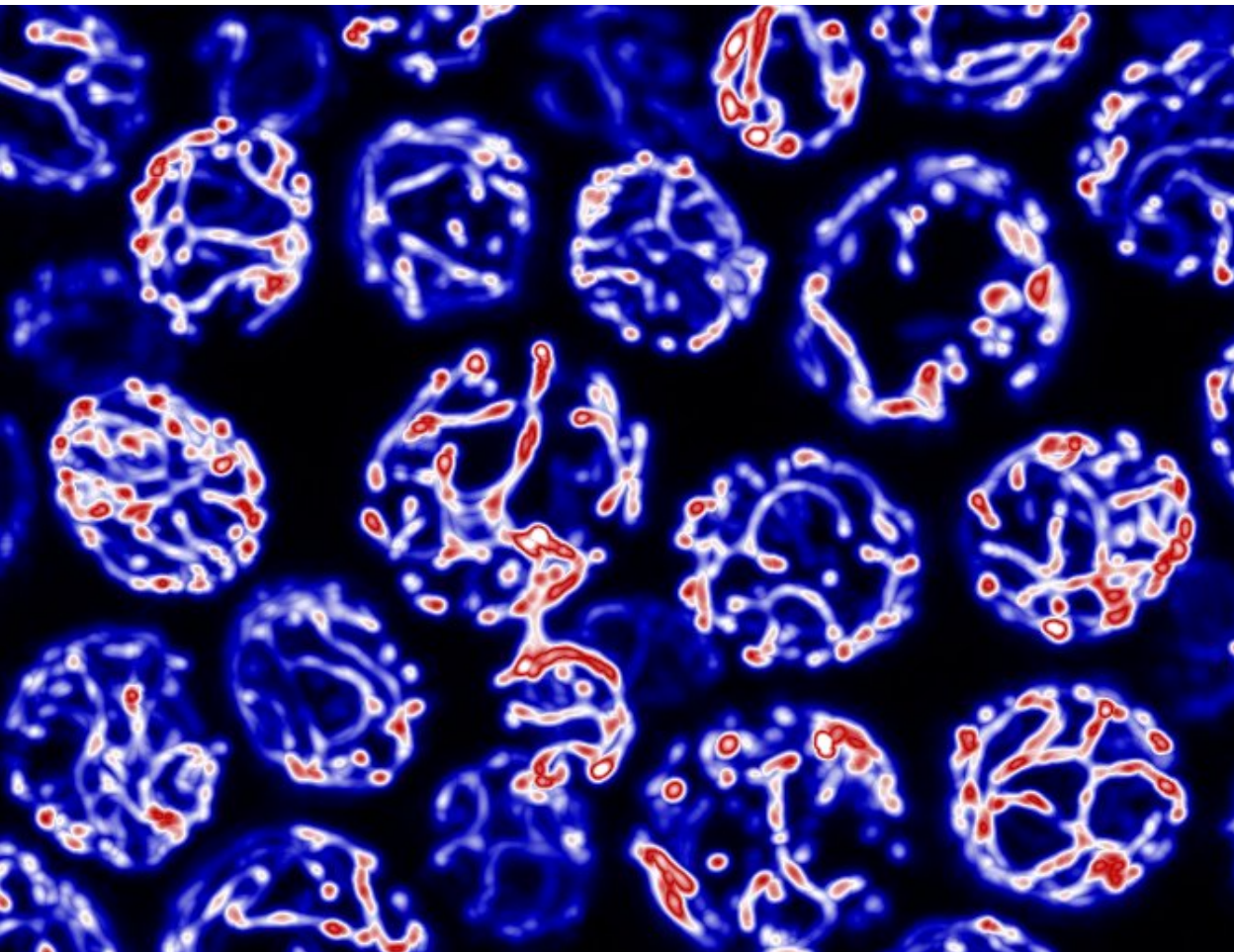


Intracellular connectivity during stress and aging

Simon Prokisch-Chalas



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Academic dissertation for the Degree of Doctor of Philosophy in Molecular Bioscience at Stockholm University to be publicly defended on Friday 13 March 2026 at 13.00 in Vivi Täckholmsalen (Q-salen), NPQ-huset, Svante Arrhenius väg 20.

Abstract

Aging is a natural, irreversible process characterized by a gradual decline in physiological functions and an increased susceptibility to diseases due to the accumulation of molecular and cellular damage over time. It is a complex, multifactorial process involving a wide range of biological changes, such as genomic instability, epigenetic alterations, and a decay in proteostasis. Understanding cellular changes caused by aging is essential for improving human health and quality of life in old age and preventing age-related diseases. To study aging processes and their effects on cellular structures and general fitness the eukaryote budding yeast (*Saccharomyces cerevisiae*) was used in this study. Despite its simplicity, yeast is an optimal model organism for aging research as many cellular processes are conserved in more complex eukaryotes, including humans. In Paper I, the ability of chronologically aged cells to re-enter the cell cycle was analyzed. Depending on whether glucose or phosphate was the limiting nutrient driving entry into stationary phase, the lipid profile of aged cells differed drastically. The alteration in lipid composition contributed to maintenance and exit of quiescent cells differentially. In Paper II, the effects of aging on the organization of the endoplasmic reticulum (ER) and its microdomains were studied. Here, we showed that partitioning into ER microdomains impacts autophagic protein turnover during cellular aging and that cellular aging causes a gradual increase of ER microdomains, in particular in the cortical ER. We also established that the transmembrane length of ER membrane proteins determines their final localization as well as their stability. Finally, in Paper III, we established a direct link between the proteolytic capacity of the vacuole and mitochondrial function. Whereas increasing this capacity was beneficial for mitochondrial functionality and abundance as well as cellular survival, decreasing it led to opposite effects. The beneficial effects were dependent on the augmented formation of membrane contact sites between the vacuole and mitochondria.

