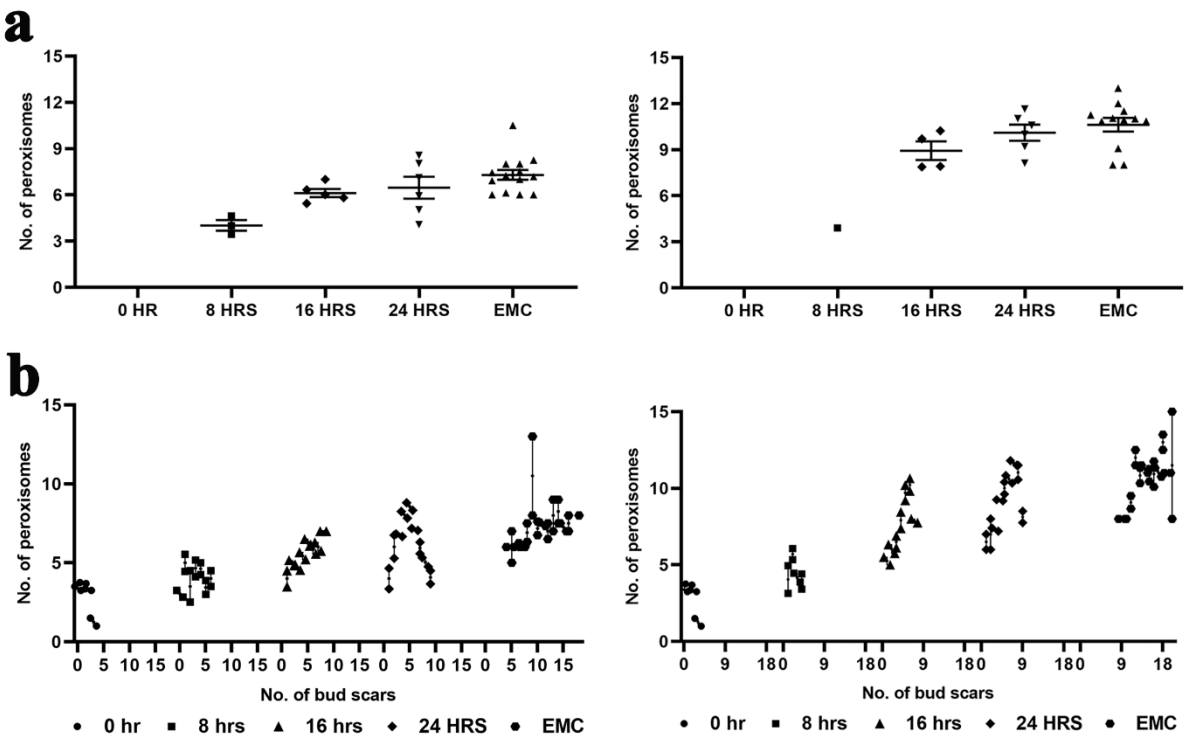

Fig. 3.4: The number of peroxisomes increase with the number of bud scars in WT cells.
(a) Variation in the number of peroxisomes with time in the OMC of WT are represented in YND and OA media. **(b)** Variation in peroxisome number with bud scars in the WT mother cells are represented in YND and OA. Error bars indicate absolute deviations from the mean value. Significance was determined using two-way ANOVA (with multiple comparisons) from two independent experiments (n = 2). Values of $P < .05$ were considered significant (*), $P < .01$ very significant (**), and $P < .001$ extremely significant (***), $P < 0.0001$ very extremely significant (****).

3.2.3 *S. cerevisiae* WT cells display extended CLS in OA media as compared to CR media

CR is a low-calorie diet that is accomplished by reducing the glucose concentration in the growth media to 0.5% or lower without compromising the supply of essential amino acids, vitamins and other nutrients (Wei et al. 2008). This dietary regimen delays ageing and results in an increase in yeast CLS (Arlia-Ciommo et al. 2018; Goldberg et al. 2009b; Kaeberlein et al. 2004; Mesquita et al. 2010). Peroxisomal proteins belonging to various classes have been studied for their effect on CLS of yeast cells in both CR and non-CR conditions. Cells lacking the peroxisome biogenesis protein Pex3 displayed CLS similar to that of WT cells in 2% glucose media (Lefevre et al. 2013). However, under CR conditions, deletion of Pex3 resulted

in reduced CLS as compared to WT (Lefevre et al. 2013). As peroxisomes are induced in OA media, to understand the role of peroxisome abundance in chronological ageing, all our CLS experiments were performed in CR and OA media.

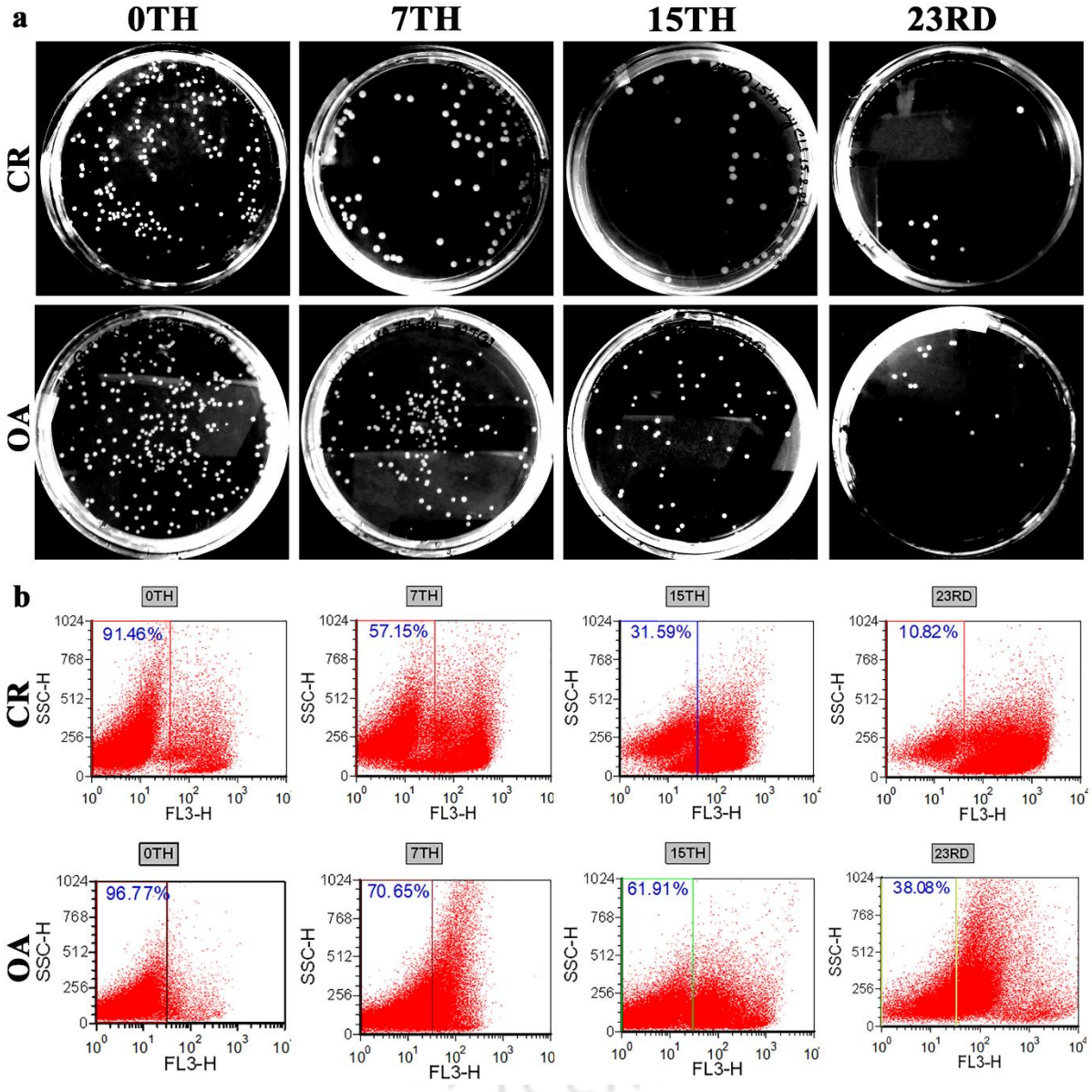

Fig. 3.5: CLS measurement by CFU assay and cell viability assessment.
(a) Representative images of the plates used for CFU assay on different days of chronological ageing in YND and OA media. Serial dilutions of the cells were prepared such that 500 cells were plated. Number of viable colonies were counted to plot the CLS curves. **(b)** Dot plots representing the percentage of viable cells at different days of chronological ageing in YND and OA media. Cells were stained with PI followed by flow cytometry and the PI negative cells (viable cells) were gated and plotted against time to assess the CLS of WT cells.

To measure the CLS of yeast cells, CFU counting and cell viability assessment of PI-stained cells were performed in this study (Fig. 3.5 and 3.6). Aliquots of cells were collected from the ageing culture, serially diluted and plated at different days of chronological ageing.

CLS curves were plotted by counting the number of viable cells (Fig. 3.5a). CLS was also measured by the assessment of cell viability by staining the cells with PI followed by flow cytometry. Percentage of viable cells were obtained from the flow cytometry plots and were plotted against time to assess the CLS (Fig. 3.5b).

WT cells displayed extended CLS in OA media when compared to YND media (Fig. 3.6a and 3.6b). The cells showed a significant increase in the mean and maximal CLS in OA (15.5 and 25 days) when compared to YND (11.5 and 21 days) (Fig. 3.6b). Similar results were also obtained from the cell viability analysis. A progressive decrease in the percentage of viable cells (PI negative) was observed on successive days of chronological ageing in both the media conditions (Fig. 3.6c). The percentage of viable cells at the end of the 27th day was 27.03% in the OA medium compared to 8.22% in CR conditions depicting extended CLS in OA-grown cells (Fig. 3.6c).

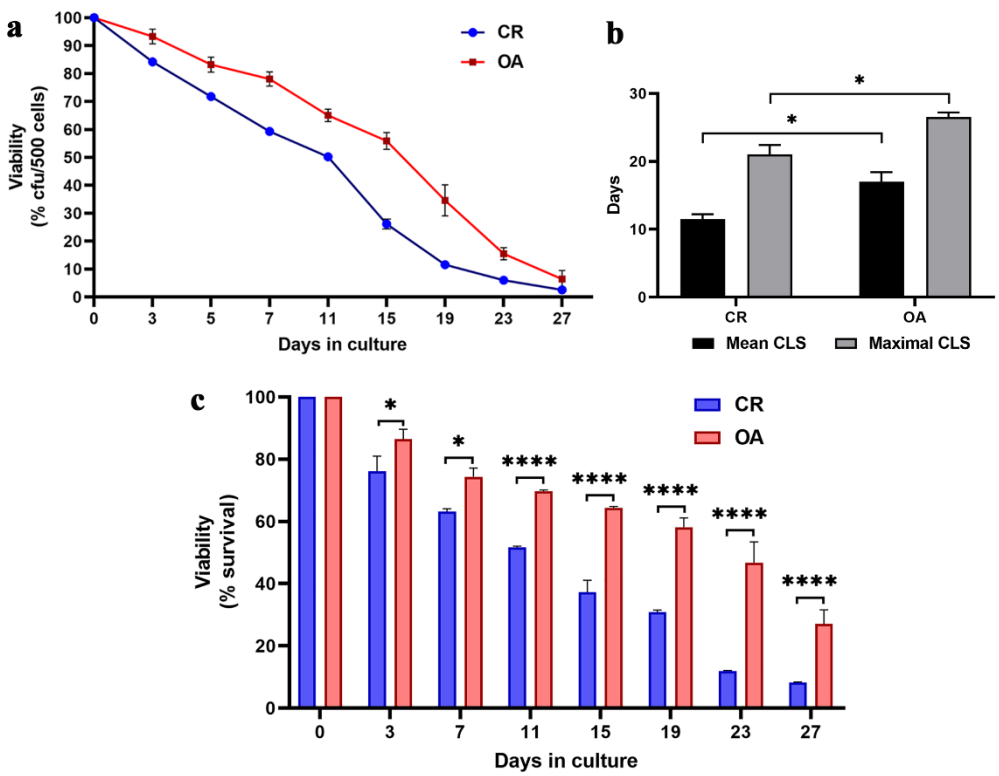

Fig. 3.6: Chronological lifespan (CLS) of WT cells in CR and OA media. (a) The CLS curves of WT cells obtained from CFU assay is represented (n=2). **(b)** The mean and maximal CLS calculated from data in (a) is depicted. **(c)** Percentage cell viability obtained from PI staining of the WT cells is represented. (*P < .05; **P < .01, ***P < .001 and ****P < 0.0001; two-way ANOVA).

3.2.4 WT cells depict altered peroxisomal phenotype upon chronological ageing

Yeast peroxisomes have an interesting ability to change their shape, size and number depending on the cellular environment (Smith and Aitchison 2013). Thus, in order to understand if peroxisome number, morphology and function are altered upon chronological ageing, WT cells expressing DsRed-SKL were analysed on different days of the CLS experiment in both CR and OA media conditions (Fig. 3.7 and 3.8). Earlier studies reported the detection of peroxisomes in WT cells upto 16 days of chronological ageing (Lefevre et al. 2013).

The number of peroxisomes in WT cells were analysed on the 0th day in both CR and OA conditions (Fig. 3.7a and 3.7b). Owing to the induction of peroxisomes in OA medium, WT cells displayed significant increase in the number of peroxisomes (Fig. 3.7b and 3.7c). The average number of peroxisomes in WT cells was 4.01 ± 0.15 peroxisomes/cell in CR and 8.55 ± 0.29 peroxisomes/cell in OA media (Fig. 3.7c).

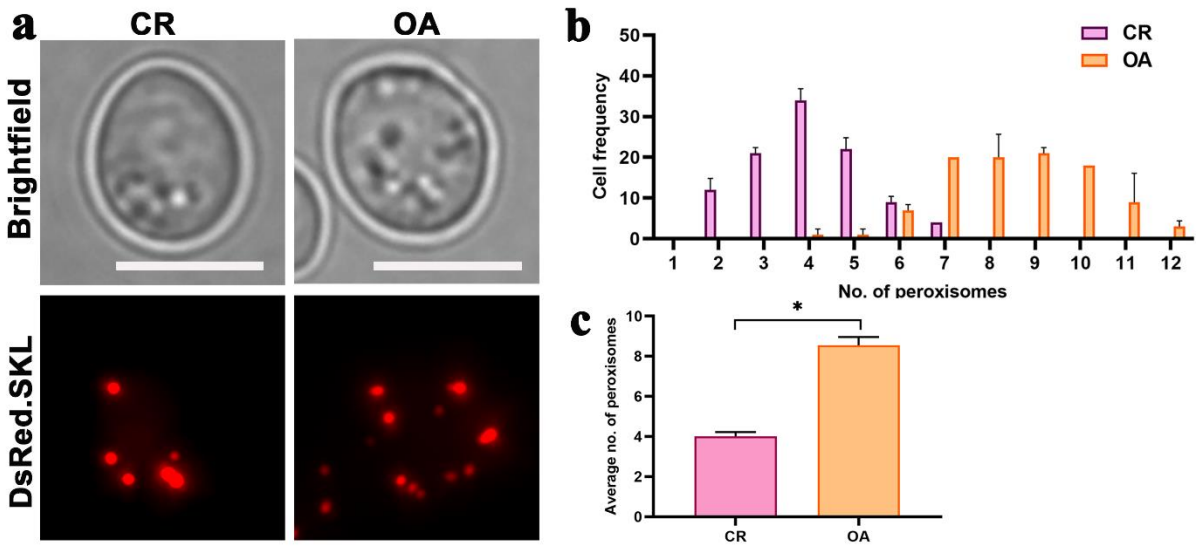

Fig. 3.7: Peroxisome number and distribution in WT cells in CR and OA media conditions on 0th day.
(a) Fluorescence microscopy images depicting peroxisome phenotype in WT cells on 0th day of chronological ageing in CR and OA media. Peroxisomes were marked with DsRed-SKL. Scale bar represents 5 µm. **(b)** and **(c)** represents the range and average number of peroxisomes in WT cells on the 0th day. Quantitative analysis of 50 cells from each experiment was performed (n =2). Error bars indicate absolute deviations from the mean value. Significance was determined using Student's t-test (with Welch's correction) from two independent experiments (n = 2). Values of P < .05 were considered significant (*), P < .01 very significant (**), and P < .001 extremely significant (***), P < 0.0001 very extremely significant (****).

We subsequently analysed the change in peroxisomal phenotype in both the media conditions during chronological ageing (Fig. 3.8). Based on the microscopic images, DsRed-SKL signal was categorized into three phenotypes: punctate, punctate + cytosolic and cytosolic (Fig. 3.8a and 3.8b). WT cells depicted mostly punctate phenotype on the 0^{th} day when cultured in both the media conditions (Fig. 3.8c). Interestingly, with the increase in chronological age, reduced number of cells with only punctate phenotype was observed in both CR and OA media conditions. On the other hand, an increase in the percentage of cells with cytosolic fluorescence was observed (Fig. 3.8c). Thus, it might be envisaged that yeast WT cells exhibit hampered peroxisomal matrix protein import upon chronological ageing. In OA media, the majority of WT cells exhibited punctate phenotype even on the 3^{rd} day (95% relative to 55% on CR) and the change of the phenotype to cytosolic was delayed when compared to CR grown cells (Fig. 3.8c). Interestingly, this is in line with the fact that WT cells also showed extended CLS in OA medium (Fig. 3.6).

3.3 Discussion

Ageing research has seen an upsurge in the identification and understanding of many molecular factors lately. Understanding these molecular and cellular mechanisms is not only essential from a basic science perspective but can also aid in engineering yeast lifespan for potential biotechnological applications. A role for subcellular compartments such as vacuole, mitochondria have been extensively studied in cellular ageing (Banerjee et al. 2020). Recent studies in various model organisms also highlighted the role of peroxisomes in ageing (Deori et al. 2018; Fanelli et al. 2013; Legakis et al. 2002). In this study, we used yeast as a model to understand the role of peroxisomes in yeast replicative and chronological ageing.

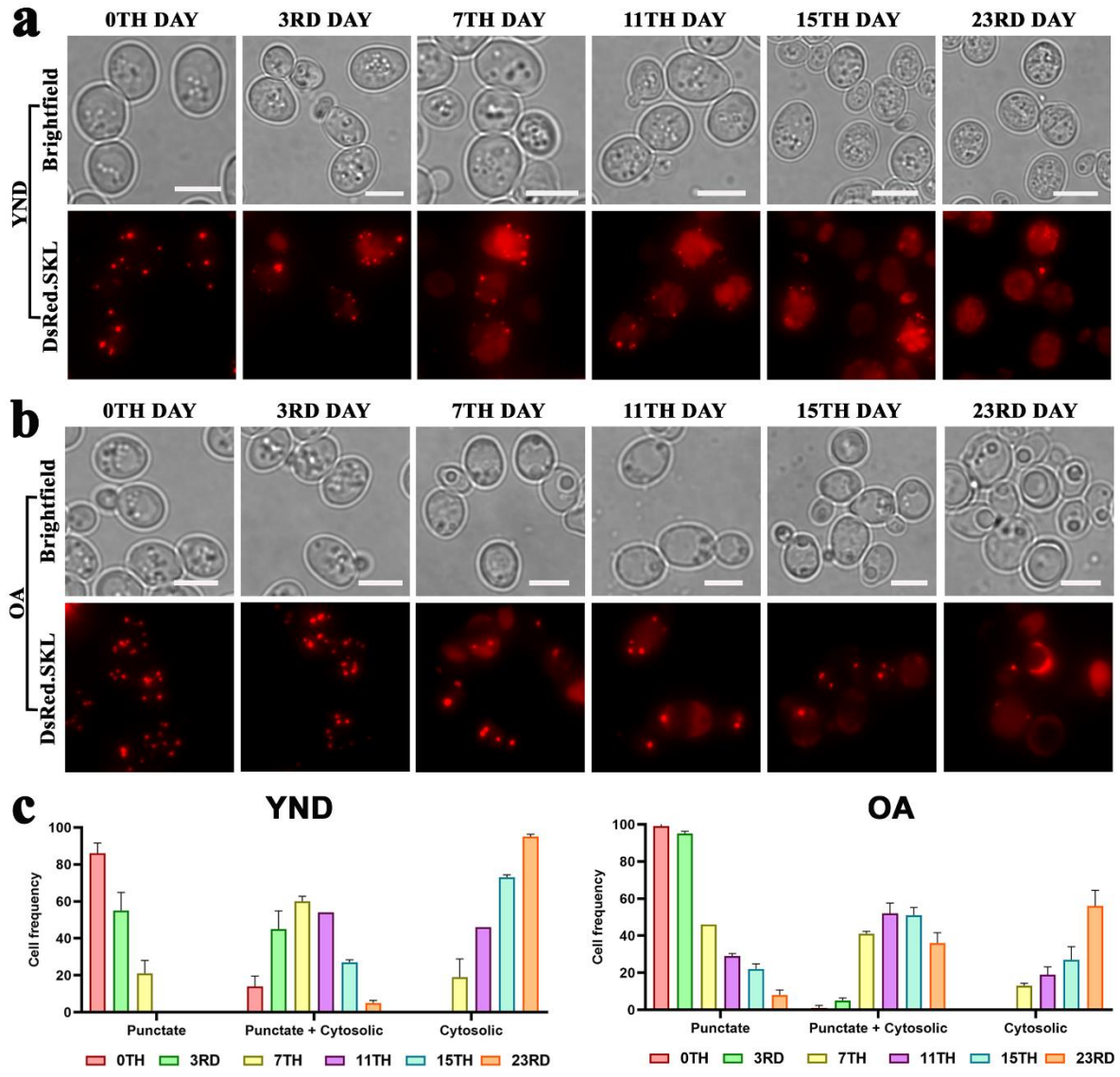

Fig. 3.8: Peroxisome phenotype is altered upon chronological ageing in CR and OA media conditions. **(a)** Representative images of punctate, punctate + cytosolic, cytosolic phenotypes. The figure represents fluorescence microscopy images depicting DsRed-SKL fluorescence as observed in WT cells on the 7th day of chronological ageing in CR media. **(b)** Representative fluorescence microscopy images depicting peroxisome phenotype in WT cells on different days of chronological ageing in CR and OA media, respectively. Scale bar represents 5 µm. **(c)** Quantitative analysis depict variation in peroxisome morphology in WT cells is represented on the 0th, 3rd, 7th, 11th, 15th and 23rd days of chronological ageing experiment.

The CLS of WT cells was also assessed in both CR and OA conditions. WT cells depicted extended mean and maximal lifespan in OA when compared to CR medium, as reported earlier (Fig. 3.6) (Lefevre et al. 2013). Interestingly, this is in line with the fact that cells cultured in OA had increased number of peroxisomes (Fig. 3.7b and 3.7c). Ageing-associated alteration in peroxisome phenotype was observed in these cells with the help of DsRed-SKL marker (Fig. 3.8a and 3.8b). With the increase in chronological age, reduced

number of cells with only punctate phenotype and increased percentage of cells with cytosolic fluorescence was observed, which might suggest defective peroxisome protein import upon ageing (Fig. 3.8c). However, the change of punctate to cytosolic phenotype was delayed in OA-grown cells, which is in line with the observation that these cells also showed extended CLS (Fig. 3.6). Thus, it would be interesting to understand whether alterations in peroxisome number has any effect on the CLS of yeast cells.

An interesting question is if there is an overlap between replicative ageing and chronological ageing and to what extent it is. Several studies have analysed various factors and pathways shared by these two ageing mechanisms. For instance, inhibition of the TOR signalling pathway has been shown to extend both RLS and CLS. Studies have also focussed on understanding how one affects the other and it is now accepted that RLS and CLS are tightly connected. Chronologically aged cells depicted reduced capacity of replication. On the other hand, mutant strains with decreased replicative potential often exhibit a limited CLS. Yeast deletion strain collection has been extensively used to identify factors/genes involved in these processes.

Chapter 4

Peroxisome number and function in replicatively aged cells is regulated by the fission proteins Pex11 and Pex25

Abstract

Peroxisomes are single membrane-bound organelles ubiquitously present in several cell types and are associated with cell and tissue-specific functions. Recent literature has highlighted the role of peroxisomes in cellular ageing in several model organisms. Metabolism of cellular reactive oxygen species is a universal function performed by these organelles. In this study, we tried to understand whether alteration in peroxisome number has any effect on replicative age of the cells in both peroxisome-inducing and non-inducing media conditions. We investigated if the increase in peroxisome number in replicatively aged cells is due to enhanced peroxisome proliferation. For this, the number of peroxisomes in replicatively aged mother cells of *pex11*, *pex25* and *pex11pex25* was analysed. Increased percentage of aged cells was observed in *pex25* and *pex11pex25* cells cultured in peroxisome-inducing oleic acid medium. Interestingly, when cultured in oleic acid, young mother cells devoid of Pex11 show reduced peroxisome proliferation compared to old mother cells. Induced activity of the antioxidant enzyme catalase and reduced accumulation of reactive oxygen species was reported in all studied strains when cultured in oleic acid medium. Further, our data also suggests that replicatively aged cells with increased peroxisome number also display mitochondrial dysfunction and fragmentation in all the strains studied. In conclusion our data suggests that the increase in peroxisome number in the replicatively aged cells seems to be partly dependent on the fission proteins.

This chapter is a part of

Deb R, Ghose S, Nagotu S (2022) Increased peroxisome proliferation is associated with early yeast replicative ageing. Curr Genet 68:207-225. https://doi.org/10.1007/s00294-022-01233-3

4.1 Introduction

Ageing is an inevitable degenerative phenomenon characterized by progressive decline in the optimum functioning of a cell, which ultimately leads to irreversible damage and cell death (Banerjee et al. 2020). RLS measures the number of daughter cells generated by a single yeast mother cell before death (Müller et al. 1980). Various methods like micromanipulation, elutriation, biotin-streptavidin affinity-based methods, microfluidic cell separation, MEP, and MAD have been developed, which enable studying yeast RLS (Banerjee et al. 2020; Hendrickson et al. 2018; Lindstrom and Gottschling 2009; Mortimer and Johnston 1959; Smeal et al. 1996; Woldringh et al. 1995; Xie et al. 2012).

Studies in yeast have identified several genetic factors associated with organelles that regulate RLS, as mentioned in section 1.4. Overexpression of histone deacetylase *SIR2* extended RLS in *S. cerevisiae* (Kaeberlein 2010; Steinkraus et al. 2008). A similar outcome was also reported in mice upon the activation of *SIRT1* (a mammalian *SIR2*-ortholog) (Finkel et al. 2009; Longo et al. 2012). A role for key ER chaperones in the regulation of RLS in yeast and fibroblasts was also reported (Matos et al. 2015; Perić et al. 2016). pH asymmetry of vacuoles is another crucial feature that distinguishes yeast mother and daughter cells (Hughes and Gottschling 2012). Loss of vacuole acidity and increased vacuolar fragmentation with ageing has also been reported (Ghavidel et al. 2018). Mitochondrial dysfunction is an important hallmark of ageing and is observed across all model systems studied (Payne and Chinnery 2015). A link between mitochondrial dynamics and its dysfunction has also been reported (Seo et al. 2010). Several proteins required to maintain mitochondrial dynamics, such as Dnm1, Mgm1, and Fis1 are also reported to regulate the RLS of yeast cells (Pernice et al. 2016; Scheckhuber et al. 2007; Scheckhuber et al. 2011). Interestingly, studies have shown that cells accumulate mitochondrial dysfunction very early during replicative ageing (Lam et al. 2011).

Yeast cells with 4-10 bud scars already show the maximum mitochondrial dysfunction, despite that the cells can undergo a maximum of 30 divisions.

Peroxisomes are cell organelles that perform important cellular functions such as β-oxidation of fatty acids and ROS homeostasis (Sibirny 2016). A recent study by Kumar and colleagues have highlighted the role of peroxisomes in yeast RLS (Kumar et al. 2018). It was observed that cells lacking Inp1 (loss of retention of peroxisomes in the mother cells) exhibited reduced RLS (Kumar et al. 2018). On the contrary, cells lacking Inp2 (affected in peroxisome inheritance to daughter cells) exhibited increased RLS (Kumar et al. 2018). The same study also reported reduced RLS in cells lacking the peroxisome biogenesis protein Pex3 emphasizing that intact and functional peroxisomes are required for maintaining yeast RLS (Kumar et al. 2018). Interestingly, a link between mitochondria and peroxisomes also seems to exist that enables the cells to cope with changes induced by replicative ageing. To compensate dysfunctional mitochondria in replicatively aged cells, retrograde (RTG) response is initiated. As a result, transcriptional upregulation of genes involved in peroxisome biogenesis and fatty acid β-oxidation has been reported (Ruetenik and Barrientos 2015).

In this study, we aim to understand whether altered peroxisome proliferation affect replicative ageing in yeast cells. Pex11 family of proteins is by far the most studied in regulating the number of peroxisomes in a cell. This family comprises of Pex11, Pex25 and Pex27 in *S. cerevisiae* (Tam et al. 2003). Previous studies have reported that Pex11 and Pex25 are predominantly involved in peroxisome division with a minor role of Pex27 (Rottensteiner et al. 2003). In order to determine whether the increased number of peroxisomes observed in the OMC of WT is due to enhanced proliferation in these cells, we analysed *pex11*, *pex25* and *pex11pex25* deletion strains which are compromised in peroxisome fission.

4.2 Results

4.2.1 Number of bud scars and percentage of aged cells increase with time in all studied strains in YND and OA grown conditions

In order to understand the effect of altered number of peroxisomes on replicative ageing, experiments similar to WT cells were performed in *pex11*, *pex25* and *pex11pex25* cells. The bud scars in the replicatively aged mother cells of the mutants were stained with calcofluor white. The number of bud scars and the percentage of YMC (0 or 1 bud scar) and OMC (≥ 4 bud scars) were quantified at different time points.

The number of bud scars in *pex11* and *pex25* increased with time in both YND and OA (Fig. 4.1a, Table 4.1). At 0 hr, 66.5% cells of *pex11* and 90% cells of *pex25* were observed to be YMC (Fig. 4.2a and 4.2b, Table 4.1). The percentage of OMC in *pex11* cells in 8, 16 and 24 hrs increased to 55.5%, 78% and 78.5% in YND media and 7%, 76% and 91% in OA medium (Fig. 4.1b, Table 4.1). Similarly, cells deleted for *PEX25* also showed an increase in the percentage of OMC with time. The OMC of *pex25* in 8, 16 and 24 hrs increased to 50%, 88% and 90.5% in YND media and 68%, 84.5% and 86% in OA medium (Fig. 4.1b, Table 4.1). Most *pex25* cells show 4 bud scars in OA media already at 8 hrs (Fig. 4.3).

Earlier studies have shown that cells lacking both Pex11 and Pex25 have only one or two peroxisomes per cell (Tam et al. 2003). *pex11pex25* cells were evaluated for the effect of this reduced number of peroxisomes on replicative age. An increase in the number of bud scars with time was also observed in these cells in both the media conditions (Fig. 4.1a). 90% of these cells were YMC at 0 hr (Fig. 4.2a and 4.2b, Table 4.1). The percentage of OMC in 8, 16 and 24 hrs cultures increased to 37.5%, 92% and 92.5% in YND media and 52.5%, 95.5% and 96.5% in OA medium (Fig. 4.1b, Table 4.1).

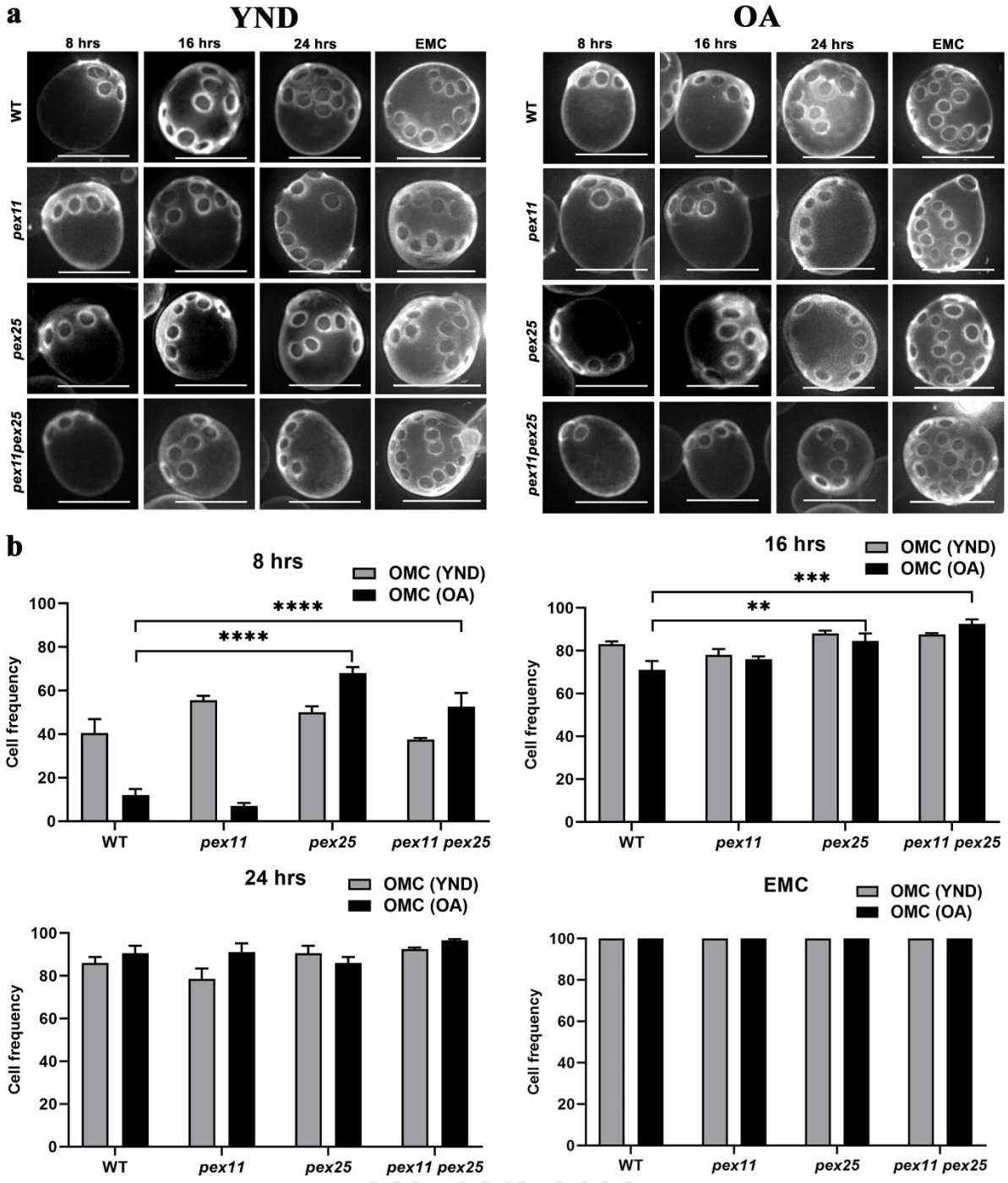

Fig. 4.1: The number of bud scars and percentage of OMC increase with time in all studied strains.
(a) Representative images depicting bud scars in biotinylated mother cells of all strains in YND and OA media at different time points are shown. Bud scars stained with calcofluor white were visualized in the replicatively aged mother cells. Scale bar represents 5 µm. Cells with ≥4 bud scars are referred to as replicatively aged OMC. **(b)** The percentage of OMC at different time points are represented in all the strains in YND and OA media. Error bars indicate absolute deviations from the mean value. Significance was determined using two-way ANOVA (with multiple comparisons) from two independent experiments (n = 2). Values of $P < .05$ were considered significant (*), $P < .01$ very significant (**), and $P < .001$ extremely significant (***), $P < 0.0001$ very extremely significant (****)

The percentage of OMC did not significantly vary between different strains grown in YND (Fig. 4.1b). However, in OA significant increase in the percentage of OMC was observed in *pex25* and *pex11pex25* strains at 8 hrs and 16 hrs (Fig. 4.1b, Table 4.1). Our data also shows that the distribution of OMC with different bud scars in all the strains are indeed comparable in cells grown in YND medium. OMC with higher number of bud scars were observed in *pex25* and *pex11pex25* cells at all the time points in OA medium (Fig. 4.3).

Table 4.1: Variation in the number of bud scars in young and old mother cells under different growth conditions. 100 random cells were selected at each time point for quantification. All values are calculated as mean ± SEM.

Strains	Time (hrs)	YMC (%)		OMC (%)		Range of bud scars	
		YND	OA	YND	OA	YND	OA
WT	0	67.5 ± 3.5	67.5 ± 3.5	0	0	0 - 3	0 - 3
	8	11 ± 0.1	29.5 ± 2.5	40.5 ± 4.5	12 ± 2	0 - 6	1 - 4
	16	1 ± 0.3	1.5 ± 0.5	83 ± 1	71 ± 3	1 – 8	1 – 7
	24	1 ± 1	1 ± 0.3	86 ± 2	90.5 ± 2.5	1 - 9	1 - 9
pex11	0	66.5 ± 4.5	66.5 ± 4.5	0	0	0 - 3	0 - 3
	8	10 ± 2	20.5 ± 1.5	55.5 ± 1.5	7 ± 1	1 - 6	1 - 4
	16	3 ± 1	3 ± 1	78 ± 2	76 ± 1	1 – 8	1 – 8
	24	4.5 ± 0.5	3 ± 1	78.5 ± 3.5	91 ± 3	1 - 9	1 - 9
pex25	0	90 ± 2	90 ± 2	0	0	0 - 3	0 - 3
	8	2.5 ± 1.5	6 ± 2	50 ± 2	68 ± 1	1 - 7	1 - 6
	16	4.5 ± 1.5	8 ± 2	88 ± 1	84.5 ± 2.5	1 – 9	1 – 9
	24	3.5 ± 1.5	8 ± 2	90.5 ± 2.5	86 ± 2	1 - 9	1 - 9
pex11pex25	0	90 ± 1	90 ± 1	0	0	0 - 3	0 - 3
	8	8.5 ± 1.5	11 ± 1	37.5 ± 0.5	52.5 ± 4.5	1 – 5	1 – 5
	16	4.5 ± 1.5	3 ± 1	92 ± 1	95.5 ± 0.5	1 – 8	1 – 8
	24	3 ± 1	2.5 ± 1	92.5 ± 0.5	96.5 ± 0.5	1 - 9	1 - 9

As mentioned earlier, 100% EMC of all the deletion strains had 4 or more than 4 bud scars (Fig. 4.1b and 4.3). In all the studied strains, the average number of bud scars was higher in OA when compared to YND. No significant change in average number of bud scars between the strains was observed in EMC in both media conditions (Table 4.2). This experiment was also performed in cells grown in YPD medium. No significant change in average number of bud scars between the strains was also observed in EMC in YPD (Table 4.2).