Keywords: *aging, lipid homeostasis, biological membranes, ER microdomains, proteolytic capacity, mitochondria, organellar connectivity.*

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Cover image: Mitochondria of cells with increased proteolytic capacity

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**To my beloved mom
Mutti Claudi**

and my siblings
Sarah, Samira,
Samuel, and Silas

L'homme n'est qu'un roseau, le plus faible de la nature, mais c'est un roseau pensant. Il ne faut pas que l'univers entier s'arme pour l'écraser. Une vapeur, une goutte d'eau, suffit pour le tuer. Mais quand l'univers l'écraserait, l'homme serait encore plus noble que ce qui le tue, parce qu'il sait qu'il meurt ; et l'avantage que l'univers a sur lui, l'univers n'en sait rien. Toute notre dignité consiste donc en la pensée. C'est de là qu'il faut nous relever, non de l'espace et de la durée, que nous ne saurions remplir. Travaillons donc à bien penser : voilà le principe de la morale. (*Blaise Pascal, Les Pensées 1670*)

List of papers

- I Peselj C, Ebrahimi M, Broeskamp F, **Prokisch S**, Habernig L, Alvarez-Guerra I, Kohler V, Vögtle FN, and Büttner S. Sterol Metabolism Differentially Contributes to Maintenance and Exit of Quiescence. *Front. Cell Dev. Biol.* **10**:788472 (2022).
- II **Prokisch S** and Büttner S. Partitioning into ER membrane microdomains impacts autophagic protein turnover during cellular aging. *Sci. Rep.* **13;14**(1):13653 (2024).
- III **Prokisch S**, Kohler V, Diessl J, Kohler A, Jastroch M, Pierrel F, and Büttner S. Vacuolar proteolytic capacity sustains mitochondrial bioenergetics via organelle connectivity. (manuscript).

Aims of thesis

Yeast cells, like all other eukaryotic cells, are sub-compartmentalized into different organelles, such as the nucleus, the lysosome-like vacuole, the mitochondria, or the endoplasmic reticulum (ER) that facilitate distinct biochemical reactions in optimal surroundings. Creating these organelles however renders an intracellular communication between them essential to guarantee that reactions are only catalyzed when environmental and cellular stimuli prompt them. This communication can either be indirect via intracellular messengers or direct via the formation of a membrane contact site (MCS) between two or more organelles. Aging, as a special and complex form of stress, leads to a continuous remodeling of organelles and alters their communication since external and internal factors change. At one point and often due to nutrient scarcity yeast cells enter a quiescent state which is a reversible, non-dividing, dormant phase that cells can exit if environmental factors change accordingly, for instance by refeeding them. Answering the question how aging influences intracellular connectivity was the main aim of this thesis. In this context not only the effects of aging on cellular structures but also the ability of differentially aged cells to exit quiescence and re-enter cell cycle were of interests.

- In Paper I, we studied the effects of phosphate and glucose starvation on the overall lipid homeostasis in yeast and how, ultimately, these alterations affected the re-entry of aged, quiescent cells into cell cycle.
- In Paper II, we addressed the question how aging changes not only the general organization of the ER membrane but also ER membrane microdomains in particular as well as the MCS between the perinuclear ER (pER) and the vacuole. In this context, we also analyzed which impact the length of the transmembrane domain of membrane proteins have in respect to their localization, stability, and turnover via autophagic processes. To address these research questions, genetically encoded, continuously expressed, artificial reporters were used.
- In Paper III, the interplay between mitochondrial as well as vacuolar functionality and the alteration of MCS between those two organelles during aging was studied. Here, we could show that augmenting the abundance of those MCS via increasing the proteolytic capacity of the vacuole led to more efficient mitochondria with higher transmembrane potential, reduced ROS production, and an increased ATP synthesis, which further prolonged cellular lifespan.

Popular summary

In times of an increasingly aging global population, aging research is of paramount importance. Unraveling the impact of aging processes have on cellular structures and biochemical pathways will pave the way to improving human health in old age and preventing age-related diseases, such as cardiovascular diseases, osteoarthritis, dementia, cancer, osteoporosis as well as diabetes. Research on humans in general, but especially aging research on humans due to their comparatively high life expectancy, is cumbersome and often ethically unfeasible or at least challenging. To circumvent this issue, the eukaryotic baker's yeast is often used as a model organism for all eukaryotes, which also includes humans. Baker's yeast is probably best known to most people as a "producer" of bread, beer and wine, but it also serves as a suitable model organism in research, since most biochemical processes are also conserved in more complex living beings, including human. Its very short lifespan is of immense advantage when it comes to aging research. This study also used yeast to investigate the effects of aging within cells.

Eukaryotic cells possess various compartments that are essential for enabling biochemical reactions under optimal conditions. Examples of such compartments include the nucleus, mitochondria, and the endoplasmic reticulum. In yeast, there is also the vacuole, which is often compared to the lysosomes found in human cells, as both organelles perform similar functions. Both the integrity of the individual organelles and their flawlessly functioning communication with each other are essential for survival and cellular thriving. The overall efficiency of all cellular processes generally decreases with age. Improving these processes cannot completely prevent aging, but it can probably delay it. Obviously, understanding these processes is a prerequisite for any potential improvement of them. This study investigated, on the one hand, the impact of aging on the structural organization of the endoplasmic reticulum and, on the other hand, the effects of different diets on aging as well as the communication between vacuole and mitochondria and their contribution to aging processes. The improvement of the latter communication did indeed lead to an extended lifespan.

In summary, this study contributes to a better understanding of aging processes, which is a prerequisite for prevention of age-related cell degeneration and hence the enabling of a more dignified life in old age.

Populärvetenskaplig sammanfattning

Med en alltmer åldrande befolkning globalt är åldrandeforskning av största vikt. Att reda ut den inverkan som åldrandeprocesser har på cellstrukturer och biokemiska vägar bidrar till att förbättra äldres hälsa och förebygga åldersrelaterade sjukdomar, såsom hjärt-kärlsjukdomar, artros, demens, cancer, benskörhet och diabetes. Forskning på människor i allmänhet, men särskilt forskning om åldrande hos människor på grund av deras jämförelsevis långa förväntade livslängd, kan vara besvärlig och etiskt problematisk. För att kringgå detta används ofta den eukaryota bagerijästen som modellorganism för alla eukaryoter, inklusive människor. Bagerijäst är förmodligen mest känd för de flesta som en "producent" av bröd, öl och vin, men den fungerar också som en lämplig modellorganism inom forskning, eftersom de flesta biokemiska processer också är bevarade i mer komplexa levande varelser, däribland människor. Dess mycket korta livslängd är en väldig fördel när det gäller åldrandeforskning. Denna studie använder därför jäst för att undersöka effekterna av åldrande i celler.