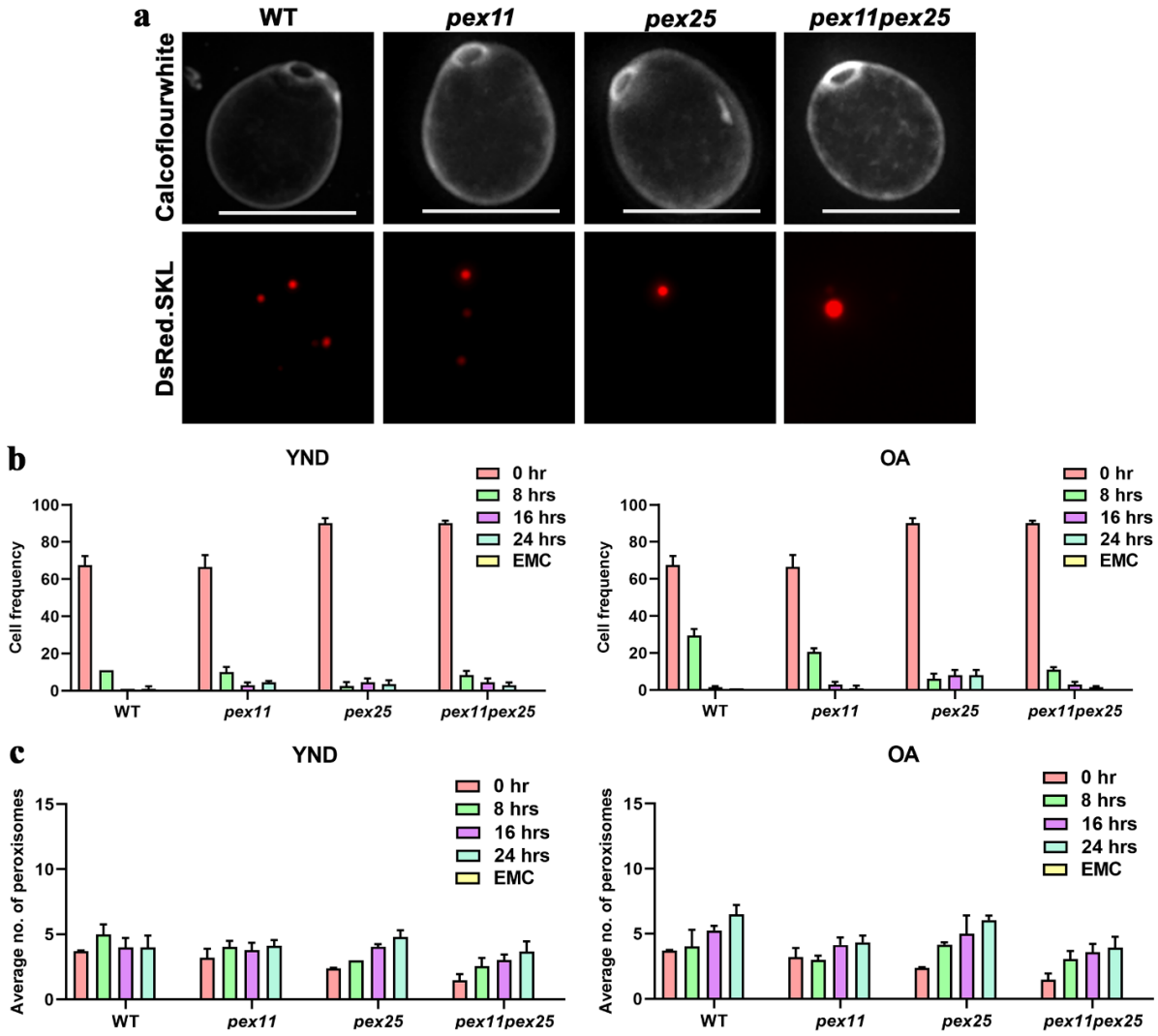

Fig. 4.2: Distribution of bud scars in the OMC of all the strains in YND and OA media.
(a) Representative images depicting bud scars and peroxisome morphology in the YMC of all studied strains. Bud scars were stained with calcofluor white and peroxisomes were marked with DsRed-SKL. Scale bar represents 5 µm. **(b)** Variation in the percentage of YMC in all the studied strains at different time points. **(c)** Average peroxisome number in these cells at different time points.

Table 4.2: Variation in the range and average number of bud scars in the EMC of all studied strains under different growth conditions. 100 random cells were selected at each time point for quantification. All values are calculated as mean ± SEM.

Strains	YND		OA		YPD	
	Range of bud scars	Average bud scar no.	Range of bud scars	Average bud scar no.	Range of bud scars	Average bud scar no.
WT	4 – 18	9.92 ± 0.22	9 – 20	14.52 ± 0.26	10-18	13.50 ± 0.30
pex11	6 – 18	11.14 ± 0.17	8 – 24	15.64 ± 0.11	10-20	13.12 ± 0.43
pex25	5 – 17	10.88 ± 0.06	8 – 24	16.11 ± 0.34	10-18	12.21 ± 0.12
pex11pex25	5 – 17	11.32 ± 0.26	7 – 25	16.07 ± 0.51	10-18	12.28 ± 0.28

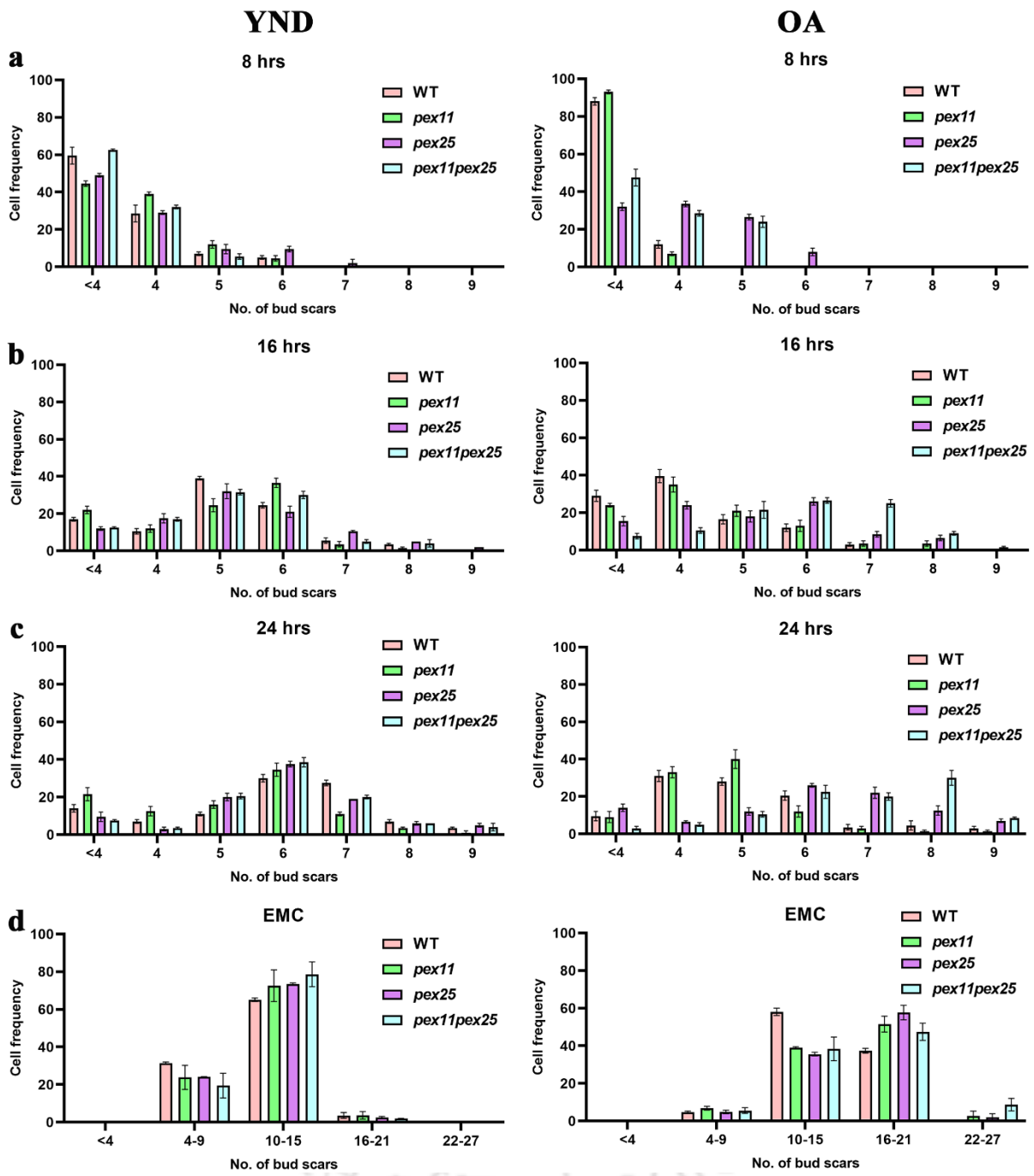

Fig. 4.3: Variation in the number of bud scars in the replicatively aged cells of all studied strains grown in YND and OA media conditions.
(a), **(b)**, **(c)** and **(d)** depicts the distribution of bud scars in the strains at 8, 16, 24 hrs and in EMC respectively.

4.2.2 Increased number of peroxisomes is observed in replicatively aged cells of all studied strains

The number of peroxisomes was analysed in the aged cells of all the deletion strains using DsRed-SKL as a marker (Fig. 4.4a). An increase in the number of peroxisomes with time was observed in *pex11* OMC in both YND and OA media conditions (Fig. 4.4b and 4.5). However,

the peroxisome number increased from 8-16 hrs and remained constant at further time points (Fig. 4.4b, Table 4.3). Interestingly, no significant increase in peroxisome number with time was observed in the YMC of *pex11* in OA highlighting the role of Pex11 in peroxisome division in these cells (Fig. 4.2c, Table 4.3).

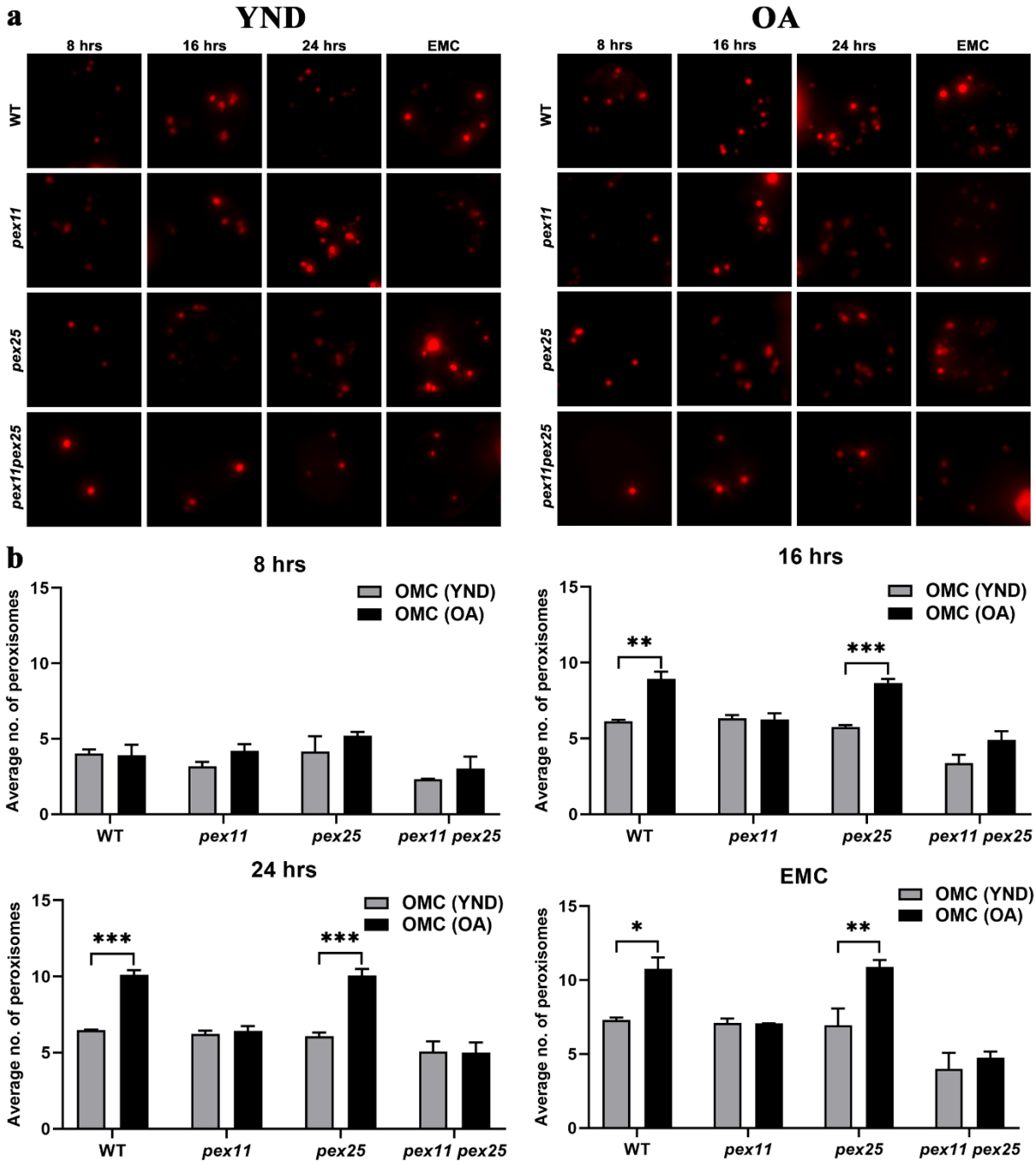

Fig 4.4: The number of peroxisomes increase with time in the mutant cells.
(a) Representative images depicting the morphology of peroxisomes in biotinylated mother cells of all the strains in YND and OA media at different time points are shown. Peroxisomes marked with DsRed-SKL were visualized in the replicatively aged mother cells. **(b)** The average number of peroxisomes in the OMC of all strains at different time points are represented in YND and OA media. Error bars show absolute deviations from the mean value.

Significance was determined using two-way ANOVA (with multiple comparisons). Values of $P < .05$ were considered significant (*), $P < .01$ very significant (**), and $P < .001$ extremely significant (***).

Cells deleted for *PEX25* also showed an increase in the number of peroxisomes in OMC with time in both the media conditions (Fig. 4.4b and Fig. 4.5). The increase in peroxisome number in OMC of *pex25* was 2.6 and 4-fold in YND and OA media compared to their respective YMC at 0 hr (Table 4.3). The average number of peroxisomes in OMC of *pex25* at 24 hrs is similar to WT. However, the increase in fold change is due to less number of peroxisomes in YMC of *pex25* at 0 hr (Fig. 4.2c, Table 4.3).

Table 4.3: Average number of peroxisomes in young and old mother cells at different time points of replicative ageing in YND and OA medium. 100 random cells were selected at each time point for quantification. All values are calculated as mean ± SEM.

Strains	Time (hrs)	Peroxisome number in YMC		Peroxisome number in OMC		Range of peroxisomes	
		YND	OA	YND	OA	YND	OA
WT	0	3.72 ± 0.3	3.72 ± 0.3	-	-	1 - 7	1 - 7
	8	5 ± 0.55	4.04 ± 0.2	4.02 ± 0.19	3.91 ± 0.5	3 - 8	3 - 8
	16	4 ± 0.5	5.25 ± 0.3	6.12 ± 0.08	8.93 ± 0.33	5 - 11	6 - 15
	24	4 ± 0.3	6.50 ± 0.5	6.47 ± 0.02	10.11 ± 0.2	4 - 13	6 - 15
	EMC	-	-	7.13 ± 0.13	10.78 ± 0.38	4 - 14	6 - 20
pex11	0	3.20 ± 0.5	3.20 ± 0.5	-	-	1 - 6	1 - 6
	8	4.03 ± 0.3	3 ± 0.22	3.16 ± 0.2	4.20 ± 0.3	1 - 7	1 - 8
	16	3.80 ± 0.4	4.14 ± 0.4	6.31 ± 0.2	6.24 ± 0.3	2 - 10	3 - 11
	24	4.13 ± 0.3	4.34 ± 0.4	6.22 ± 0.2	6.43 ± 0.3	2 - 11	3 - 15
	EMC	-	-	6.93 ± 0.18	6.95 ± 0.05	2 - 9	3 - 11
pex25	0	2.38 ± 0.4	2.38 ± 0.4	-	-	1 – 4	1 – 4
	8	3 ± 0.1	4.18 ± 0.1	4.14 ± 0.7	5.21 ± 0.2	1 – 6	2 - 7
	16	4.05 ± 0.2	5 ± 1	5.75 ± 0.1	8.66 ± 0.2	3 – 8	5 – 11
	24	4.80 ± 0.4	6.05 ± 0.3	6.68 ± 0.2	10.06 ± 0.3	3 – 10	6 – 13
	EMC	-	-	6.96 ± 0.8	11.14 ± 0.36	3 – 11	6 – 17
pex11pex25	0	1.47 ± 0.3	1.47 ± 0.3	-	-	1 - 3	1 – 3
	8	2.57 ± 0.4	3.07 ± 0.4	2.32 ± 0.03	3.03 ± 0.6	1 – 6	1 – 6
	16	3.02 ± 0.3	3.58 ± 0.5	3.37 ± 0.4	4.89 ± 0.4	1 – 8	2 – 8
	24	3.69 ± 0.6	3.94 ± 0.6	5.07 ± 0.5	5.01 ± 0.5	2 – 8	3 – 9
	EMC	-	-	4.02 ± 0.36	4.85 ± 0.43	2 – 8	2 – 8

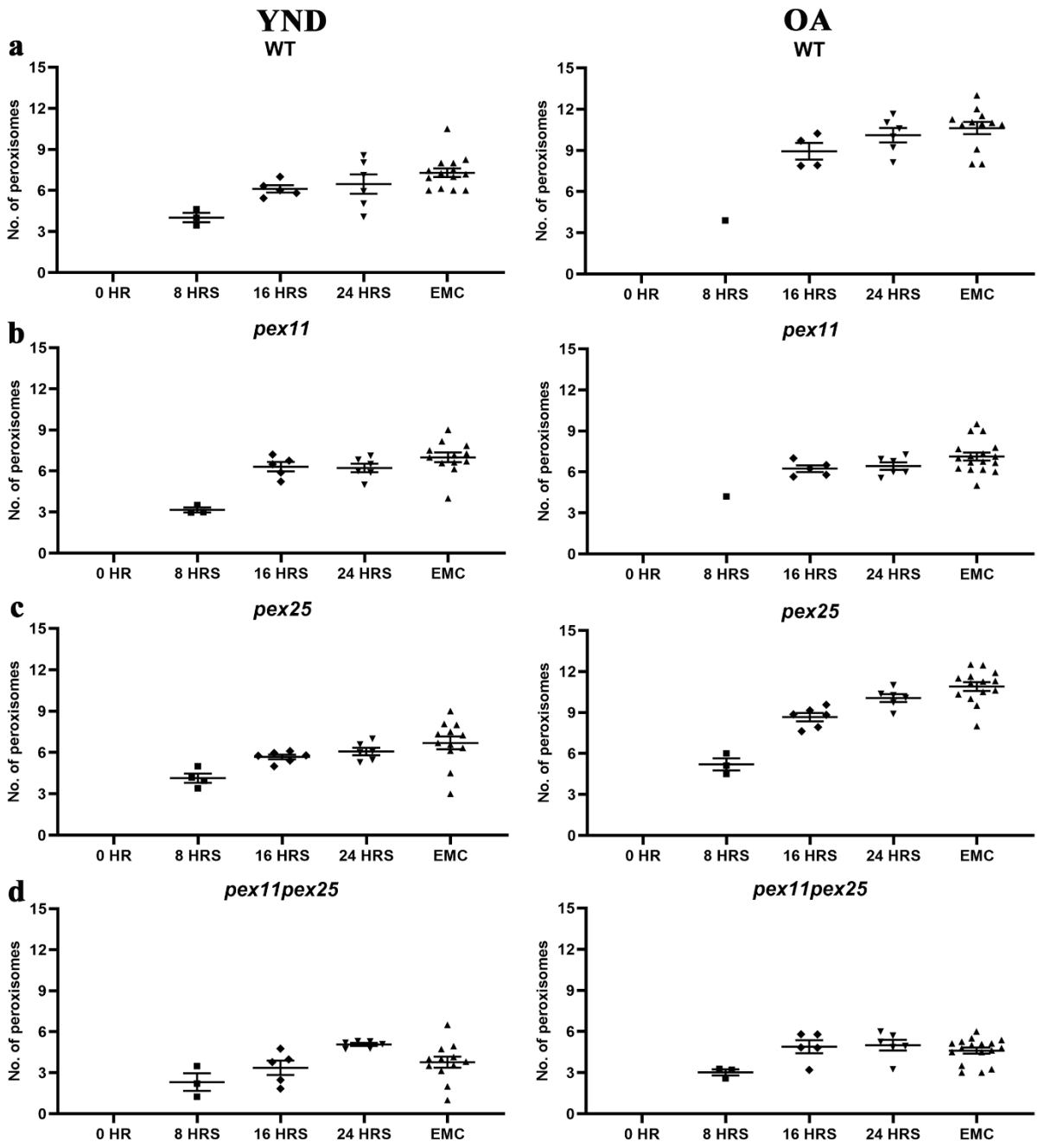

Fig. 4.5: Variation in the number of peroxisomes with time in the OMC of all the strains grown in YND and OA media conditions.
(a), **(b)**, **(c)** and **(d)** depicts the distribution of peroxisomes in WT, *pex11*, *pex25* and *pex11pex25* cells, respectively.