Eukaryota celler har olika delar som är viktiga för att möjliggöra biokemiska reaktioner under optimala förhållanden. Exempel på sådana organeller är cellkärnan, mitokondrierna och endoplasmiskt retikulum. Hos jäst finns även vakuolen, som ofta jämförs med lysosomerna i mänskliga celler, eftersom båda organellerna utför liknande funktioner. Både de enskilda organellernas integritet och deras felfritt fungerande kommunikation med varandra är avgörande för överlevnad och utveckling. Den totala effektiviteten hos alla cellulära processer minskar generellt med åldern. Att förbättra dessa processer kan inte helt förhindra åldrande, men det kan säkerligen fördröja det. Att förstå dessa processer är uppenbarligen en förutsättning för eventuella förbättringar av dem. Denna studie undersöker å ena sidan åldrandets inverkan på endoplasmiskt retikulums strukturella organisation och å andra sidan effekterna av olika dieter på åldrande samt kommunikationen mellan vakuol och mitokondrier och deras bidrag till åldrandeprocesser. Förbättringen av den senare kommunikationen påvisade i studien en förlängd livslängd.

Sammanfattningsvis bidrar denna studie till en bättre förståelse av åldrandeprocesser, vilket är en förutsättning för att förebygga åldersrelaterad celldegeneration och därmed möjliggöra ett mer värdigt liv i ålderdomen.

Kurzzusammenfassung

Angesichts einer zunehmend alternden Weltbevölkerung ist die Altersforschung von größter Bedeutung. Die Aufklärung der Auswirkungen von Alterungsprozessen auf Zellstrukturen und biochemische Stoffwechselwege trägt dazu bei, die menschliche Gesundheit im Alter zu verbessern und altersbedingten Erkrankungen wie Herz-Kreislauf-Erkrankungen, Arthrose, Demenz, Krebs, Osteoporose und Diabetes vorzubeugen. Forschung am Menschen im Allgemeinen, insbesondere aber die Altersforschung am Menschen aufgrund seiner vergleichsweise hohen Lebenserwartung, ist aufwendig und oft ethisch nicht vertretbar oder zumindest herausfordernd. Um dieses Problem zu umgehen, wird häufig die eukaryotische Bäckerhefe als Modellorganismus für alle Eukaryoten, einschließlich des Menschen, verwendet. Bäckerhefe ist den meisten Menschen wahrscheinlich am besten als „Produzent“ von Brot, Bier und Wein bekannt, dient aber auch als geeigneter Modellorganismus in der Forschung, da die meisten biochemischen Prozesse auch in komplexeren Lebewesen, wie dem Menschen, konserviert sind. Ihre sehr kurze Lebensdauer ist ein immenser Vorteil in der Altersforschung. Auch in dieser Studie wurde Hefe eingesetzt, um die Auswirkungen des Alterns in Zellen zu untersuchen. Eukaryotische Zellen besitzen verschiedene Kompartimente, die für biochemische Reaktionen, die dort unter optimalen Bedingungen ablaufen, unerlässlich sind. Beispiele hierfür sind der Zellkern, die Mitochondrien und das endoplasmatische Retikulum. In Hefezellen findet sich zudem die Vakuole, die oft mit den Lysosomen menschlicher Zellen verglichen wird, da beide Organellen ähnliche Funktionen erfüllen. Sowohl die Integrität der einzelnen Organellen als auch ihre reibungslose Kommunikation untereinander sind für das Überleben und die Entwicklung der Zellen essenziell. Die Gesamteffizienz aller zellulärer Prozesse nimmt jedoch im Verlaufe der Zeit ab. Eine Verbesserung dieser Prozesse kann das Altern zwar nicht vollständig verhindern, aber sicherlich verzögern. Das Verständnis dieser Prozesse ist daher Voraussetzung für jede potenzielle Verbesserung derer. Diese Studie untersuchte einerseits den Einfluss des Alterns auf die Struktur des endoplasmatischen Retikulums und andererseits die Auswirkungen verschiedener Ernährungsweisen auf das Altern sowie die Kommunikation zwischen Vakuole und Mitochondrien und deren Beitrag zu Alterungsprozessen. Die Verbesserung der letztgenannten Kommunikation führte tatsächlich zu einer verlängerten Lebensspanne. Zusammenfassend trägt diese Studie zu einem besseren Verständnis der Alterungsprozesse bei, was eine Voraussetzung für die Prävention altersbedingter Zelldegeneration und damit auch für das Ermöglichen eines würdevolleren Lebens im Alter ist.

Resumen popular

Ante el creciente envejecimiento de la población mundial, la investigación sobre el envejecimiento reviste una importancia fundamental. Comprender el impacto de los procesos de envejecimiento en las estructuras celulares y las vías bioquímicas contribuye a mejorar la salud humana en la vejez y a prevenir enfermedades relacionadas con la edad, como las enfermedades cardiovasculares, la osteoartritis, la demencia, el cáncer, la osteoporosis y la diabetes. La investigación en humanos en general, y especialmente la investigación sobre el envejecimiento humano debido a nuestra relativamente larga esperanza de vida, es compleja y a menudo éticamente inviable, o al menos presenta un desafío significativo. Para superar este problema, puede utilizarse la levadura de panadería eucariota como organismo modelo para todos los eucariotas, incluidos los humanos. La levadura de panadería es probablemente más conocida por su uso en la producción de pan, cerveza y vino, pero también constituye un organismo modelo ideal para la investigación, ya que la mayoría de sus procesos bioquímicos se conservan en seres vivos más complejos, como los humanos. Su corta vida útil representa una ventaja significativa para la investigación sobre el envejecimiento. Este estudio también utilizó levadura para investigar los efectos del envejecimiento en las células.

Las células eucariotas poseen diversos compartimentos esenciales para que las reacciones bioquímicas se lleven a cabo en condiciones óptimas. Ejemplos de estos compartimentos son el núcleo, las mitocondrias y el retículo endoplásmico. En la levadura, también está presente la vacuola, que a menudo se compara con los lisosomas de las células humanas, ya que ambos orgánulos desempeñan funciones similares. Tanto la integridad de los orgánulos individuales como su comunicación fluida entre sí son esenciales para la supervivencia y el desarrollo celular. La eficiencia general de todos los procesos celulares generalmente disminuye con la edad. Si bien mejorar estos procesos no puede prevenir completamente el envejecimiento, sí puede retrasarlo. Evidentemente, comprender estos procesos es un requisito previo para cualquier posible mejora. Este estudio investigó, por un lado, el impacto del envejecimiento en la organización estructural del retículo endoplásmico y, por otro, los efectos de diferentes dietas en el envejecimiento, así como la comunicación entre la vacuola y las mitocondrias y su contribución a los procesos de envejecimiento. La mejora de esta última comunicación sí condujo a una mayor longevidad.

En resumen, este estudio contribuye a una mejor comprensión de los procesos de envejecimiento, lo cual es un requisito previo para la prevención de la degeneración celular relacionada con la edad y, por lo tanto, para posibilitar una vida más digna en la vejez.

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1. Biological aging – wear and tear

Biological aging is a normal process of life and can be defined as the time-dependent decline in function of any living system. Aging is not limited to multicellular but also occurs in unicellular organisms and happens at the level of both the cell and the organism as a whole. Interestingly, aging not only occurs in eukaryotic but also in prokaryotic cells (Stewart *et al.*, 2005), originally believed not to age. Even though the prokaryote *Escherichia coli* that usually is found in the intestine of warm-blooded organisms (reviewed in Tenaillon *et al.*, 2020), divides symmetrically, both cells display different replicative capacities (Stewart *et al.*, 2005). The cell obtaining the newly synthesized pole during cell division shows a higher replicative capacity than the one keeping the old one (Stewart *et al.*, 2005).

However, in this study, the Baker's yeast was used as a model organism for all eukaryotic organisms. During aging different biochemical processes and cellular structures can be impacted. To provide an overview of all of them, so-called hallmarks of aging in eukaryotic cells were described that will be discussed in the following.

1.1 Hallmarks of aging in eukaryotic cells

So far 12 hallmarks of aging that need to fulfill three established criteria, have been described and grouped into three categories: primary, antagonistic, and integrative (Fig. 1). Primary sources of aging are genomic instability, telomeric attrition, epigenetic alteration, loss of proteostasis as well as disabled macroautophagy. Instead, cellular senescence, mitochondrial dysfunction, and deregulated nutrient-sensing belong to antagonistic hallmarks, whereas integrative ones are composed of stem cell exhaustion, altered intercellular communication, chronic inflammation as well as dysbiosis (López-Otín *et al.*, 2023). The three criteria that every hallmark of aging needs to meet are the time-dependent manifestation of alteration during aging, the possibility to accelerate aging by accentuating it, and the opportunity to decelerate, halt, or even reverse aging by influencing it positively.

Every day and throughout the entire life of an organism both nuclear (nDNA) and mitochondrial DNA (mtDNA) suffer from intrinsic and extrinsic stimuli that can lead to genomic alterations. Evolution generated many repair mechanisms to detect and reverse DNA damage that mostly function immaculately in young, healthy cells but lose their efficiency during aging. Excessive DNA damage and thus overburdening of DNA repair mechanisms can cause premature aging. Telomeres at the end of eukaryotic chromosomes are repeated

nucleotide sequences that protect them (Allshire *et al.*, 1988; Moyzis *et al.*, 1988; Brown, 1989; Cross *et al.*, 1990). Every DNA replication causes a shortage of telomeres that leads to structural changes and genomic instability (Stansel *et al.*, 2001). Once cells reach the so-called Hayflick Limit (Hayflick, 1999) and telomeres become too short, they can no longer replicate and induce senescence (Mathon and Lloyd, 2001; Fitzgerald *et al.*, 1999; Harley *et al.*, 1997) or undergo apoptosis (López-Otín *et al.*, 2023). In general, any form of telomeric attrition is linked to aging processes (Iskandar *et al.*, 2025). To counteract this telomerase can elongate telomeres, which rejuvenates cells (Chakravarti *et al.*, 2021; Blasco, 2005); however most somatic cells do not express them.

Besides direct changes on DNA level that cause aging, epigenetic alterations such as modifications of methylations (Seale *et al.*, 2022) and acetylations and thus histones (Lu *et al.*, 2021; Oh and Petronis, 2021; Blasco, 2007) also impact cellular aging. Moreover, changes of levels of non-coding RNAs are also linked to aging (Jusic *et al.*, 2022; Weigelt *et al.*, 2020; Kumar *et al.*, 2021)

Not only DNA alterations and modifications influence aging processes but also proteins and their stability. A reduction of protein quality control mechanisms shortens cellular lifespan, and a complete collapse of the proteostasis network causes cell death (Hetz *et al.*, 2020; Hipp *et al.*, 2019). In general, aging is a risk factor for neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's, and amyotrophic lateral sclerosis (Hipp *et al.*, 2019; Vilchez *et al.*, 2019), which are caused by an accumulation of protein aggregates (Hommen *et al.*, 2021). In accordance with this, knockdown or knockout of proteases that are involved in the removal of misfolded or aggregated proteins in *Drosophila* or mice reduce survival (Tsakiri *et al.*, 2013; Gordon *et al.*, 2014). When autophagic processes that not only remove protein aggregates but also other macromolecules and even entire organelles (Levine and Kroemer, 2019) are inhibited cells usually die faster (Cassidy *et al.*, 2020; Klionsky *et al.* 2021). Stimulation of autophagic processes however extends cellular survival (Lu *et al.*, 2021; Pyo *et al.*, 2013).