An increase in the number of peroxisomes with time was also observed in OMC of *pex11pex25* in both growth conditions (Fig. 4.4b and 4.5). However, the increase in peroxisome number in the *pex11pex25* OMC at 24 hrs was 1.9 and 2.2-fold in YND and OA medium respectively, compared to their YMC at 0 hr (Table 4.3). Fig. 4.4b represents the average number of peroxisomes in the OMC of all the studied strains both on YND and OA media

conditions at various time points. The OMC of *pex25* cells in OA medium showed a significant increase in the number of peroxisomes when compared to YND. In line with the function of Pex11 in peroxisome fission, reduced peroxisome number was observed in the OMC of *pex11* and *pex11pex25* cells in OA (Fig. 4.4b).

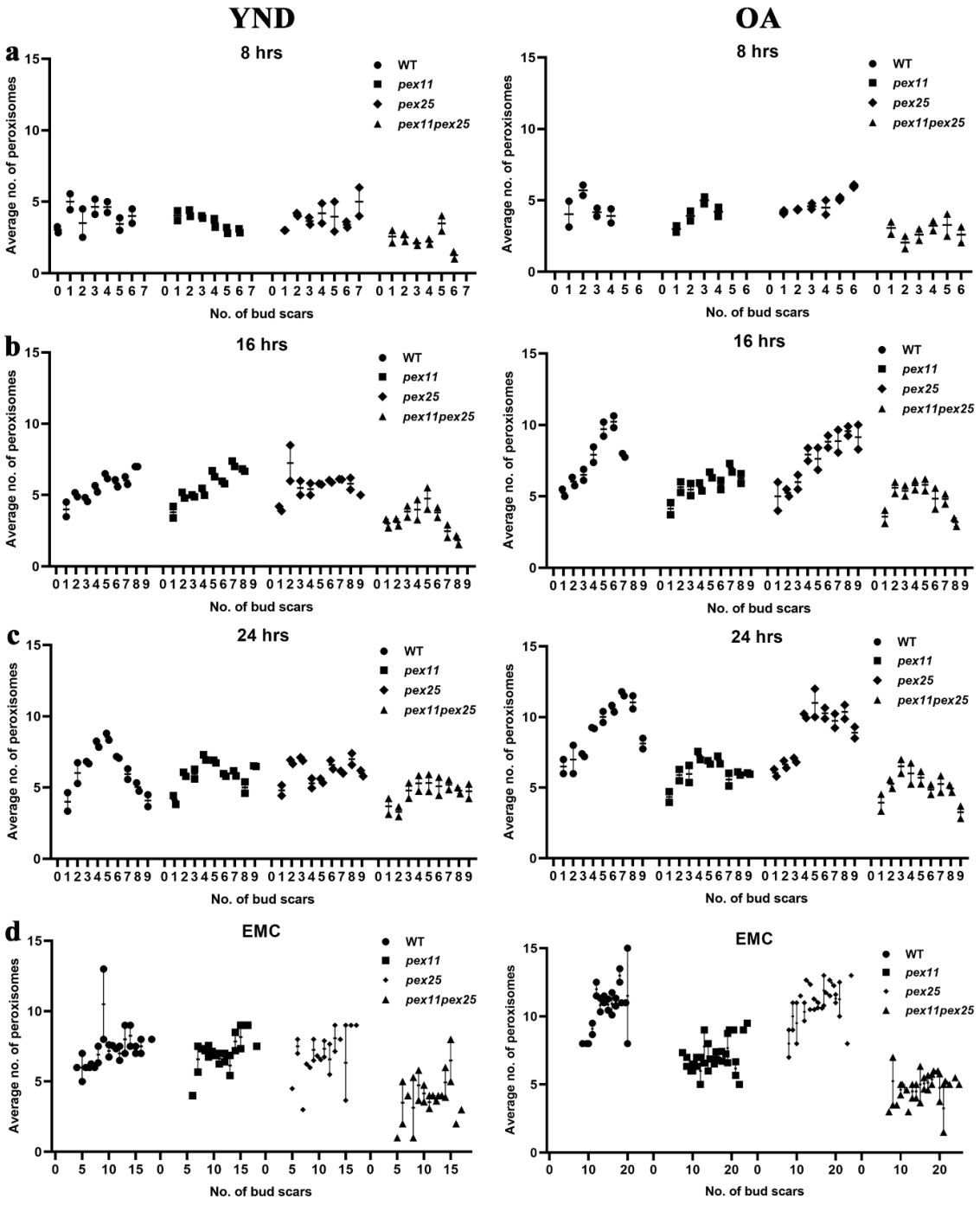

Fig. 4.6: Variation in the number of peroxisomes with bud scars in the mother cells of all the studied strains in YND and OA.

(a), **(b)**, **(c)** and **(d)** represents the average number of peroxisomes with bud scars at 8, 16, 24 hrs and in the EMC, respectively.

Similar to WT cells, the EMC of all the studied strains did not show a significant increase in peroxisome number as compared to their respective OMC at either 16 or 24 hrs in YND and OA media (Fig. 4.4b and 4.5b). However, the increase in peroxisome number in OMC was found to be partly dependent on the peroxisome fission protein Pex11, as OMC of *pex11* and *pex11pex25* strains contained less number of peroxisomes as compared to WT OMC even in the peroxisome-inducing conditions (Fig. 4.4b and 4.5). Fig. 4.6b represents the variation in the number of peroxisomes with an increase in the number of bud scars in all the strains cultured in both the media conditions, thereby suggesting a correlation between peroxisome number and replicative age in the yeast cells.

4.2.3 Oxidative stress in cells lacking Pex11 and Pex25

Often mitochondria are highlighted as the main producers of ROS in cells. Apart from mitochondria, the other significant sites for ROS production in a cell are peroxisomes, ER and the plasma membrane (Zhang and Wong 2021). Among the non-mitochondrial sources of cellular ROS, peroxisomes contribute a considerable amount (Deori et al. 2018). To understand if the altered number of peroxisomes in cells lacking Pex11 and Pex25 results in a change in oxidative stress, intracellular ROS was analysed in exponentially grown cells stained with H_2-DCFDA (James et al. 2015). These cells were grown in YND and OA till they reached mid-log phase and were subsequently stained with PI. Only viable cells were considered for ROS measurement (Fig. 4.7a and 4.7c). Flow cytometry analysis revealed reduced ROS in *pex25* cells compared to WT cells in YND media (Fig. 4.7a). Cells lacking *CTT1* and *CTA1* were taken as controls. In *S. cerevisiae*, *CTT1* and *CTA1* encodes the cytosolic catalase T and the peroxisomal catalase A, respectively (Cohen et al. 1988; Hartig and Ruis 1986). *ctt1* and *cta1* cells did not show a significant difference in ROS staining in YND. On the other hand, in OA medium, *cta1* showed significantly enhanced ROS compared to WT cells. However, no significant change in ROS was observed in *pex11*, *pex25*, *pex11pex25* and *ctt1* cells grown in

OA (Fig. 4.7c). The activity of the ROS scavenging enzyme catalase was also measured in these cells and is represented as fold change with respect to WT. As shown in fig. 4.7b and 4.7d, a significant increase in catalase activity was observed in *pex25* cells grown in both YND and OA medium. *cta1* cells showed the least catalase activity in OA grown cells (Fig. 4.7d).

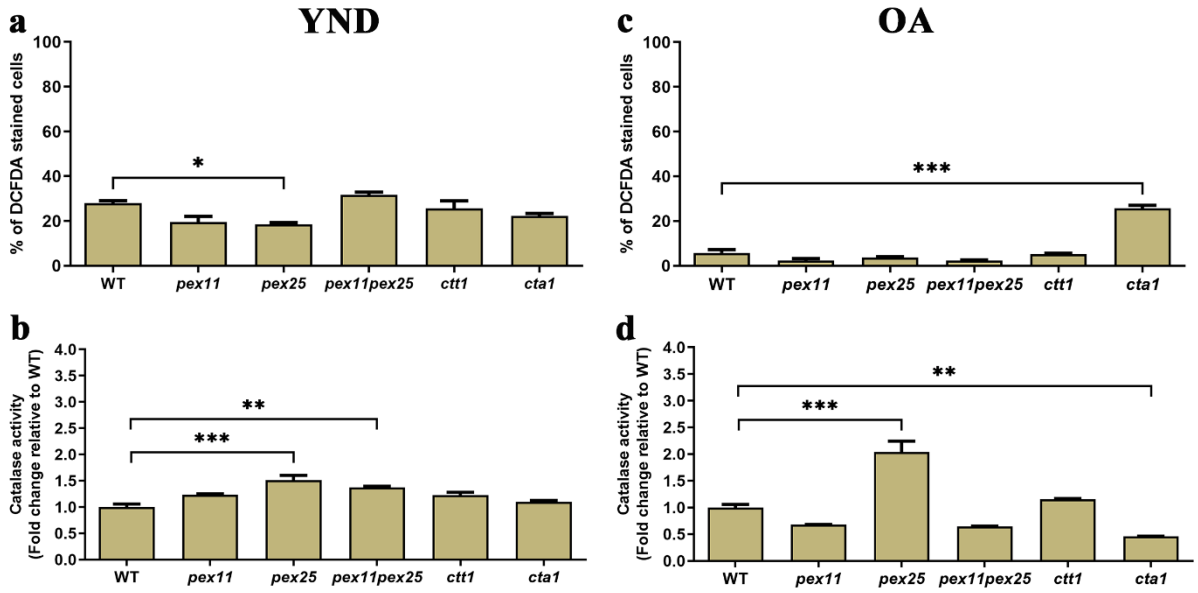

Fig. 4.7: Peroxisome fission mutants do not show significant increase in ROS.
ROS production was measured using the fluorescent probe H_2-DCFDA in exponentially grown viable cells in YND **(a)** and OA **(c)**. 50,000 cells per sample were analysed from three independent flow cytometry experiments (n =3). The catalase activity in YND and OA media is represented in **(b)** and **(d)** respectively. Results were represented as fold change in activity as compared to WT cells from three independent experiments (n = 3). *ctt1* and *cta1* cells were taken as control. Significance was determined using one-way ANOVA (with multiple comparisons). Values of $P < .05$ were considered significant (*), $P < .01$ very significant (**), and $P < .001$ extremely significant (***)

4.2.4 Oxidative stress in replicatively aged mother cells

To compare oxidative stress in young and old cells grown in both YND and OA medium, OMC were collected after 16 hrs of culturing. The percentage of OMC was considerably increased at this time in all the strains studied and no significant increase in peroxisome number in OMC was observed after 16 hrs (Fig. 4.1b and 4.5). Intracellular ROS and catalase activity were analysed as described in 4.2.3. An interesting finding was reduced ROS and increased catalase activity in OMC of all strains grown in OA medium (Fig. 4.8). *pex11* and *pex11pex25* depicted the strongest phenotype. Surprisingly, this corresponded to an

increase in peroxisome number only in WT and *pex25* cells. However, no induction of peroxisomes was observed in OMC of *pex11* and *pex11pex25* in OA (Fig. 4.4b). A significant increase in the level of intracellular ROS (Fig. 4.8a) and decreased catalase activity (Fig. 4.8b) was observed in OMC of *pex11* and *pex11pex25* grown in YND compared to the young cells. This is in line with earlier studies that reported increased oxidative stress very early during replicative ageing (Lam et al. 2011). However, no significant difference in catalase activity was observed between OMC of different strains in YND (Fig. 4.8b). A relationship between the increased number of peroxisomes in OMC and their functionality needs to be further evaluated.

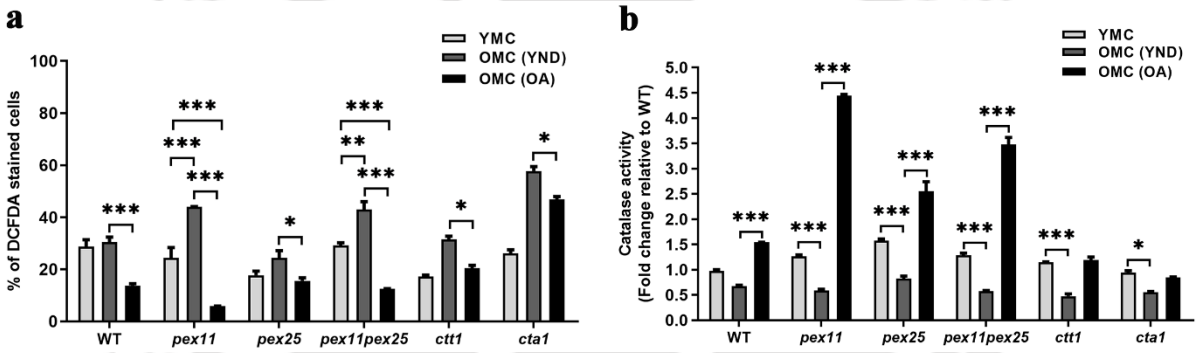

Fig. 4.8: Replicatively aged mother cells in OA exhibit reduced ROS and increased catalase activity.
(a) ROS accumulation in YMC (light grey) and OMC of WT, *pex11*, *pex25* and *pex11pex25* cells cultured for in YND (dark grey) and OA (black) is represented. ROS production was measured with the fluorescent probe H_2-DCFDA in viable cells using flow cytometry. 50,000 cells per sample were analysed from two independent experiments (n =2). **(b)** The catalase activity in YND and OA is represented as fold change compared to WT cells from two independent experiments (n = 2). *ctt1* and *cta1* cells were taken as control. Significance was determined using two-way ANOVA (with multiple comparisons). Values of $P < .05$ were considered significant (*), $P < .01$ very significant (**), and $P < .001$ extremely significant (***).

4.2.5 Mitochondrial dysfunction upon replicative ageing

Mitochondrial dysfunction results in petite colonies in *S. cerevisiae* and increased expression of peroxisomal proteins in these colonies has been reported (Epstein et al. 2001). Mitochondrial dysfunction has been reported in early replicatively aged cells (>4 bud scars) (Lam et al. 2011). To analyse if there is reduced mitochondrial function in aged cells of the strains used in our study, pooled YMC (0 hr) and EMC from YND grown cultures were spotted onto YND, YPD and YPDG plates in different dilutions. YMC of all the strains showed similar

growth on all the media conditions. EMC plated onto YPDG plates depicted the most reduced growth compared to YMC suggesting reduced mitochondrial function in these cells (Fig. 4.9a). Interestingly, growth of WT and all the deletion cells was very similar on YND, YPD and YPDG plates indicating no additional dysfunction in the mutants (Fig. 4.9a).

To further assess mitochondrial functionality, YMC (0 hr) and EMC of all the strains were stained with TMRE and MitoTracker Orange which enables staining of mitochondria depending on their membrane potential (Dhar et al. 2019; Pozniakovsky et al. 2005). Changes in the MMP in these cells were assessed using fluorescence microscopy (Fig. 4.9b and 4.9c) and flow cytometry (Fig. 4.9d). Reduced mitochondrial staining upon using both the dyes and mitochondrial fragmentation was observed in OMC of all the strains (Fig. 4.9b and 4.9c). The histogram profiles obtained from flow cytometry also showed similar intensity distribution for both the dyes (Fig. 4.9d). No significant difference between strains was observed in these experiments suggesting no additional mitochondrial dysfunction in the mutant cells. These results suggest that we cannot rule out the contribution of mitochondrial dysfunction to the increase in peroxisome number in OMC of all studied strains. Albeit this may not be the only reason for the increase in peroxisome number.

4.3 Discussion

Ageing is proposed to be the primary risk factor for chronic diseases such as neurodegeneration, cancer, diabetes and cardiovascular disease (Niccoli and Partridge 2012). Peroxisome abnormalities with age have been proposed and are under investigation with respect to such age-associated disorders (Cipolla and Lodhi 2017). Recent studies have highlighted the importance of functional peroxisomes in another class of age-related disease called HGPS. Abnormalities of peroxisomes and catalase deficiency have been reported in HGPS. The authors report poorly assembled peroxisomes may result in increased oxidative

stress which further contributes to the premature senescent phenotype in HGPS (Mao et al. 2020).

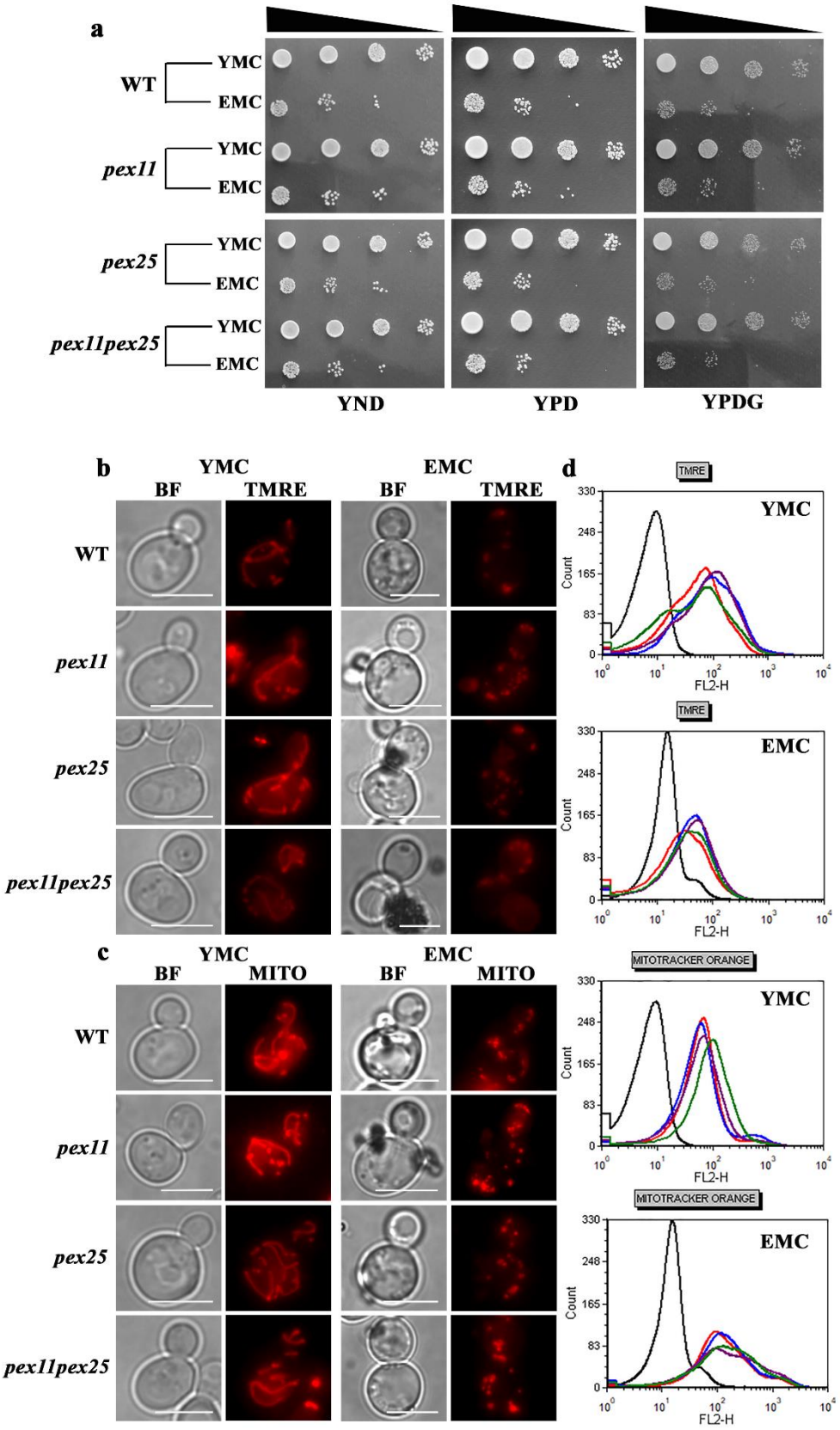

Fig. 4.9: Mitochondrial function in aged mother cells of peroxisome fission mutants is similar to WT cells. **(a)** Spot assay depicting growth of YMC and EMC of all the strains on selective glucose media (YND), complete glucose media (YPD) and glycerol (YPDG) medium. Columns in each plate represent various dilutions. **(b)** and **(c)** depict representative images of mitochondria in the YMC and EMC of all the strains stained with TMRE and MitoTracker Orange respectively. Scale bar represents 5 μm. **(d)** Histogram profiles of the same stained samples representing the shift in the fluorescence intensity of TMRE and MitoTracker Orange (acquired in FL2-H laser channel) analysed by flow cytometry. In the graph, green -WT, *pex11* – blue, *pex25* – purple, *pex11pex25* – red and black represents blank.

In this study, we tried to investigate if peroxisome division proteins can regulate the increase in the peroxisome number in OMC. For this, we analysed cells lacking proteins involved in peroxisome fission i.e. Pex11, Pex25 and both the proteins. An increased number of peroxisomes in *pex11* OMC compared to young cells was observed in both YND and OA media (Fig. 4.10). However, this increase in OA was not in par with the WT cells. When compared to the WT a significant increase in peroxisome number was not observed in YMC (from 0 to 24 hrs) of *pex11* in OA. This leads to the speculation that peroxisome proliferation in YMC is primarily dependent on the fission of pre-existing organelles mediated by Pex11. Interestingly, the increase in peroxisome number in aged cells seems to be partly dependent on the fission proteins as replicatively aged *pex11* and *pex11pex25* cells show reduced peroxisome number when compared to WT. However, increased number of peroxisomes was observed in aged cells of all strains studied when compared to their respective young cells.

Interestingly, increased catalase activity and reduced ROS was observed in OMC compared to YMC in all strains cultured on OA. This could be due to the adaptation of cells to β-oxidation and the need to scavenge excess ROS produced due to this pathway. Surprisingly, the reduced number of peroxisomes characteristic of *pex11* and *pex11pex25* mother cells in OA did not influence the catalase activity. However, it is known that *S. cerevisiae* has two catalase enzymes (Lushchak and Gospodaryov 2005). It cannot be ruled out that the enhanced activity in these cells could be due to the increased activity of cytosolic catalase in response to the change in the cellular environment. Unlike in the OA medium, a significant decrease in catalase activity in OMC compared to YMC of *pex11* and *pex11pex25* was observed in YND though

an increase in peroxisome number in these cells could be seen. A role for Pex11 in inter-organelle membrane contact sites has also been reported (Ušaj et al. 2015). Loss of such contacts may also result in an imbalance of ROS homeostasis in OMC. On the same lines, Kumar and colleagues also analysed RLS of cells lacking Inp1 required for retention of functional peroxisomes in the mother cells cultured on a glucose-containing medium. The authors reported decreased RLS (increased replicative age) in these cells (Kumar et al. 2018). On the other hand, cells lacking Inp2 required for the inheritance of peroxisomes exhibited increased RLS.

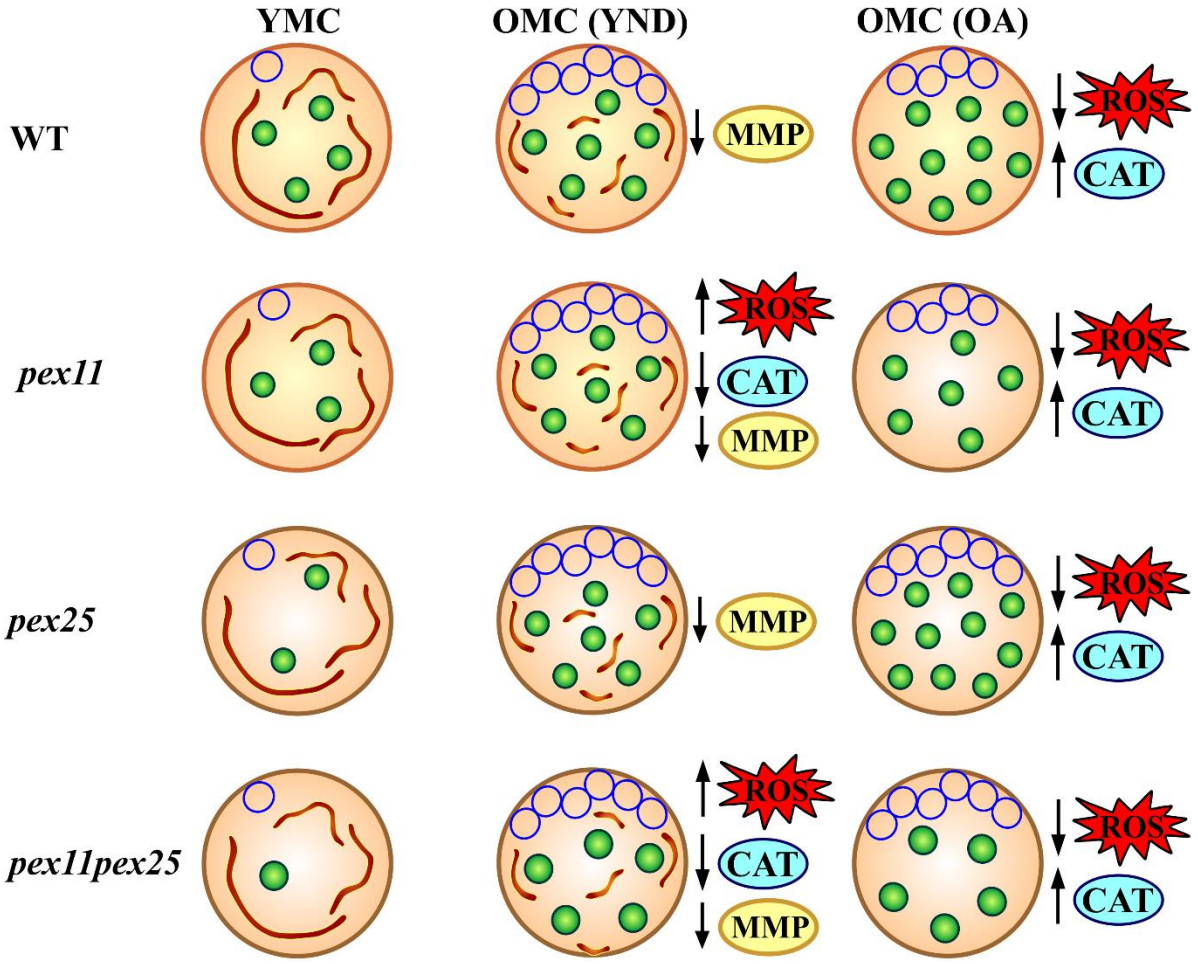

Fig. 4.10: A schematic summarising key findings of this study
YMC, OMC in YND and OMC in OA for all the strains studied are represented. Significant increase in peroxisome number was observed in the OMC in YND compared to YMC in all studied strains. Peroxisome induction and increase in number was observed only in OMC of WT and *pex25* in OA compared to OMC in YND. Reduced ROS accumulation was observed in OMC in OA in all the studied strains. Concomitantly increased catalase activity in OMC in OA in all the strains was also observed. Increased ROS and reduced catalase activity were observed in OMC of *pex11* and *pex11pex25* cells in YND. Reduced mitochondrial function as suggested by

reduction in mitochondrial membrane potential was observed in the OMC of all strains grown in YND compared to their respective YMC.
Green filled circles - peroxisomes, Blue empty circles – bud scars, Brown structures – mitochondria

Extensive research is carried out to understand the role of mitochondria in cellular ageing and ROS homeostasis. One intriguing aspect is the accumulation of fragmented mitochondria in aged mother cells (Laun et al. 2001). An intricate link between the structure and function of peroxisomes and mitochondria is now accepted. A role for the loss of function of mitochondria resulting in increased peroxisome number via mechanisms such as RTG signalling has also been hypothesized. However, this does not seem to be the only reason of increase in peroxisome number in old cells in our study, as *pex11pex25* cells that exhibit similar mitochondrial dysfunction as in WT, do not exhibit similar increase in number of peroxisomes in OA medium.

In our study, though the increase in peroxisome number in OMC of *pex11* and *pex11pex25* is not in par with WT cells, albeit an increase was observed. Hence, we speculate that the increase in peroxisome number in OMC especially in OA medium may not be solely due to increased proliferation. ROS and catalase activity measured in the OMC in OA also suggests no accumulation of dysfunctional peroxisomes. However, it still needs to be investigated in detail what is the contribution of dysfunctional mitochondria to this increase in peroxisome number.

In conclusion, our data clearly shows an increase in peroxisome number in OMC/EMC and this increase may be partly dependent on peroxisome proliferation mediated by Pex11 and also as a response to mitochondrial dysfunction in these cells. Our study suggests that increased peroxisome number can be a marker for early replicative ageing. Interestingly, this increase in number does not result in increased ROS. It would be interesting to know if catalase import is altered in these cells and thereby results in increased activity. On the similar lines, recent

studies suggest that cellular redox levels can affect protein import into peroxisomes (Okumoto et al. 2020).

Understanding conserved mechanisms that govern yeast lifespan will enable us to translate the findings to higher eukaryotes. Yeast ageing can also be used as a tool to identify mechanisms of compounds with antioxidant properties (Stępień et al. 2020). Such studies can also help decipher changes in virulence in pathogenic fungi that may depend on ageing (Bhattacharya et al. 2020).

Chapter 5

Altered peroxisome dynamics upon chronological ageing is associated with the fission proteins Pex11 and Pex25

Abstract

Ageing is characterized by changes in several cellular processes, with dysregulation of peroxisome function being one of them. Interestingly, the most conserved function of peroxisomes, ROS homeostasis, is strongly associated with ageing and age-associated pathologies. Previous studies have identified a role for peroxisomes in the regulation of chronological lifespan in yeast. In this study, we report the effect of altered peroxisome number on the chronological lifespan of yeast in two different growth media conditions. Three mutants, *pex11*, *pex25* and *pex27*, defective in peroxisome fission, have been thoroughly investigated for the chronological lifespan. Reduced chronological lifespan of all the mutants was observed in peroxisome-inducing growth conditions. Furthermore, the combined deletion *pex11pex25* exhibited the most prominent reduction in lifespan. Interestingly altered peroxisomal phenotype upon ageing was observed in all the cells. Increased ROS accumulation and reduced catalase activity was exhibited by chronologically aged mutant cells. Interestingly, mutants with a reduced number of peroxisomes concomitantly also exhibited an accumulation of free fatty acids and increased number of lipid droplets. Taken together, our results reveal a previously unrealized effect of fission proteins in the chronological lifespan of yeast.

This chapter is a part of

Deb R, Nagotu S (2022) The nexus between peroxisome abundance and chronological ageing in *Saccharomyces cerevisiae*. Biogerontology. https://doi.org/10.1007/s10522-022-09992-9

5.1 Introduction

Peroxisomes are single membrane-bound organelles characteristic of eukaryotic cells. The adaptability of size, shape and function to the cell type and cellular environment is a unique feature of these organelles (Smith and Aitchison 2013). Among their numerous cell and tissue-specific functions, fatty acid β-oxidation and ROS homeostasis are the core and conserved functions (He et al. 2021). Apart from being one of the major contributors to ROS production, peroxisomes also help in scavenging the produced ROS by housing several antioxidant enzymes and thus maintaining redox balance within the cell (Fransen and Lismont 2018). Increased oxidative stress in the cell due to the accumulation of ROS is one of the primary causes of ageing and results in the loss of cell viability (López-Otín et al. 2013; Shields et al. 2021).

Ageing is one of the greatest challenges faced by mankind and is associated with physiological decline and deterioration at the cellular and molecular level (Khan et al. 2017). Yeast serves as one of the apt models for studying conserved phenomena associated with ageing (Banerjee et al. 2020; Kaeberlein 2010; Krisko and Kennedy 2021). These single-celled organisms are an important source of discovery of conserved mechanisms of two ageing processes, namely replicative and chronological ageing (Longo et al. 2012). CLS measures the survival time of yeast cells after entering into the stationary phase (Fabrizio and Longo 2003; Mirisola and Longo 2022).

Earlier studies have provided evidence of a connection between impairment of peroxisome functions and chronological ageing in yeast (Lefevre et al. 2013; Mesquita et al. 2010; Petriv and Rachubinski 2004). ROS scavenging mediated by antioxidant enzyme catalase, housed in the peroxisomes, is one such function (Wang et al. 2015). *S. cerevisiae* encodes for two isoforms of catalase, the peroxisomal catalase Cta1 and the cytosolic catalase Ctt1 (Seah and Kaplan 1973). Cells deleted for peroxisomal catalase showed reduced CLS,

whereas cells lacking both the isoforms showed extended CLS (Mesquita et al. 2010; Petriv and Rachubinski 2004). Another important function of peroxisomes is β-oxidation of fatty acids, for which they are the sole sites in yeast (Poirier et al. 2006). Complete or partial block in this pathway resulted in shortened CLS of *S. cerevisiae* cells (Lefevre et al. 2013).

Proteins that play a vital role in regulating peroxisome abundance in a cell belong to the Pex11 family (Carmichael and Schrader 2022). In *S. cerevisiae* Pex11, Pex25 and Pex27 belong to this family (Rottensteiner et al. 2003; Tam et al. 2003). A role for proteins involved in peroxisome biogenesis in the regulation of yeast CLS has been reported by earlier studies (Lefevre et al. 2015; Lefevre et al. 2013; Scheckhuber 2020). Interestingly, the lack of peroxisomal proteins such as Pex3/Pex25/Pex27 did not show any change in the CLS in cells cultured in 2% glucose medium (Chalermwat et al. 2020; Lefevre et al. 2013). In this study, we aim to understand how altered peroxisome number regulates the CLS of *S. cerevisiae* under both CR and oleic acid OA media conditions. Furthermore, the effect of the altered number of peroxisomes on oxidative stress, free fatty acid accumulation, lipid bodies, and metabolism was also analysed in chronologically aged cells. Our results suggest a variable role for the Pex11 family proteins in regulating CLS and associated cellular changes in OA cultured yeast cells.

5.2 Results

5.2.1 *S. cerevisiae* peroxisome fission mutants do not exhibit altered CLS in CR conditions

Previous studies highlighted the role of peroxisomes in yeast chronological ageing under CR conditions (Arlia-Ciommo et al. 2018; Lefevre et al. 2015; Lefevre et al. 2013; Mesquita et al. 2010). Proteins involved in peroxisome biogenesis and matrix protein import, such as Pex3, Pex5 and Pex7, when deleted, resulted in reduced CLS under CR conditions (Lefevre et al. 2013). Similarly, cells lacking enzymes required for β-oxidation, such as Fox1/Fox2/Fox3/Pot1 showed reduced CLS (Arlia-Ciommo et al. 2018). Cells lacking

peroxisomal catalase required for ROS scavenging in *S. cerevisiae* depicted extended CLS (Mesquita et al. 2010). Interestingly, Lefevre and colleagues showed that specifically blocking peroxisome fission and not mitochondrial fission resulted in extended CLS (Lefevre et al. 2015).

The peroxins which play a vital role in peroxisome fission belong to the Pex11 family. In *S. cerevisiae*, this family comprises Pex11, Pex25 and Pex27 (Tam et al. 2003). Interestingly, cells lacking both Pex11 and Pex25 show the most significant reduction in peroxisome number and a role for Pex25 in the formation of peroxisomes from the ER has also been proposed (Huber et al. 2012; Tam et al. 2003). Thus, to understand the effect of altered peroxisome abundance on chronological ageing in yeast, CLS experiments similar to WT (Chapter 3) were also performed in *pex11*, *pex25*, *pex27* and *pex11pex25* cells.

pex11, *pex25*, *pex27* and *pex11pex25* cells showed similar mean lifespan as WT cells when cultured in CR medium (Table 5.1). However, *pex27* cells displayed a significant increase (p=0.02) in the maximal lifespan (25 days) compared to WT cells (21 days) (Fig. 5.1a and 5.1b, Table 5.1). In line with this, *pex27* cells also showed a significant increase in cell viability upon ageing when compared to WT cells (Fig. 5.1c). On the other hand, *pex25* cells displayed a significant decrease in cell viability on the 11th, 15th and 19th day when compared to WT (Fig. 5.1c).

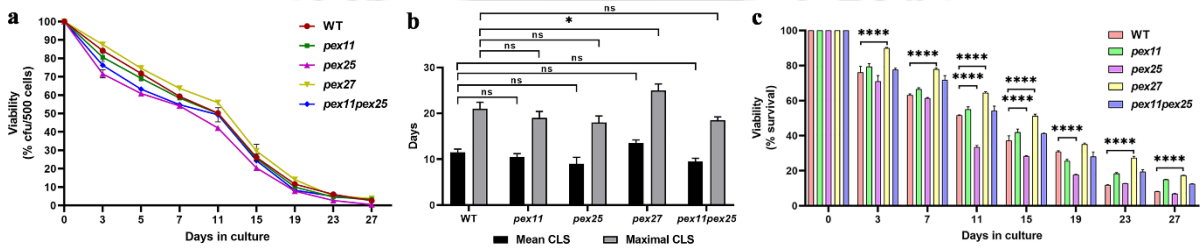

Fig. 5.1: Chronological lifespan (CLS) of the studied strains in CR condition.
(a) The CLS curves obtained from CFU assay is represented (n=2). **(b)** The mean and maximal CLS calculated from data in (b) is depicted. **(c)** Percentage cell viability obtained from PI staining of the cells is represented. (*P < .05; **P < .01, ***P < .001 and ****P < 0.0001; two-way ANOVA).

5.2.2 Glucose and ethanol utilization in chronologically aged cells under CR conditions

Yeast cells cultured in standard glucose culture conditions accumulate fermentation products, such as ethanol (Fabrizio et al. 2005). On the other hand, yeast cells cultured under CR conditions (0.5% glucose) consume the produced ethanol (Fig. 5.2a) (Arlia-Ciommo et al. 2018). Earlier studies reported ethanol accumulation in the culture media due to slower consumption by the cells and resulted in accelerated chronological ageing (Arlia-Ciommo et al. 2018). To assess the effect of deletion of Pex11 family proteins on the metabolic status of the cells, the concentrations of glucose and ethanol were measured in the CR culture medium (containing 0.5% glucose) over different days of chronological ageing by HPLC. Our analysis showed that by 0^{th} day glucose was undetectable in the culture medium and the rate of ethanol utilization was similar in WT and all the deletion strains studied (Fig. 5.2b and 5.2c). These data are in line with the results of the chronological ageing experiment where no significant difference between the mean life span of WT, *pex11*, *pex25*, *pex27* and *pex11pex25* was observed (Fig. 5.1).

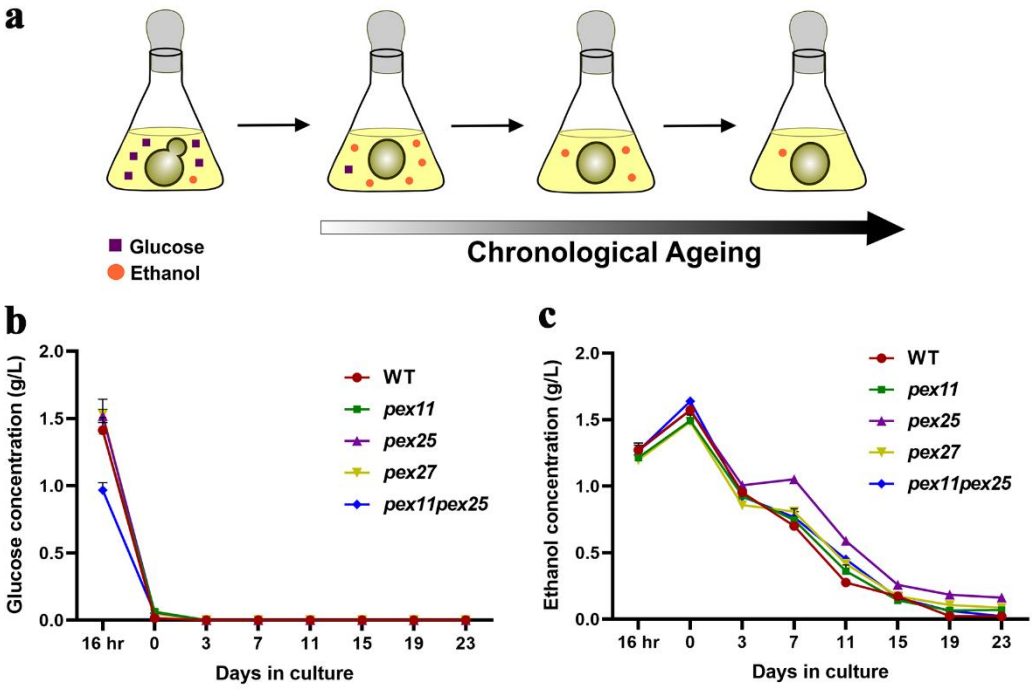

Fig. 5.2: Glucose and ethanol detection upon chronological ageing in yeast mutants.
(a) Schematic representation of glucose and ethanol utilization by the chronologically aged yeast cells. Yeast cells cultured in glucose produce ethanol as a by-product of fermentation. Under CR conditions (0.5% glucose), the

produced ethanol is consumed by the cells upon chronological ageing. **(b)** and **(c)** represent the concentrations of glucose and ethanol as measured using HPLC in the CR culture medium of WT and deletion strains at different days of chronological ageing. Error bars indicate absolute deviations from the mean value (n=2).