The second category of hallmarks of aging are factors that are considered antagonistic (López-Otín *et al.*, 2023). One example for an antagonistic source of aging is a deregulation of nutrient-sensing. Whereas reduction of the nutrient-sensing network leads to an extended lifespan, its activation reduces cellular survival (Slack *et al.*, 2015; Singh *et al.* 2019). Also, mitochondrial functionality belongs into this category. Most likely through their increased production of reactive oxygen species (ROS), dysfunctional mitochondria cause premature cell aging

(Amorim *et al.*, 2022). In line with this, studies in *C. elegans* and *Drosophila* also revealed the involvement of mitochondrial functionality in cellular aging (Lionaki *et al.*, 2022; Owusu-Ansah *et al.*, 2013). The last hallmark of aging in this category is cellular senescence. Senescent cells have permanently lost their ability to divide but do not die and often disrupt normal tissue function (Campisi, 2013; Munoz-Espin and Serrano, 2014; van Deursen, 2014; Sharpless and Sherr, 2015). Studies in mice (Xu *et al.*, 2018) as well as clinical trials (Robbins *et al.*, 2021) revealed that removal of senescent cells, also termed senolysis, prolonged lifespan. Senolysis in mouse models of human diseases, for instance, alleviated the symptoms of atherosclerosis, cataracts, cardiac hypertrophy, renal dysfunction, sarcopenia, lipodystrophy, and osteoarthritis (Baker *et al.*, 2008, 2011, 2016; Jeon *et al.*, 2017; Childs *et al.*, 2016).

The third category of hallmarks of aging are integrative factors (López-Otín *et al.*, 2023). Stem cell exhaustion is one hallmark of aging in this category. Aging is tightly linked to tissue renewal and injured-induced plasticity as well as de-differentiation, processes that decline with age (Tata and Rajagopal, 2017; Lin *et al.*, 2017; Murata *et al.*, 2020). In studies with mice, transient reprogramming partially reversed reduced visual acuity (Lu *et al.*, 2020) as well as improved long-term memory (Rodríguez-Matellán *et al.*, 2020) caused by natural aging. Intercellular communication mainly through factors present in the blood impacts cellular aging as well. Whereas transfusions of old blood to young mice result in their premature aging (Rebo *et al.*, 2016), transfusions of young blood to old mice rejuvenate them (Rando and Jones, 2021). In this context, the extracellular matrix (ECM) as another way of intercellular communication is worth mentioning. Since the ECM is composed of long-lived proteins it is more prone to age-dependent changes (Fedintsev and Moskalev, 2020). Once these proteins change their conformation or aggregate tissue fibrosis can be triggered (Selman and Pardo, 2021). Lastly, both chronic inflammation and dysbiosis, two complex forms of biological states, are hallmarks of aging. Whereas chronic inflammation is rather a combination of all hallmarks (López-Otín *et al.*, 2023), dysbiosis is caused by age-dependent alterations of the gut microbiota that generally favor a decrease of ecological diversity (Biagi *et al.*, 2016).

Unquestionably, aging is a complex biological process that is influenced by many different intrinsic and extrinsic factors. In this study, the budding yeast *Saccharomyces cerevisiae* was used to unravel how intracellular connectivity is impacted by aging and stress. Yeast can age in three different ways, which enables studies on aging from different angles. In the following, these three ways will be described.

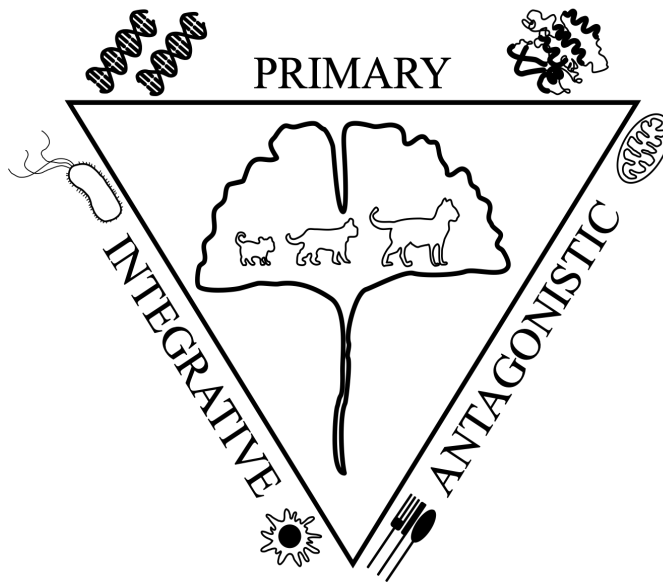


Fig. 1: Simplified representation of the three categories of hallmarks of aging

1.2 Aging in *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* has long been serving as a model organism for all eukaryotic organisms to study diverse cellular processes since it harbors many technical and methodical advantages compared to other organisms. Evolutionary conservation of many biochemical processes combined with these conveniences made yeast a powerful organism to examine different cellular pathways. Even though yeast as a unicellular organism and due to its simplicity might at first seem to be rather disadvantageous than advantageous to address different biologically relevant questions, a large number of fundamental insights into diverse eukaryotic phenomena were first shown in yeast.

Also, in aging research yeast has long been used. Both its short lifespan and the fact that yeast can age in three different ways – replicative, chronological, and in colonies – also made yeast a powerful model organism in this respect.

1.2.1 Aging in colonies

In nature, budding yeast usually grows in colonies on top of suitable substances, such as normal soil, decayed leaves and tree bark (Banno and Mikata, 1981) as well as ripe grapes in vineyards (Torok *et al.*, 1996; Mortimer and Polsinelli, 1999; Redzepovic *et al.*, 2002). In these

environments, cells in the middle of the colony enter into stationary conditions, whereas those at the border of the colony keep dividing (Meunier and Choder, 1999). Both types of cells show distinct expression patterns. Cells in the periphery are characterized by repressed mitochondrial respiration and stress response as well as increased rates of fatty acid oxidation, amino acid metabolism and peroxisome biogenesis, which prolongs cellular survival (Palková *et al.*, 2002). Cells in the middle of the colony however show little activity and eventually even exhibit programmed cell death, similarly to apoptosis in higher eukaryotes (Váchová and Palková, 2005). Those cells that undergo programmed cell death liberate both nutrients and ammonia. The secreted nutrients can be exploited by the remaining cells and the ammonia alters transcription in such a way that programmed cell death is limited to cells in the middle of the colony (Váchová and Palková, 2005). These events are crucial for the entire colony since removal of cells from the middle of the colony impacts the peripheral cells negatively and compromises survival of the entire colony (Váchová and Palková, 2005).

Studies in yeast cells that age in colonies, a form that in some aspects display multicellular characteristics, can lead to a better understanding of aging processes in defined regions, such as epithelial, connective, muscle or nervous tissues, in higher eukaryotes.