5.2.3 *S. cerevisiae* peroxisome fission mutants exhibit altered CLS when cultured in OA medium

Previous studies have reported that cells with defects in peroxisome biogenesis (*pex3*), β-oxidation (*pot1*), peroxisome fission (*pex11*) and inheritance (*inp1*) depicted reduced CLS when cultured in OA medium (Lefevre et al. 2013; Scheckhuber 2020). The CLS of the fission mutants were also analysed in the OA medium. *pex11* cells in our study depicted a significant reduction in the mean and maximal CLS when compared to WT cells (11.5 and 20 days) (Fig. 5.3a and 5.3b, Table 5.1). A significant decrease in cell viability in these cells was observed from 11th day onwards (Fig. 5.3c). On the other hand, no significant difference in the mean and maximal lifespan of *pex25* and *pex27* cells, when compared to WT was observed (Fig. 5.3a and 5.3b, Table 5.1). A similar result was observed with respect to the cell viability of these strains (Fig. 5.3c). Interestingly, the double deletion *pex11pex25* cells displayed the shortest CLS in OA medium with a mean and maximal lifespan of 7 and 18 days (Fig. 5.3a and 5.3b, Table 5.1). On the 27th day, only 2.15% of *pex11pex25* cells were viable compared to 27.03% of WT cells (Fig. 5.3c).

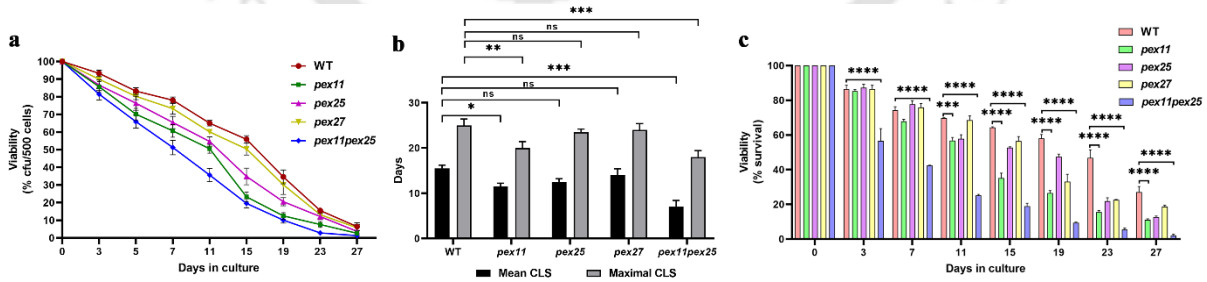

Fig. 5.3: Chronological lifespan (CLS) of the studied strains in OA condition
(a) CLS curves obtained from CFU assay. **(b)** The mean and maximal CLS of the cells calculated from data in (a) is represented. **(c)** Percentage cell viability obtained from PI staining of the cells is represented. (*P < .05; **P < .01, ***P < .001 and ****P < 0.0001; two-way ANOVA).

5.2.4 Chronologically aged cells depict altered peroxisomal phenotype

In order to understand if peroxisome morphology in the fission mutants are altered upon chronological ageing, all the strains expressing DsRed-SKL were analysed on different days of the CLS experiment in both CR and OA media conditions (Fig. 5.4 and 5.5). The number of peroxisomes on the 0^{th} day in both CR and OA conditions was analysed in different strains (Fig. 5.4a and 5.4b). Fig. 5.4c represents the distribution of peroxisomes at 0^{th} day in CR and OA media (Fig. 5.4c). In CR condition, a significant reduction in peroxisome number was observed in *pex25* (2.41 ± 0.19 peroxisomes/cell) and *pex11pex25* (2.36 ± 0.12 peroxisomes/cell) cells, whereas *pex11* cells displayed an increase in peroxisome number (4.99 ± 0.15 peroxisomes/cell) as compared to WT cells (Fig. 5.4d, Table 5.2). In OA media, *pex11*, *pex25* and *pex11pex25* cells showed significantly less number of peroxisomes when compared to WT cells with 4.9 ± 0.06, 6.82 ± 0.02 and 4.53 ± 0.01 peroxisomes/cell, respectively (Fig. 5.4d, Table 5.2). No significant change in peroxisome number was observed in *pex27* cells in both CR and OA conditions (Fig. 5.4d).

Table 5.1: Mean and maximal CLS of the studied strains under different growth conditions. The mean and maximal CLS are defined as the time points where 50% and less than 10% of the cells are viable (able to form colony), respectively. Data are represented as mean ± SEM from two independent experiments. The statistical difference between the strains and WT is represented as p-values.

Strains	Mean CLS (p-value)	Maximal CLS (p-value)
CR (0.5% glucose)		
WT	11.5 ± 0.5	21 ± 1
pex11	10.5 ± 0.5 (0.8634)	19 ± 1 (0.3553)
pex25	9 ± 1 (0.1832)	18 ± 1 (0.0887)
pex27	13.5 ± 0.5 (0.3553)	25 ± 1 (0.0200)
pex11pex25	9.5 ± 0.5 (0.3553)	18.5 ± 0.5 (0.1832)
OA		
WT	15.5 ± 0.5	25 ± 1
pex11	11.5 ± 0.5 (0.0225)	20 ± 1 (0.0059)
pex25	12.5 ± 0.5 (0.0890)	23.5 ± 0.5 (0.5488)
pex27	14 ± 1 (0.5488)	24 ± 1 (0.8127)
pex11pex25	7 ± 1 (0.0001)	18 ± 1 (0.0005)

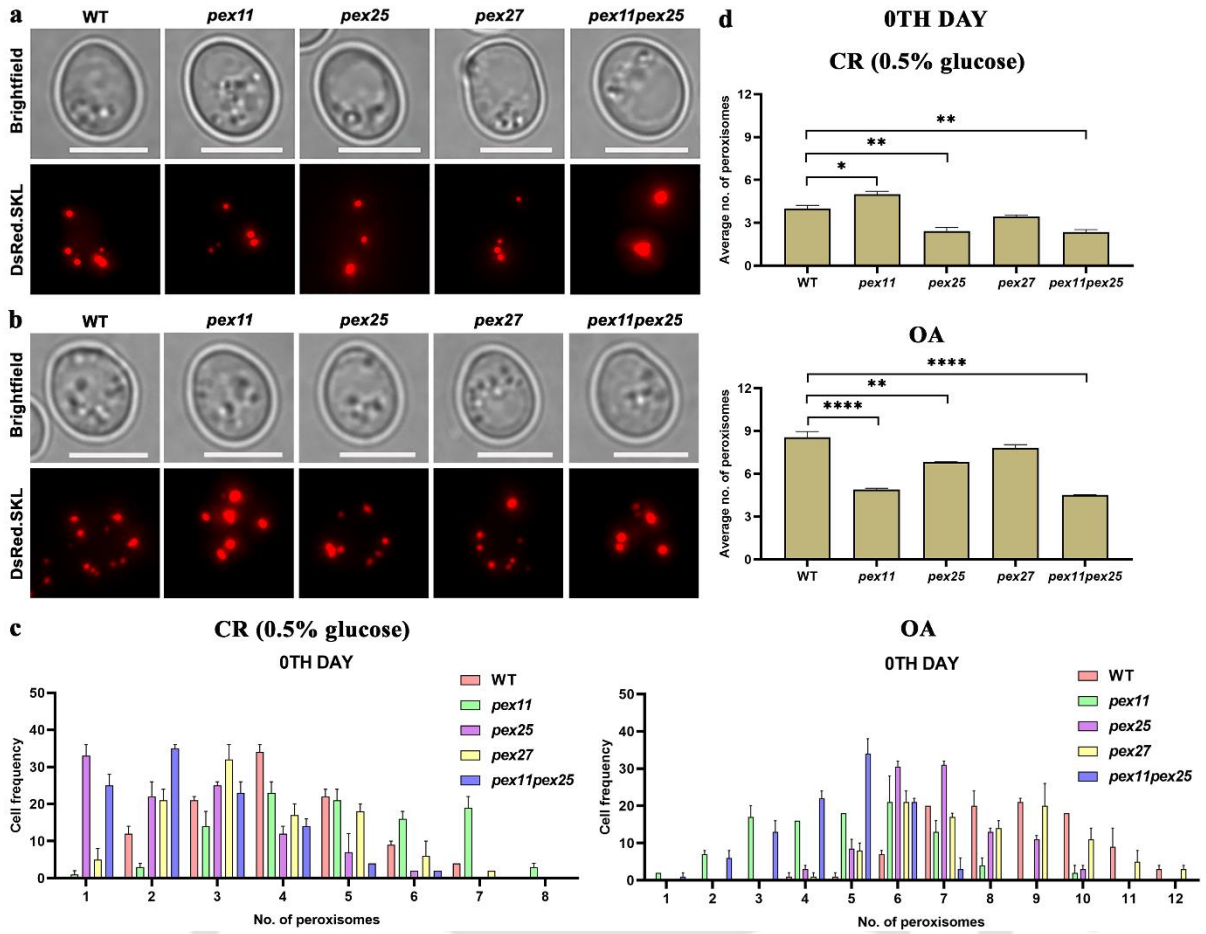

Fig. 5.4: Altered peroxisome number in cells lacking Pex11 and Pex25 in CR and OA media conditions. **(a)** and **(b)** Fluorescence microscopy images depicting peroxisomes in WT and deletion cells on the 0th day of chronological ageing in CR and OA media, respectively. Peroxisomes were marked with DsRed-SKL and visualized by fluorescence microscopy. Scale bar - 5 µm. **(c)** and **(d)** represents the average number and range of peroxisomes in different strains on the 0th day. (*P < .05; **P < .01, ***P < .001 and ****P < 0.0001; one-way ANOVA).

Peroxisome phenotype (DsRed-SKL signal) was also analysed in all the strains at different days of chronological ageing (Fig. 5.5, 5.6 and 5.7). Cells of all studied strains depicted mostly punctate phenotype on the 0th day when cultured in both the media conditions (Fig. 5.5, 5.6 and 5.7). All the studied strains displayed reduced number of cells with only punctate phenotype and increased percentage of cells with cytosolic fluorescence upon chronological ageing in both the media conditions (Fig. 5.7).

In OA media, most of the *pex11*, *pex25* and *pex11pex25* cells displayed significantly less punctate (38%, 28% and 25%) phenotype when compared to WT cells in the OA medium as early as on 3rd day (Fig. 5.7b). Among the deletion strains, *pex27* cells showed the highest

percentage of cells (70%) with punctate fluorescence and resembled WT cells in the phenotype. *pex11* and *pex11pex25* cells exhibited the highest percentage of cells with cytosolic phenotype on all days analysed (Fig. 5.7). Interestingly, this is in line with the fact that these mutants also showed reduced CLS in cells grown in OA medium (Fig. 5.3). Previous studies have also reported reduced CLS in cells defective for peroxisomal matrix protein import under CR conditions (Lefevre et al. 2013). Thus, it might be suggested that *pex11* and *pex11pex25* cells exhibit hampered peroxisomal matrix protein import.

Table 5.2: Average number of peroxisomes in the studied strains quantified at 0th day of chronological ageing. 50 random non-budding cells cultured in CR and OA media were selected for quantification. All values are calculated as mean ± SEM. The statistical difference between the strains and WT is represented as p-values.

Strains	CR (0.5% glucose) (p-value)	OA (p-value)
WT	4.01 ± 0.15	8.55 ± 0.29
pex11	4.99 ± 0.15 (0.0136)	4.9 ± 0.06 (<0.0001)
pex25	2.41 ± 0.19 (0.0016)	6.82 ± 0.02 (0.0014)
pex27	3.44 ± 0.08 (0.1010)	7.82 ± 0.16 (0.0529)
pex11pex25	2.36 ± 0.12 (0.0014)	4.53 ± 0.01 (<0.0001)

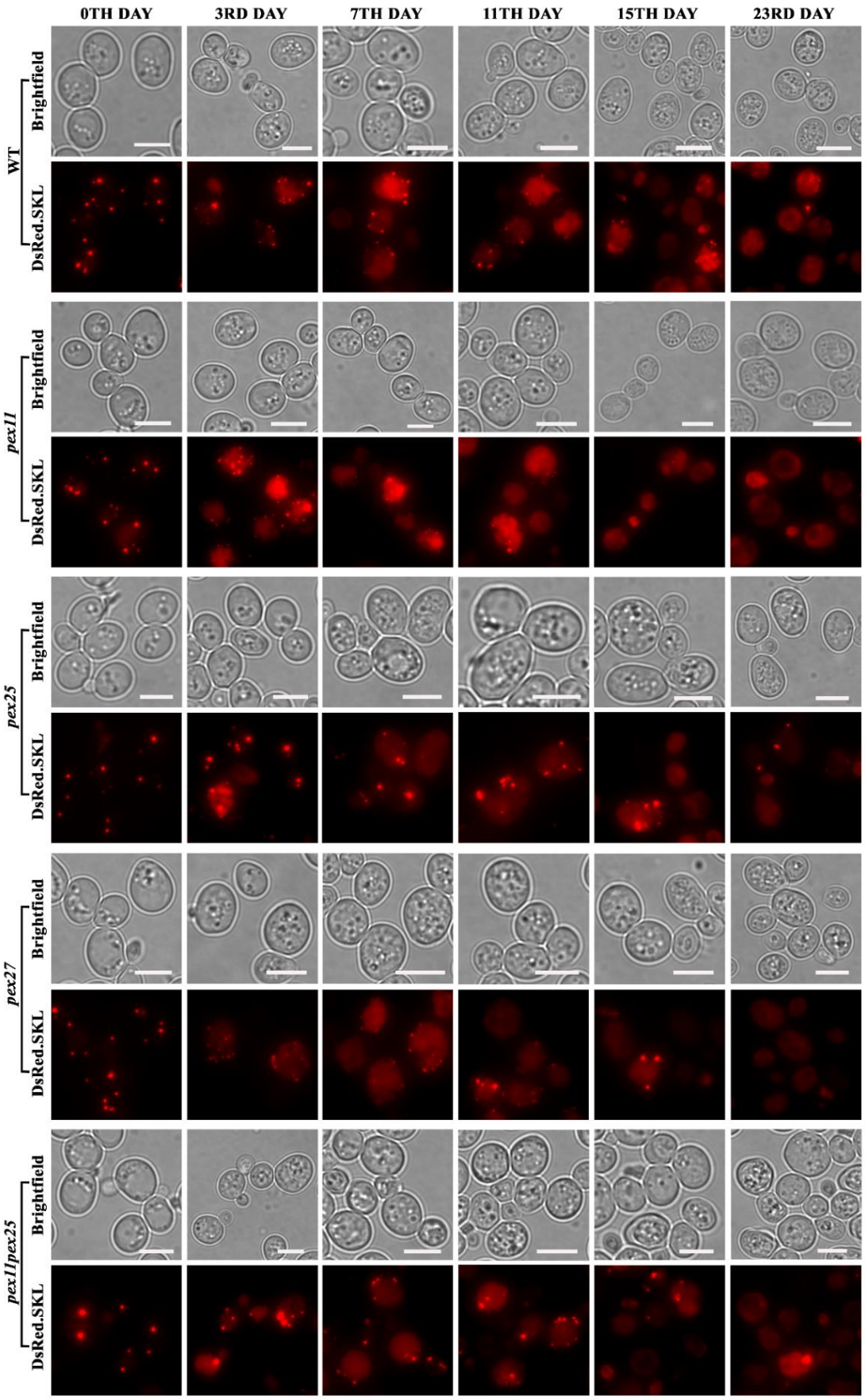

Fig. 5.5: Fluorescence microscopy images of cells expressing DsRed-SKL on different days of chronological ageing in CR media. Scale bar - 5 µm.

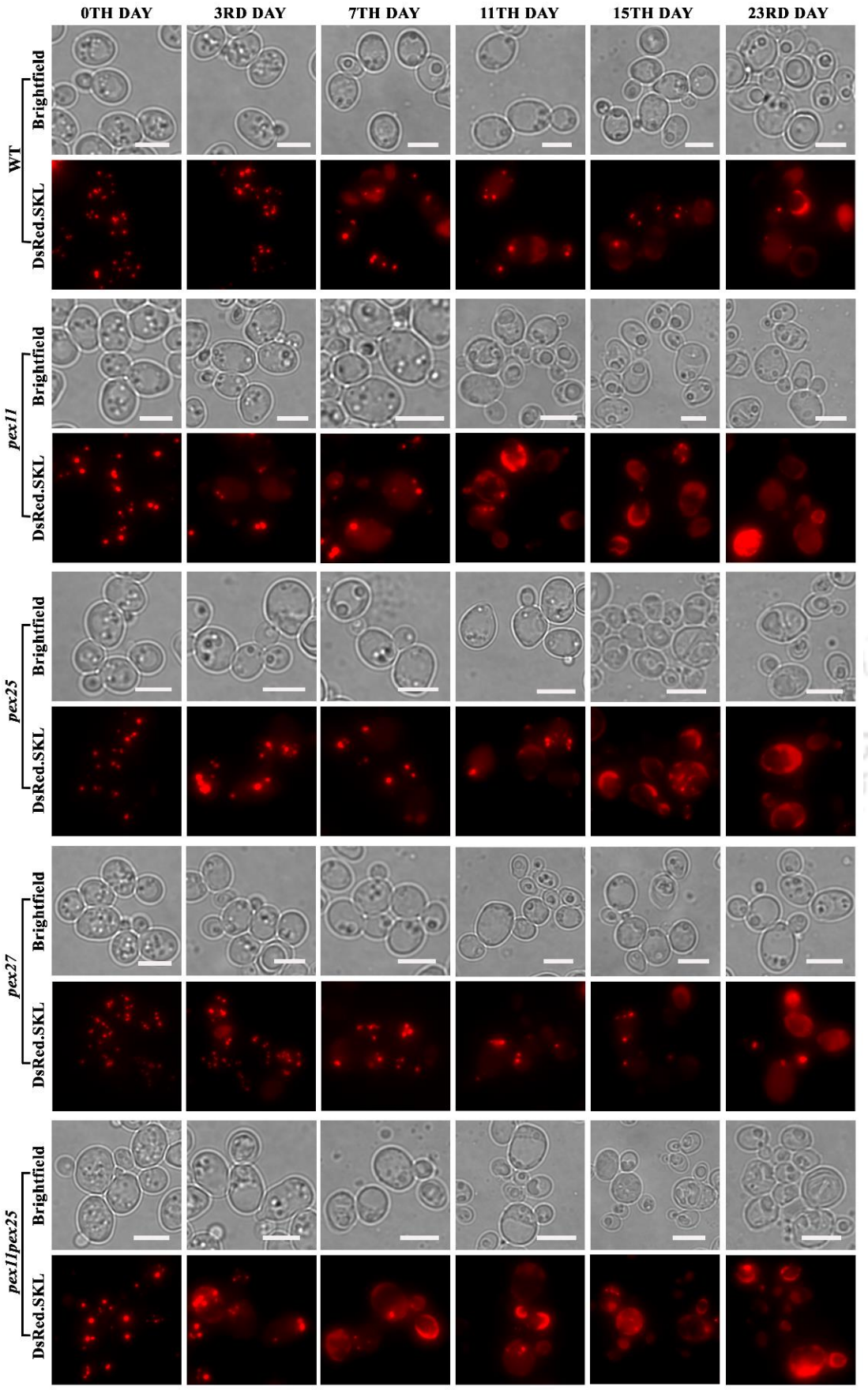

Fig. 5.6: Fluorescence microscopy images of cells expressing DsRed-SKL on different days of chronological ageing in OA media. Scale bar - 5 µm.

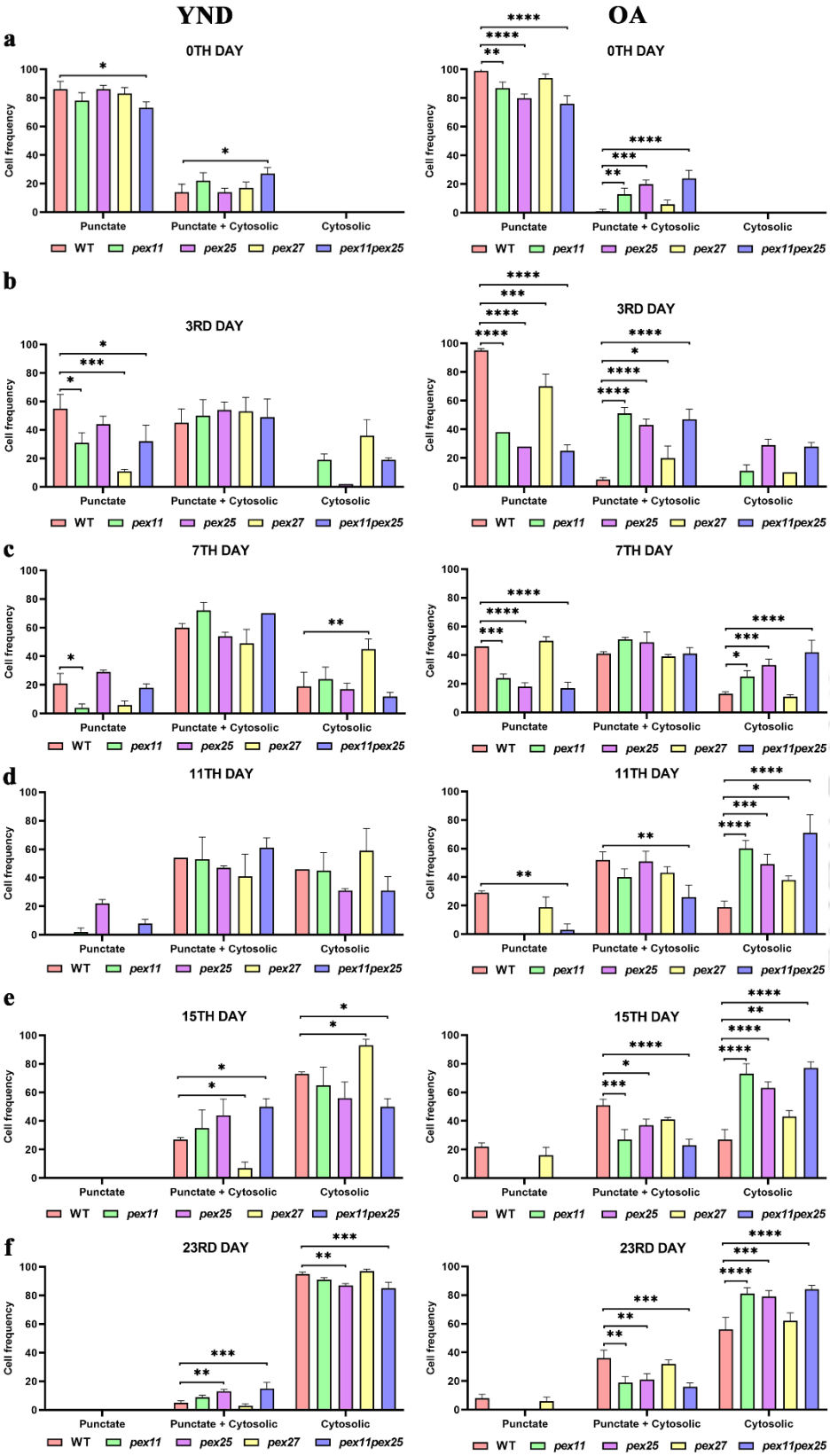

Fig. 5.7: Peroxisomal phenotype is altered upon chronological ageing in CR and OA media conditions. Peroxisomes were marked with DsRed-SKL and the DsRed-SKL signal was categorized into three phenotypes *i.e.* punctate, punctate + cytosolic and cytosolic. **(a)**, **(b)**, **(c)**, **(d)**, **(e)** and **(f)** represent the variation in peroxisome morphology in the cells on the 0^{th}, 3^{rd}, 7^{th}, 11^{th}, 15^{th} and 23^{rd} days of chronological ageing experiment, respectively.

Quantitative analysis of 50 cells from each experiment was performed (n =2). (*P < .05; **P < .01, ***P < .001 and ****P < 0.0001; two-way ANOVA).

5.2.5 Increased LD number and FFA accumulation is observed in cells with reduced number of peroxisomes upon chronological ageing

LDs are specialized organelles that maintain storage lipids in the cytosol (Brasaemle and Wolins 2012). They are made up of a neutral lipid core and are enclosed in a phospholipid monolayer (Beller et al. 2010). The neutral lipids, triacylglycerols (TAG) and ergosteryl esters (EE) are first synthesized in the ER and then subsequently get deposited in the LDs. TAG lipolysis in LD produces FFAs which then undergo β-oxidation in the peroxisomes (Fig. 5.8a) (Kohlwein et al. 2013). Efficient oxidation of FFAs in the peroxisomes was reported to assure longevity in chronologically aged yeast cells (Arlia-Ciommo et al. 2018). Earlier studies have reported that mutants with defective β-oxidation when cultured in OA media depicted shortened CLS and this was most likely due to the lipotoxicity caused by higher levels of FFA in these cells (Lefevre et al. 2013). Moreover, studies also reported the involvement of Pex11 in fatty acid oxidation in *S. cerevisiae* and maintenance of lipid homeostasis in *Y. lipolytica* (Dulermo et al. 2015; van Roermund et al. 2000). Thus, in order to test if reduced CLS in the peroxisome fission mutants was associated with FFA accumulation, the concentration of FFA was determined in the cells grown in OA media on the 0^{th} and 7^{th} day of ageing experiments (Fig. 5.8b). 7^{th} day was chosen as this was the mean CLS of *pex11pex25* cells in OA medium (Table 5.1). As shown in fig. 5.8b, all the deletion mutants displayed a similar concentration of FFA as WT cells on the 0^{th} day. On the 7^{th} day, WT cells showed a reduction in concentration of FFA when compared to 0^{th} day (Fig. 5.8b, Table 5.3). However, on the 7^{th} day, all the fission mutants exhibited significantly higher FFA concentration when compared to the WT cells with the highest in *pex25* and *pex11pex25* cells (Fig. 5.8b, Table 5.3).

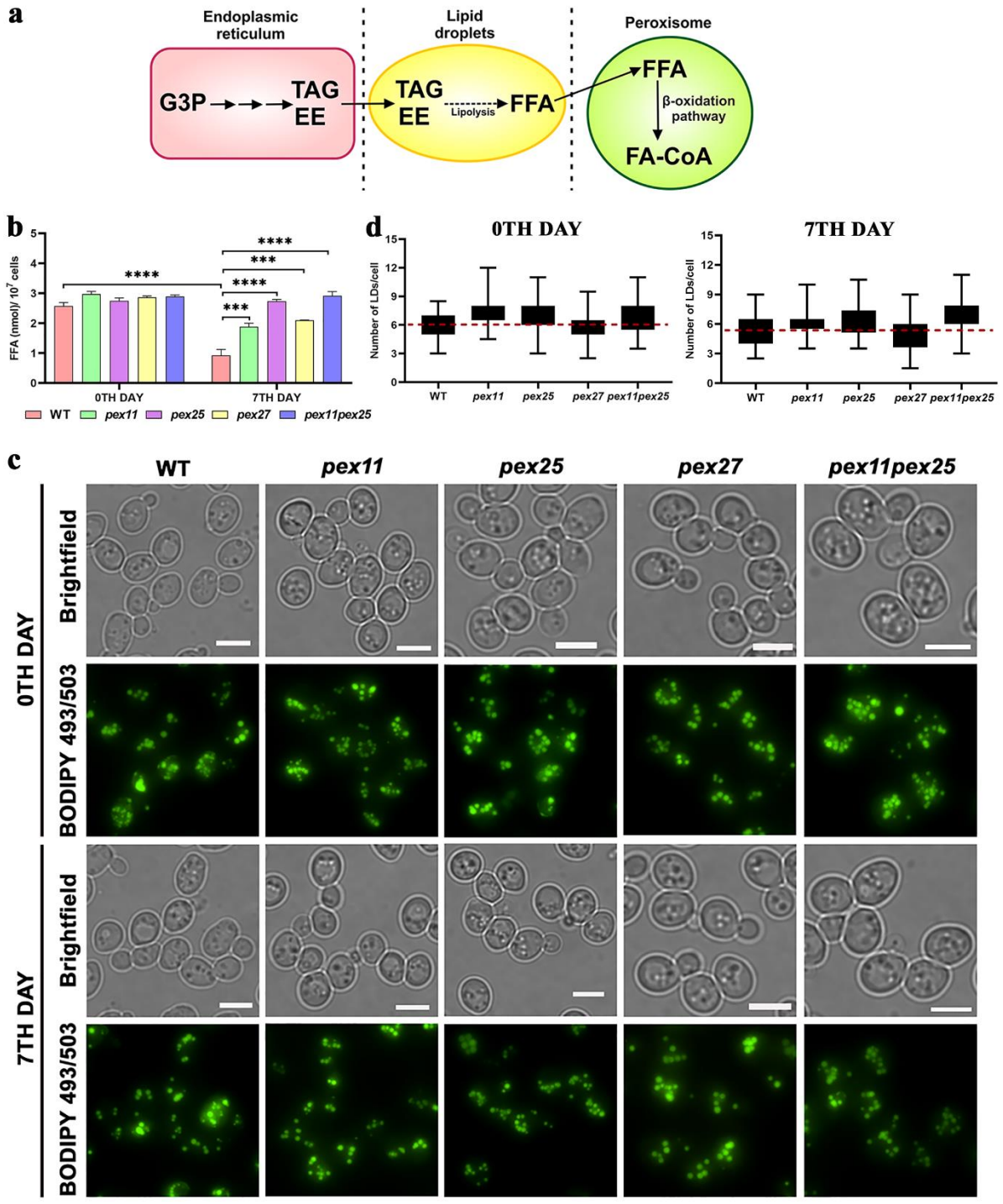

Fig. 5.8: Chronologically aged cells depict altered free fatty acid (FFA) accumulation and lipid droplet (LD) number.

(a) Schematic representing the synthesis and oxidation of the FFAs. The neutral lipids, triacylglycerols (TAG) and ergosteryl esters (EE), synthesized in the ER from glycerol-3-phosphate (G3P) are stored in the LDs. TAG further undergoes lipolysis and results in the formation of FFAs in the LDs, which subsequently enters peroxisomes to undergo β-oxidation and result in the formation of FA-CoA. **(b)** The bar graph represents the amount of FFAs in WT and deletion cells measured on the 0^{th} and 7^{th} day of chronological ageing in the OA medium. **(c)** Fluorescence microscopy images depict LDs in different deletion cells stained with BODIPY 493/503. **(d)** The box and whisker plot illustrates the distribution range of LD number in the strains. The mean of LDs in WT cells is denoted by the red dashed line. Quantification data were obtained from two independent

experiments (n =2) and LDs were counted in 50 cells in each experiment. Scale bar represents 5 µm. (*P < .05; **P < .01, ***P < .001 and ****P < 0.0001; two-way ANOVA).

To further confirm the accumulation of FFA, LDs in cells cultured in OA media were labelled with BODIPY 493/503 and the number of LDs were quantified on the 0^{th} and 7^{th} day of CLS (Fig. 5.8c and 5.8d). A significant increase in the average number of LDs was observed in *pex11* and *pex11pex25* cells as compared to WT on the 0^{th} day (Fig. 5.8c and 5.8d, Table 5.3). Interestingly, on 7^{th} day, increased number of LDs was observed in *pex25* and *pex11pex25* cells when compared to WT (Fig. 5.8c and 5.8d, Table 5.3).

Table 5.3: Average number of lipid droplets (LDs) and amount of FFAs in all studied strains at the 0^{th} and 7^{th} days of chronological ageing in OA medium. For quantification, 50 random non-budding cells were selected at each time point. All values are calculated as mean ± SEM. The statistical difference between the strains and WT is represented as p-values.

Strains	Average no. of LDs		Amount of FFA (nmol)/10^7 cells	
	Day 0 (p-value)	Day 7 (p-value)	Day 0 (p-value)	Day 7 (p-value)
WT	6.02 ± 0.01	5.37 ± 0.06	2.58 ± 0.11	0.92 ± 0.20
pex11	7.35 ± 0.08 (0.0046)	5.83 ± 0.37 (0.3966)	2.97 ± 0.09 (0.0734)	1.88 ± 0.12 (0.0003)
pex25	6.85 ± 0.41 (0.0631)	6.37 ± 0.06 (0.0251)	2.75 ± 0.09 (0.6180)	2.73 ± 0.06 (<0.0001)
pex27	5.95 ± 0.07 (0.9976)	4.90 ± 0.30 (0.4032)	2.87 ± 0.05 (0.2273)	2.10 ± 0.01 (0.0003)
pex11pex25	7.04 ± 0.08 (0.0231)	6.79 ± 0.08 (0.0029)	2.89 ± 0.06 (0.1791)	2.92 ± 0.14 (<0.0001)

5.2.6 Altered ROS homeostasis in chronologically aged cells

Apart from mitochondria, peroxisomes are one of the major contributors of ROS in the cell (Deori et al. 2018). Oxidative stress in the cells due to ROS accumulation is considered one of the hallmarks of ageing (Liguori et al. 2018). Earlier studies have shown increased oxidative stress upon chronological ageing in mutants defective in peroxisome biogenesis (Lefevre et al. 2013). To understand whether deletion of Pex11 family proteins alters the oxidative stress, intracellular ROS was measured in the cells cultured in CR and OA on different days of chronological ageing (Fig. 5.9a). Increased ROS accumulation with

chronological ageing in the cells grown in both the media conditions was observed. However, this increase was higher in OA than in CR medium and no significant change in accumulation was observed between the strains in CR conditions (Fig. 5.9a). In OA medium *pex11pex25* cells showed a significant increase in ROS accumulation as compared to WT from 15th day onwards (Fig. 5.9a).

S. cerevisiae has two catalases encoded by Ctt1 (cytosolic) and Cta1 (peroxisomal). Earlier studies have reported that Ctt1 is expressed constitutively except for conditions such as oxidative stress (Jamieson 1998; van der Klei et al. 1990). Cta1, on the other hand, is induced in peroxisome-inducing media such as OA. Change in the overall catalase activity upon chronological ageing was estimated and is represented as the fold change relative to the 0th day WT (Fig. 5.9b). No significant change in the catalase activity upon chronological ageing was observed in the CR medium and also no difference between strains was noted (Fig. 5.9b). On the other hand, WT, *pex11*, *pex25* and *pex11pex25* cells showed a significant decrease in catalase activity with ageing on the OA medium. The *pex11* and *pex11pex25* cells depicted the most reduction in catalase activity among these. Interestingly, *pex27* cells did not show any significant reduction in catalase activity with age (Fig. 5.9b).

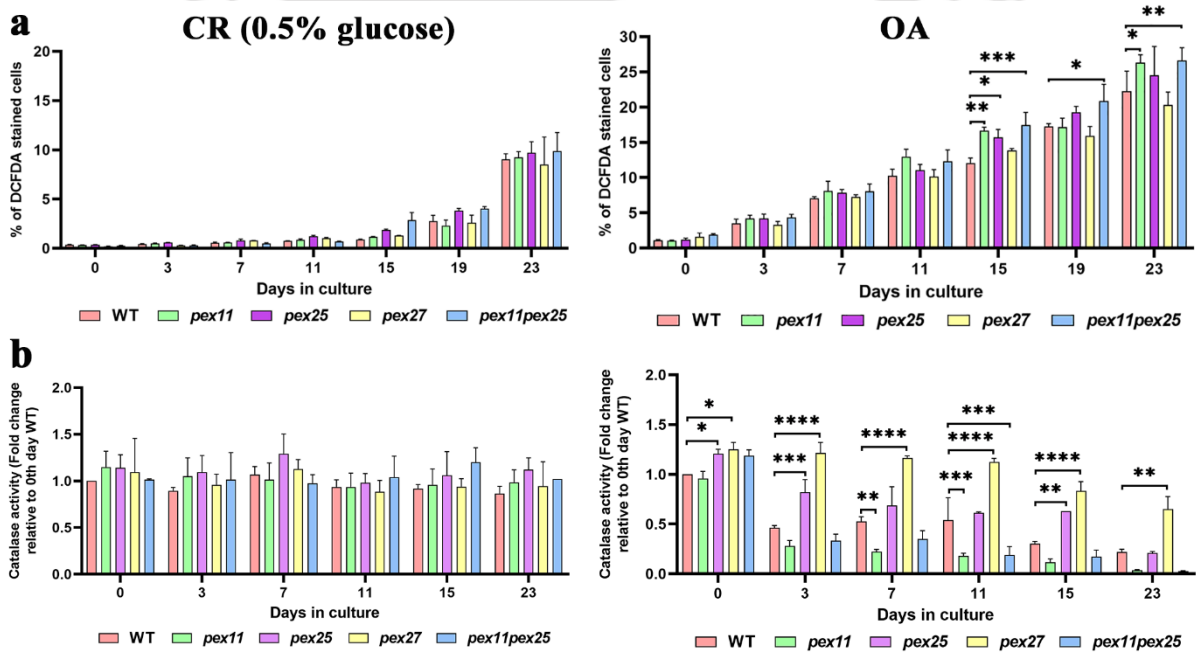

Fig. 5.9: Chronologically aged cells in OA media show enhanced ROS and reduced catalase activity.
(a) Flow cytometry analysis of ROS accumulation in cells at different days of chronological ageing using the fluorescent probe H_2-DCFDA. **(b)** Catalase activity measured on different days is represented as fold change relative to the activity of WT cells on the 0^{th} day (n =2). (*P < .05; **P < .01, ***P < .001 and ****P < 0.0001; two-way ANOVA).

5.3 Discussion

Several recent studies have highlighted the role of subcellular compartments such as vacuole, mitochondria *etc.* in cellular ageing (Banerjee et al. 2020). The role of peroxisomes in ageing have also been extensively studied in various model organisms (Deori et al. 2018; Fanelli et al. 2013; Legakis et al. 2002). In this study, we aim to take forward our understanding of the role of peroxisomes in yeast chronological ageing. Peroxisome abundance in a cell requires division proteins that belong to the Pex11 family (Huber et al. 2012). Cells lacking both Pex11 and Pex25 depict a reduced number of peroxisomes in both peroxisome-inducing and non-inducing growth conditions (Tam et al. 2003). In the current study, the CLS of these mutants was assessed in both CR and OA conditions.

Our results suggest that reduced number of peroxisomes does not affect the lifespan of cells in CR conditions evident from the data of *pex11pex25* cells. Mean CLS did not alter between WT and mutants studied in these conditions (Fig. 5.1). Upon HPLC analysis, no significant change in the glucose and ethanol utilization in all the studied strains was observed (Fig. 5.2). However, *pex11*, *pex25* and *pex11pex25* cells showed shortened CLS compared to WT when grown in OA (Fig. 5.3, Table 5.1). *pex27* cells that exhibited similar peroxisome number and phenotype as that of WT cells also depicted CLS similar to WT in OA conditions (Fig. 5.3; Table 5.2). Interestingly, earlier studies in yeast mutants defective in peroxisome biogenesis and protein import have shown reduced CLS in both CR and OA medium (Lefevre et al. 2013). On the other hand, Lefevre and colleagues showed that maximum CLS and not the mean CLS is extended upon blocking peroxisome fission (Lefevre et al. 2015). However, in our study, no such extension of maximum CLS was observed under CR conditions.