1.2.2 Replicative aging

Replicative aging reflects the number of divisions one mother cell can undergo before cellular death. During every asymmetric division damaged material is retained in the mother cell whereas the daughter cells only obtain newly synthesized, undamaged material. It is therefore not surprising that, due to the accumulation of more and more damaged material after each division, the mother cell eventually loses the ability to divide and becomes senescent (Carmona-Gutierrez and Büttner, 2014). Hence, replicative aging in yeast serves as a model to understand the aging of mitotically active cells in higher eukaryotes.

Research on replicative aging in yeast led to a better understanding how organisms maintain their germline (Unal *et al.*, 2011) which is of great importance in the face of aging. In general, studies in this context consequently give insights on aging processes of for instance skin cells, epithelial cells of the intestinal mucosa as well as stem cells.

1.2.3 Chronological aging

Another form of yeast aging is chronological aging that describes the time a yeast culture survives in stationary phase. Usually, yeast that is inoculated freshly in new media displays a lag phase when they need to adapt to the new environment. Depending on the genetic background of the yeast as well as the provided media this phase varies in its duration. After adaption yeast starts growing exponentially by fermenting organic products. Once these products become depleted and/or toxic byproducts exceed a certain threshold cellular growth slows down and yeast cells experience a diauxic shift with changes in gene expression to re-adapt to the new environment. After this shift the yeast culture enters a post-diauxic growth phase where it can still divide but already respire. This phase however is characterized by a reduced growth rate. After some time, the culture eventually reaches the stationary phase and cells stop dividing. The longer this phase lasts the more cells start to die (Fig. 2). Interestingly, yeast cells in stationary phase differentiate into two different subpopulations, quiescent and non-quiescent cells. Whereas quiescent cells mainly are composed of healthy daughter cells, replicative older cells are mostly non-quiescent cells (Allen *et al.*, 2006). Quiescent cells are dormant but still have the capacity of entering the cell cycle again upon addition of fresh media. Studies on yeast chronological aging can lead to the gain of understanding of factor that are involved in aging processes of postmitotic tissues, such as muscle and brain.

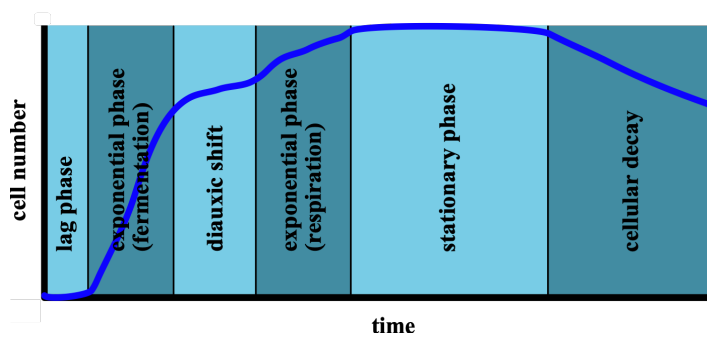


Fig. 2: Representation of yeast growth in liquid culture illustrating six different phases: the lag phase and first adaption to the new environment, exponential phase with rapid growth, diauxic shift and re-adaption, decelerated exponential phase, stationary phase, and cellular decay

In summary, chronological aging in yeast not only models aging in non-dividing, terminally differentiated human cells, such as mature neurons, cardiac muscles cells, and skeletal muscle, but also serves as a simple model for organismal aging (Longo and Fabrizio, 2014).

2. The endoplasmic reticulum (ER) – the cell factory

In yeast, like in every other eukaryotic cell, the endoplasmic reticulum (ER) plays fundamental roles in many different cellular processes, such as lipid synthesis (Carman and Henry, 2007; Henry *et al.*, 2012), calcium homeostasis (Strayle *et al.*, 1999), and protein translocation and folding (Lyman and Schekman, 1996). Unlike other organelles, where the targeting mostly happens post-translationally (Kunze and Berger, 2015), proteins are mainly inserted into the ER membrane co-translationally. Due to the specific signal peptide involved in this pathway this process is called signal recognition particle (SRP)-dependent translocation (Akopian *et al.*, 2013). Nevertheless, there are also several SRP-independent processes, such as the guided entry of tail-anchored (TA) proteins (GET) pathway (Ast and Schuldiner, 2013), and the Hsp70-dependent post-translational process (Zimmermann *et al.*, 2011) that both enable the translocation of proteins across the ER membrane.

In yeast, approximately 30% of all newly synthesized proteins are sorted by the secretory pathway and thus need to be translocated across or inserted into the ER membrane at one point (Ghaemmaghami *et al.*, 2003; Shoa and Hegde, 2011). ER membrane proteins, like all other membrane proteins, are characterized by transmembrane domains (TMDs), such as α helices for instance that enable a protein to be integrated into the ER membrane (Killian and von Heijne, 2000). To fulfill their functions adequately and reach their final destination, ER proteins have, among other things, α helices with different lengths and in varying numbers. On the one hand, different TMD lengths help proteins to travel along the secretory pathway since membranes of organelles involved in this process vary in their mean hydrophobic length (Singh and Mittal, 2016). While membrane proteins with long TMDs are preferentially transported to the plasma membrane, those with shorter TMDs are relocated to the vacuole but also to other organelles of the secretory pathway (Rayner and Pelham, 1997). On the other hand, the TMD length also determines whether a protein is excluded from regions of reduced thickness (RRTs), where only membrane proteins with short TMDs can localize or part of regions of increased thickness (RITs), such as lipid rafts, where only membrane proteins with long TMDs can be incorporated (Simons and Ikonen, 1997; Pike, 2006). In lipid rafts that are mainly associated with the plasma membrane, membrane proteins execute a plethora of crucial cellular functions, such as ion homeostasis (Gaber *et al.*, 1988; Madrid *et al.*, 1998; Mitsui *et al.*, 2009) and nutrient transport (Maliska *et al.*, 2013; Umebayashi and Nakano, 2003).

Due to different localizations, functionalities and properties of proteins in general distinct processes have evolved to insert them into the ER membrane or to translocate them across the ER membrane. Some of these processes overlap in certain aspects and can even replace each other if one translocation system is lacking. A selection is described in more detail below.