To further investigate this reduced CLS of cells that lack Pex11 family proteins in OA, we chose to analyze various parameters that may be affected in these cells. Ageing-associated alteration in peroxisome number and matrix protein import was observed in these cells (Fig. 5.4 and 5.7). This is in line with studies by Lefevre and colleagues, who also reported reduced CLS in cells lacking receptors for peroxisome matrix protein import (Lefevre et al. 2013). The *pex11pex25* cells showed the least number of peroxisomes when cultured in both CR and OA conditions on 0^{th} day (Table 5.2). Most cells showed completely cytosolic DsRed-SKL fluorescence by the end of the CLS experiment indicating a collapse of import-competent peroxisomes (Fig. 5.7). Interestingly, increased oxidative stress and FFA accumulation were observed in OA-induced mutant cells, indicating a decline in peroxisome function in aged cells (Fig. 5.8 and 5.9). Earlier studies have demonstrated that TAG accumulation and enlarged LDs are associated with CLS (Goldberg et al. 2009a; Handee et al. 2016). The accumulated FFA are proposed to result in necrotic cell death resulting in the observed reduced viability upon chronological ageing. An association of the increased number of LDs with an extension of CLS has also been recently reported (Kovacs et al. 2021). The *pex11pex25* cells showed the most significant reduction in catalase activity and, at the same time, showed the highest accumulation of FFA (Fig. 5.8 and 5.9).

Several large-scale CLS screens have been performed to identify proteins that are associated with the regulation of CLS. However, many such identified candidates have turned out to be false-positive hits when confirmed experimentally (Burtner et al. 2011; Fabrizio et al. 2010; Garay et al. 2014; Matecic et al. 2010; Powers et al. 2006). Pex25 was also identified as one such candidate in a screen by Fabrizio and colleagues (Fabrizio et al. 2010). Recent studies by Pogoda and colleagues also identified Pex11 as one of the genes whose downregulation may extend CLS (Pogoda et al. 2021). However, our experimental analysis shows no role for both Pex25 and Pex11 in CLS regulation under CR conditions (Fig. 5.1). On the other hand, our

study shows a significant role of both the proteins in CLS of OA-grown cells (Fig. 5.3). Interestingly, in line with our study Chalermwat and colleagues also reported an extended lifespan of cells upon overexpression of Pex11 in WT cells (Chalermwat et al. 2020).

To summarize, in this manuscript we have assessed various cellular parameters associated with peroxisomes and their effect on yeast CLS. Interestingly, the analysed mutants did not show any effect on CLS in CR conditions. Cells lacking Pex11 that depict reduced number of functional peroxisomes, increased accumulation of FFAs and LDs, reduced catalase activity and increased ROS accumulation exhibit reduced CLS in peroxisome-inducing growth conditions. *pex25* cells on the other hand depicted reduced number of peroxisomes albeit with no change in catalase activity and ROS accumulation. Accumulation of FFAs and LDs was also observed in these cells that resulted in reduction of CLS but to a lesser extent compared to *pex11* cells. Interestingly, a synergistic effect was observed in the double deletion *pex11pex25* cells which exhibited the greatest reduction in CLS and depicted the maximum alteration in the studied parameters (Fig. 5.10). Cells lacking the paralog of Pex25, Pex27 had the least effect on the CLS with no significant changes associated with peroxisome function. These cells displayed no change in catalase activity upon chronological ageing that can also be correlated to the higher number of cells with punctate peroxisome phenotype on all days. Surprisingly, FFA accumulation observed in these cells was similar to *pex11* cells.

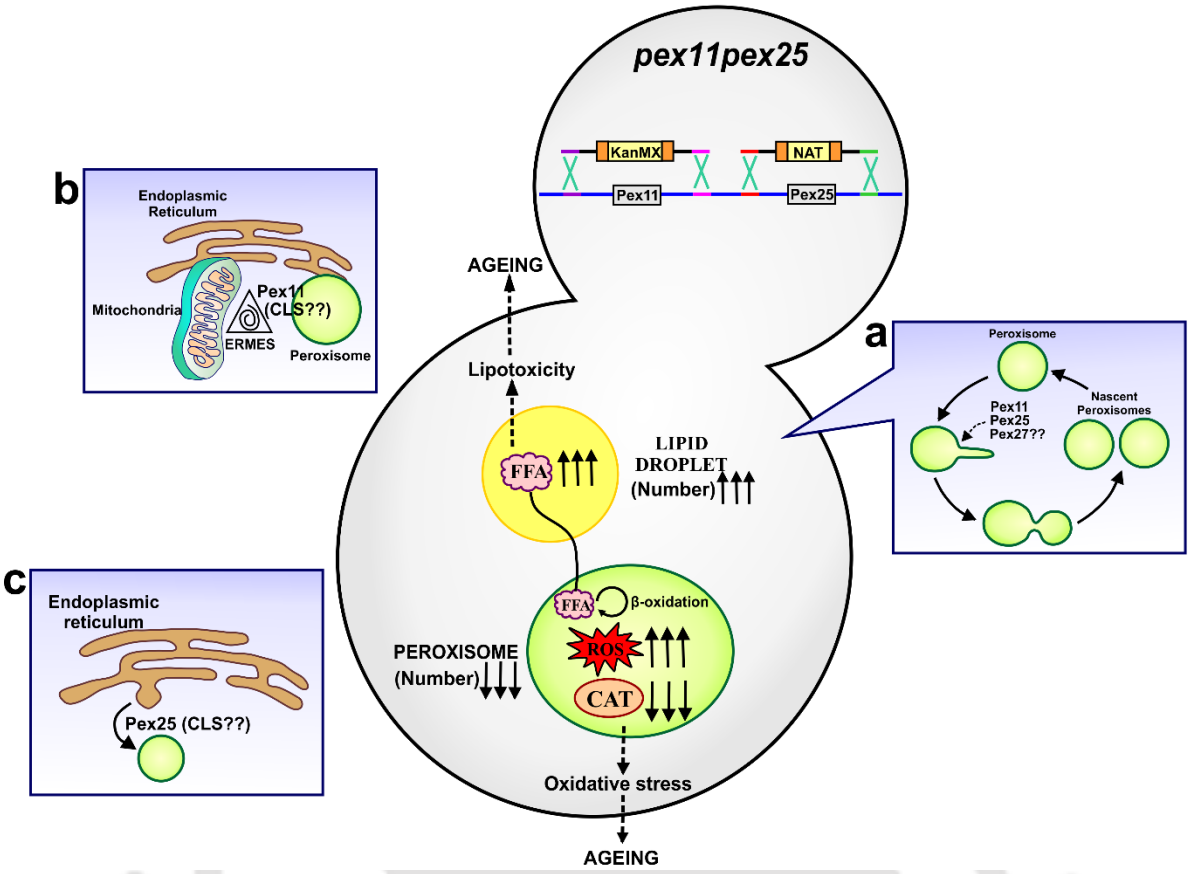

Fig. 5.10: Schematic representing age-associated changes in *pex11pex25* cells identified in this study. Cells lacking both Pex11 and Pex25 depicted reduced number of peroxisomes and increased number of LDs in OA medium. Free fatty acids (FFAs) formed in the lipid droplets (LDs) are metabolized by the β-oxidation pathway in peroxisomes. This metabolic pathway helps in maintaining the levels of FFAs in the cell. The reduced peroxisome number most likely results in the accumulation of FFAs in the LDs. These cells also exhibited increases ROS and reduced overall catalase activity suggesting loss of functional peroxisomes. Both lipotoxicity resulted due to accumulation of FFAs and increased oxidative stress may contribute to the shortened CLS of these cells. Pex11, Pex25 and Pex27 have been earlier reported to be involved in peroxisome proliferation (Rottensteiner et al. 2003). However, the role of Pex27 in this process is not yet understood clearly (a) On the other hand, Pex11 and Pex25 are reported to have additional functions other than their role in peroxisome proliferation. Hence, a role for these functions in the regulation of CLS needs further investigation. For instance, a role for Pex11 in formation of membrane contact sites (MCS) between mitochondria and peroxisomes via the ER-mitochondria encounter structure (ERMES) complex has been reported (Ušaj et al. 2015) (b) Alterations in these MCS may also influence the CLS of these cells. Interestingly, a role for Pex25 in the formation of peroxisomes from ER has also been reported (Huber et al. 2012) (c) If *de novo* biogenesis is altered upon chronological ageing needs to be studied.

Though all three proteins belong to the same family, the extent of effect generated due to lack of each of these proteins is different. If this observed connection between lifespan and peroxisomes is majorly due to the altered number of peroxisomes in mutant cells needs further investigation. Interestingly, a recent study by Wróblewska and colleagues also suggests differences in localization of these proteins in cells lacking Pex3. Pex25 was observed to be

localized to small peroxisomal vesicles, Pex11 was localized to mitochondria and Pex27 was localized to the cytosol (Wróblewska et al. 2017). Yofe and colleagues also identified Pex35 another protein that most likely regulates peroxisome abundance. Pex35 was observed to be present in close proximity with Pex11 and Pex25 and not with Pex27 (Yofe et al. 2017). This suggests these proteins may have dynamic changes in their localization and function depending on the complex/ regulatory unit they are a part of and hence may have unidentified functions. Recent studies suggest a requirement of interactions between peroxisomes and other organelles of the cells for the exchange of metabolites and proteins resulting in optimum functioning of peroxisomes. Pex11 also has been reported to be an important factor in such interactions (Schrader et al. 2015; Ušaj et al. 2015; Wanders et al. 2016). Hence, it would be interesting to understand if the physical interactions between peroxisomes and other organelles are altered upon ageing. Along the same lines, recently, it was reported that an increase in the number of LDs results in more fitter mitochondria in aged cells (Kovacs et al. 2021). Recent studies also highlight the role of peroxisome degradation in ageing and thereby emphasizes the importance of peroxisome homeostasis in ageing (Dolese et al. 2021).

Chapter 6

Conclusion
&
Future perspectives

6.1 Conclusion

6.1.1 Yeast helping to reshape ideas on ageing

Ageing is an inevitable process and is controlled by various genetic and molecular factors which are interconnected. Although the effects of age are easily recognizable, but several questions regarding the regulation of this process is still a mystery. According to World Population Prospects 2019 (United Nations, 2019), scientists have predicted a faster pace of population ageing in future and by 2050, the number of persons with age 65 years or over will double to 1.5 billion worldwide. This will result in a challenging situation, as population ageing will be accompanied by an increase in health problems and disease. Thus, research should focus on promoting healthy ageing *i.e.* the development and maintenance of functional ability for the wellbeing of people in older age. The phenomenon of ageing is ubiquitous in all organisms and the hallmarks of ageing were proposed nearly a decade ago (López-Otín et al. 2013). However, the molecular causes associated with these hallmarks are not completely known. Advancement in ageing research has come from studies involving model systems such as *D. melanogaster*, *C. elegans*, mammalian cells, mouse models, yeast, *etc*. Various cellular pathways involved in ageing are conserved across all the model systems (Pitt and Kaeberlein 2015). Yeast, in general, and *S. cerevisiae* in particular, is an interesting model organism for ageing studies as yeast ageing phenomenon is very similar and comparable to the ageing in higher eukaryotes (Denoth Lippuner et al. 2014). In yeast, there are two models of ageing, namely chronological and replicative ageing (Fabrizio and Longo 2003; Müller et al. 1980). Several studies have highlighted the role of subcellular organelles in the regulation of ageing process in yeast (Hughes and Gottschling 2012; Kaeberlein 2010; Kumar et al. 2018; Lefevre et al. 2013; Payne and Chinnery 2015; Perić et al. 2016; Scheckhuber et al. 2011). Peroxisomes are one such single membrane-bound organelle which maintain ROS homeostasis in the cells. These organelles have the ability to adapt their number and size depending on the type of cells and

media conditions (Smith and Aitchison 2013). The peroxisomal proteins or peroxins which regulate peroxisome number and size in a cell belong to the Pex11 family proteins and comprise of Pex11, Pex25 and Pex27 in *S. cerevisiae* (Akşit and van der Klei 2018; Koch and Brocard 2011). Thus, in order to establish a link between peroxisome abundance and ageing in yeast, our study aimed at understanding the following:

- Peroxisome dynamics in WT cells cultured in different media conditions upon replicative and chronological ageing.
- Changes associated with peroxisomes in replicatively aged mother cells.
- Effect of altered peroxisome number on the CLS of yeast cells.

6.1.2 Old yeast: new peroxisome clues

To establish an association between peroxisome dynamics and longevity, we first analysed possible changes in peroxisome number and morphology during replicative and chronological ageing in WT yeast cells cultured in different media. We report for the first time an increase in peroxisome number upon replicative ageing of WT cells cultured in both YND and OA media (Fig. 3.3). However, owing to peroxisome induction, WT OMC grown in OA depicted increased average peroxisome number as compared to YND medium (Fig. 3.3b). The CLS of the WT cells was assessed in CR and OA media (Fig. 3.6). Chronologically aged cells culture in OA medium displayed an increase in peroxisome number upon chronological ageing (Fig. 3.7). A significant increase in the mean and maximal CLS was observed in OA medium as compared to CR (Fig. 3.6). On the similar lines, the change in the peroxisome phenotype from punctate to cytosolic was also delayed in OA-grown cells (Fig. 3.8). Taken together, our results suggest altered peroxisome number and morphology upon ageing in yeast.

In order to understand if the increase in peroxisome number in replicatively aged cells is due to peroxisome fission, *pex11*, *pex25* and *pex11pex25* cells were analysed. Quantitative analysis revealed a significant decrease in peroxisome number in *pex11* and *pex11pex25* OMC

when compared to WT in both YND and OA media. This indicates a role for Pex11 in the regulation of peroxisome number in replicatively aged cells (Fig. 4.4). However, despite reduced peroxisome number, *pex11* and *pex11pex25* OMC showed an interesting increase in catalase activity in OA medium (Fig. 4.8). This might be due to enhanced activity of cytosolic catalase in response to the excess ROS generated due to the β-oxidation pathway in OA medium. Further our data suggests that this increase in peroxisome number may not be due to RTG signalling. However, this needs further investigation. Altogether our data clearly indicates that altered peroxisome dynamics in the replicatively aged cells is dependent on Pex11.

The effect of altered peroxisome number upon chronological ageing of yeast cells was also studied. The CLS of cells lacking Pex11 family proteins was analysed in both CR and OA media. Interestingly, reduction in peroxisome number in the fission mutants had no effect on CLS in CR conditions (Fig. 5.1). However, in OA medium, reduced CLS was observed in *pex11*, *pex25* and *pex11pex25* cells as compared to WT (Fig. 5.3). This is in line with the observation that these cells also displayed a significant decrease in peroxisome number as compared to WT in OA medium (Fig. 5.4, Table 5.2). *pex27* cells which had similar peroxisome number and phenotype as that of WT cells also depicted CLS similar to WT in OA conditions (Fig. 5.3 and 5.7; Table 5.2). *pex11pex25* cells which displayed the least mean and maximum CLS in OA medium, also exhibited increased ROS accumulation and reduced catalase activity (Fig. 5.3 and 5.9). Highest accumulation of FFA was also observed in these cells (Fig. 5.8). In summary, our findings demonstrate a significant role for peroxisome proliferation in the regulation of CLS of yeast cells cultured in OA medium.

6.2 Future perspectives

- In this study, we analysed mutant cells with reduced number of peroxisomes for their effect on both replicative and chronological ageing. Overexpression of these proteins

result in increased number of peroxisomes and hence would be interesting to observe the effect on ageing.

- Peroxisomes work in co-ordination with other organelles in order to maintain cellular homeostasis. Recent studies have identified that peroxisome fission protein Pex11 plays a major role in mitochondria-peroxisome communication. Therefore, it would be interesting to understand whether the physical interaction of peroxisomes with other organelles may also be affected and influences cellular ageing.
- Another important pathway to achieve peroxisome homeostasis in a cell is pexophagy or the autophagic degradation of peroxisomes. Future studies can also look at how pexophagy and ageing process are interlinked.
- Yeast has emerged as an ideal model to understand the process of ageing in humans. This model organism also has the potential to help in the discovery of compounds and drugs that regulate the ageing process through high-throughput screens.
- A role for PEX11β (human homologue of yeast PEX11) in disease conditions has been reported. Molecules that upregulate PEX11β and restore peroxisome dynamics has been proposed to be beneficial in certain disease conditions. As the peroxisome fission pathway is conserved between yeast and humans, novel molecules/compounds can be identified using yeast screens.
- Genome-wide RLS and CLS screens in yeast have led to the identification of several genes associated with the regulation of yeast ageing (Fabrizio et al. 2010; McCormick et al. 2015). However, the molecular mechanisms by which many of these genes regulate the process of ageing are still unknown. Moreover, many candidates identified in these screens were found to be false-positives upon experimental confirmation (Burtner et al. 2011; Fabrizio et al. 2010; Garay et al. 2014). Hence, individual candidates need to be investigated for their role in CLS/RLS.

- Future studies can also aim to understand the inter-dependence between RLS and CLS. Interestingly, opposite effects in both ageing types have been reported in cells lacking protein such as Sirtuin2 (Fabrizio et al. 2005; Kaeberlein et al. 1999).

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List of Publications, Conferences & Workshops attended, Awards and Achievements

Publications from Ph. D thesis

- **Deb R**, Nagotu S (2022) The nexus between peroxisome abundance and chronological ageing in *Saccharomyces cerevisiae*. Biogerontology. doi:10.1007/s10522-022-09992-9
- **Deb R**, Ghose S, Nagotu S (2022) Increased peroxisome proliferation is associated with early yeast replicative ageing. Current Genetics. 68(2):207-225. doi:10.1007/s00294-022-01233-3
- **Deb R**, Joshi N, Nagotu S (2021) Peroxisomes of the Brain: Distribution, Functions, and Associated Diseases. Neurotoxicity Research. 39(3):986-1006. doi:10.1007/s12640-020-00323-9
- **Deb R**, Nagotu S (2017) Versatility of peroxisomes: An evolving concept. Tissue and Cell. 49 (2 Pt B):209-226. doi:10.1016/j.tice.2017.03.002

Publications from collaborative research

- Infant T*, **Deb R***, Ghose S, Nagotu S (2021) Post-translational modifications of proteins associated with yeast peroxisome membrane: An essential mode of regulatory mechanism. Genes and Cells. 26(11):843-860. doi:10.1111/gtc.12892. * - equal contribution
- Glingston S, Rajpoot J, Deori NM, **Deb R**, Kumar S, Nagotu S (2021) Characterization of nucleocapsid and matrix proteins of Newcastle disease virus in yeast. 3 Biotech. 11(2):65. doi:10.1007/s13205-020-02624-4
- Banerjee R*, **Deb R***, Roy K*, Chatterjee S, Nagotu S (2019) Uptake and intracellular fate of nona-arginine peptide in yeast. Peptide Science. 111(4), e24101. * - equal contribution
- Glingston RS, **Deb R**, Kumar S, Nagotu S (2019) Organelle dynamics and viral infections: at cross roads. Microbes and Infection. 21(1):20-32. doi:10.1016/j.micinf.2018.06.002
- Deori NM*, **Deb R***, Banerjee R*, Nagotu S. Yeast: A Multifaceted Eukaryotic Microbe and its Biotechnological Applications. *Advances in Microbial Biotechnology*. 2018. doi:10.1201/9781351248914-7. * - equal contribution

Conferences and workshop attended

Conferences:

- North-East Research Conclave-2022, Indian Institute of Technology Guwahati, Guwahati, India (2022) (Oral presentation)
- Research and Industrial Conclave-2022, Indian Institute of Technology Guwahati,

Guwahati, India (2022) (Poster presentation)

- National Conference MiCON-2022, Yenepoya Research Centre, Mangalore, India (2022) (Oral presentation)
- 3rd National Biomedical Research Competition-2021, Society of Young biomedical Scientists, India (2021) (E-poster presentation)
- International Conference on Yeast Biology and Filamentous Fungi, University of Hyderabad, Hyderabad, India (2019) (Poster presentation)
- Research Conclave-2019 organized by Students' Academic Board, Indian Institute of Technology Guwahati, Guwahati, India (2019) (Poster presentation)
- 10th Conference on Yeast Biology-2018, Jawaharlal Nehru University, New Delhi(Poster Presentation)
- Research Conclave-2017 organized by Students' Academic Board, Indian Institute of Technology Guwahati, 2017 (Poster presentation)

Workshop attended:

- 20th Indo-US flow cytometry symposium cum workshop on "Applications of flow cytometry in Biotechnology"

Awards and achievements

- Received best poster presentation award at **Research and Industrial Conclave,** IIT-Guwahati, Guwahati, India (2022)
- Received best poster presentation award at **Research and Industrial Conclave**, IIT-Guwahati, Guwahati, India (2019).