2.1 Co- and post-translational translocation across and into the ER

To reach their final destination and fulfill their desired function membrane proteins have to be synthesized, folded, and translocated into the ER membrane properly (Fig. 3) and different kinds of stress can disturb this process. The GET pathway, for instance, enables TA proteins to be inserted into the ER membrane post-translationally (Ast and Schuldiner, 2013). TA proteins are characterized by their single TMD at the C terminus and are involved in many different cellular processes, such as translocation of other proteins across the ER membrane, vesicular transportation, apoptosis, and protein quality control (Charton *et al.*, 2012; Hegde and Keenan, 2011). Newly synthesized TA proteins are captured by a dimer of the chaperon Sgt2 that shields its TMD and binds through a complex formed by Get4/5 (Rao *et al.*, 2016; Wang *et al.*, 2010) to Get3, an ATPase (Jonikas *et al.*, 2009; Wang *et al.*, 2010). Thereafter, the entire complex migrates to the ER surface where its receptors Get1/2 reside and eventually, the TA protein is inserted into the ER membrane (Schuldiner *et al.*, 2005, 2008).

SRP-independent (SND) proteins (Snd1, Snd2, and Snd3) most likely provide another mechanism of inserting TA proteins into the ER membrane since they exhibit overlapping properties to the GET pathway (Aviram *et al.*, 2016). While independent depletion of the GET pathway or the SND translocation in yeast can be tolerated, a simultaneous absence of both pathways leads to lethality (Chio *et al.*, 2017) suggesting that both pathways can compensate at least to some extent for each other.

In contrast to both the GET and SND pathway, the SRP-dependent co-translational translocation involves the signal peptide SRP that binds to the nascent, secretion signal bearing polypeptide, stops its further translation and brings it to the ER membrane by interaction with the SRP receptor (SR) on the ER surface where the translation is re-initiated (Hann and Walter, 1991; Hann *et al.*, 1992; Stirling and Hewitt, 1992; Willer *et al.*, 2003). When proteins are inserted into the ER membrane post-translationally and GET pathway-independently they firstly interact with a heterotrimeric complex (Sec62, Sec66, and Sec67) and are then transported to the ER membrane (Deshaies and Schekman, 1989; Feldheim *et al.*, 1993;

Feldheim and Schekman, 1994). In both cases, for successful continuation of translation as well as folding and insertion into the ER membrane the heterotrimeric Sec61 complex, also called the translocon, is needed (Zimmermann *et al.*, 2011). This complex interacts with many additional factors and proteins, such as chaperones and co-chaperones (e.g.; Kar2/BiP and Sec63 (Römisch, 1999; Rothblatt *et al.*, 1989; Young *et al.*, 2001)), nucleotide exchange factors (Sil1 and Lhs1 (Tyson and Stirling, 2000)), and SND proteins (Snd1, Snd2, and Snd3) in case ER membrane proteins are translocated to the ER membrane that do not harbor SRP (Aviram *et al.*, 2016).

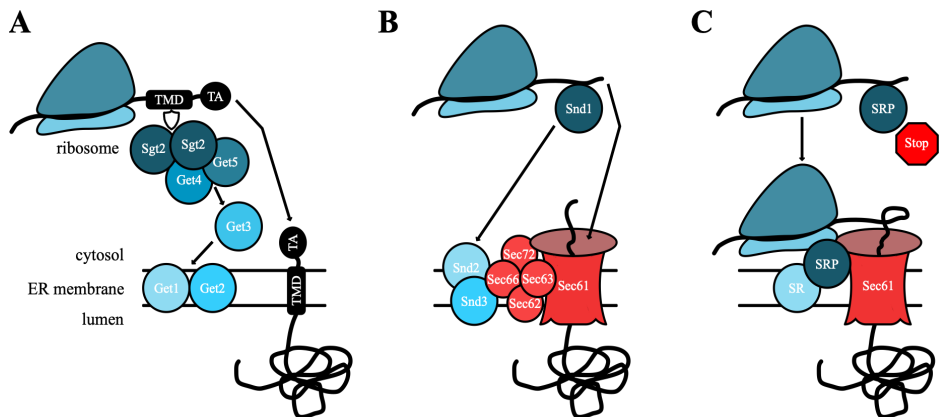


Fig. 3: Representation of post- and co-translational translocation of newly synthesized proteins: (A) GET pathway. (B) SND pathway. (C) SRP-dependent pathway. The GET pathway enables the insertion of TA proteins post-translationally. A dimer of Sgt2 recognizes the TMD of a TA protein and shields it. This dimer binds through Get4/5 to Get3 and the entire complex migrates to the surface of the ER where its receptors Get1/2 reside. Finally, the TA protein is inserted into the ER membrane. The SND pathway enables the co-translational translocation of a newly synthesized protein across the ER membrane. Here, Snd1/2/3 are involved to facilitate the translocation of the protein into or across the ER membrane using the translocon. The SRP-dependent pathway depends on the signal peptide SRP that binds to the nascent polypeptide chain and halts its further translation. The entire complex of polypeptide chain, ribosome, and SRP migrates to the surface of the ER membrane where the translation is re-initiated. Similar to the SND pathway, the SRP-dependent pathway also relies on the translocon to enable the translocation of the newly synthesized protein into or across the ER membrane.

Besides their correct insertion into the ER membrane or their translocation across the ER membrane, their turnover needs to be regulated, too. Under certain circumstances, proteins can also exhibit folding problems and misfold. Systems of the protein quality control, such as the unfolded protein response as well as the ER-associated degradation machinery and autophagy, contribute to maintaining proteostasis by removing excess or misfolded proteins.

2.2 Unfolded protein response (UPR)

The Sec61 complex not only is involved in the correct insertion of proteins into the ER membrane and their folding but also in their removal since it communicates through Kar2/BiP with the unfolded protein response (UPR). UPR is way to maintain cellular integrity and to initiate the removal of misfolded proteins and protein aggregates by altering the transcription rate of multiple target genes (Fig. 4).

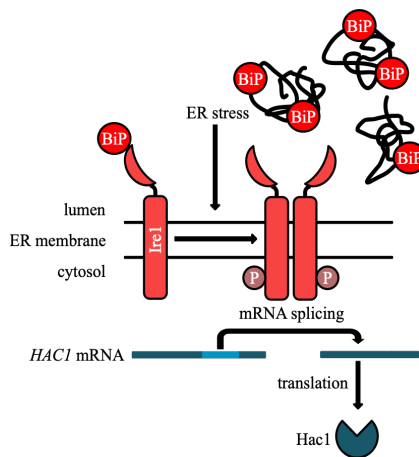


Fig. 4: Representation of the unfolded protein (UPR) response in yeast: under normal conditions without protein aggregates BiP is bound to Ire1. Upon protein aggregation BiP dissociates from Ire1 and binds to them. Ire1 now auto-phosphorylates, oligomerizes and activates its RNase domain. *HAC1* pre-mRNA is cleaved and Hac1 can be synthesized. Hac1 is a transcription factor that then migrates to the nucleus where it regulates the transcription of several target genes.

Upon ER stress that leads to misfolded proteins Kar2/BiP dissociates from Ire1 and binds to them (Okamura *et al.*, 2000). As a consequence, Ire1 auto-phosphorylates and oligomerizes which in turn activates its RNase domain (Shamu and Walter, 1996). Activated Ire1 cleaves *HAC1* pre-mRNA to facilitate its translation and synthesizes Hac1 (Cox *et al.*, 1993). Hac1 is a transcription factor that then migrates to the nucleus and activates about 400 UPR target genes; for example, genes for ER chaperones, lipid biosynthesis enzymes and proteins of the UPS (Thibault *et al.*, 2011). The UPR does not always follow the same pattern and adapts to diverse stresses differently, rendering it very likely that other regulatory, yet-unknown factors are involved (Wu *et al.*, 2014). Ire1 also seems to bind misfolded proteins directly and thus prevents the generation of protein aggregates (Promlek *et al.*, 2011) but this fact remains controversial. The kinase domain of Ire1 is important for its inactivation but not involved in UPR per se (Rubio *et al.*, 2011).

Overall, UPR enables the removal of misfolded proteins that otherwise would aggregate which consequently would lead to cellular disturbance and malfunction. Yeast lacking Ire1 and hence UPR shows a reduced chronological life span (Chadwick *et al.*, 2020) as well as growth rate in exponential phase (Das *et al.*, 2023) and an abnormal ER morphology (Ren *et al.*, 2020).

However, the two main mechanisms of cellular degradation and protein quality control system are the ER-associated degradation machinery which is coupled to the proteasome and autophagy (Reggiori and Klionsky, 2013).

2.3 ER-associated degradation and the proteasome

Besides its involvement in co- and post-translational translocation of proteins across and into the ER membrane and in UPR, the Sec61 complex also interacts directly with the ER associated degradation (ERAD) machinery (Fig. 5). In this case, it transports misfolded proteins in a retrograde manner back to the cytosol where the proteasome degrades them (Pilon *et al.*, 1997; Plemper *et al.*, 1997; Willer *et al.*, 2008; Schäfer and Wolf, 2009). Due to the localization of folding problems within proteins ERAD is classified into three distinguishable, yet partially overlapping machineries: ERAD-L (luminal proteins with folding problems), ERAD-M (TM proteins with folding problems within the membrane), and ERAD-C (TM proteins with folding problems in the cytosolic domain) (Lemberg and Strisovsky, 2021). Although these processes differ, they all share one of the two E2 ubiquitin-conjugating enzymes, Ubc6. Ubc7 additionally is part of ERAD-C (Xu *et al.*, 2009; Christianson and Ye, 2014; Lips *et al.*, 2020; Lemberg and Strisovsky, 2021). While ERAD-L and ERAD-M are also called E3 ubiquitin ligase Hrd1 complex since their main channel protein is Hrd1 (Hampton *et al.*, 1996), ERAD-C is either named E3 ubiquitin ligase Doa10 complex due to the channel protein Doa10 that is involved in this subclass of ERAD (Swanson *et al.*, 2001) or Asi ubiquitin ligase complex when it is formed among other things by the heterodimer of Asi1, Asi2, and Asi3 and located in the inner nuclear membrane (INM) where it keeps the INM intact (Foresti *et al.*, 2014). It ubiquitinates for instance misfolded INM proteins, proteins involved in sterol biosynthesis as well as unassembled complex subunits (Foresti *et al.*, 2014; Natarajan *et al.*, 2020). To activate and hence, initiate ERAD in all three cases so-called cytoplasmic E1 ubiquitin-activating enzymes are needed (e.g.; Uba1 (McGrath *et al.*, 1991)). The ubiquitinated substrates are extracted from the membrane by the AAA+ ATPase Cdc48 and thus transported to the cytoplasm or nucleoplasm, (Ye *et al.*, 2001), and finally degraded by the 26S proteasome

(Finley *et al.*, 2016). Due to the involvement of both the ubiquitination and the proteasome, this degradation pathway is also called ubiquitin-proteasome system (UPS).

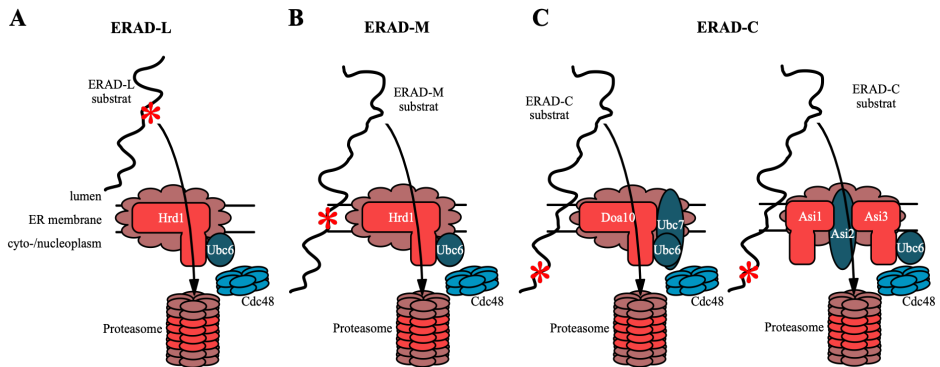


Fig. 5: Representation of the different types of ERAD: (A) ERAD-L that recognizes misfolded luminal proteins, (B) ERAD-M for TM proteins with folding problems within the membrane and (C) the two variants of ERAD-C for TM proteins that display folding problems in the cytoplasmic or nucleoplasmic domain. All ERAD machineries transport misfolded proteins back to the cytoplasm/nucleoplasm in a retrograde manner. Cdc48 is involved in the release of polyubiquitinated proteins that get then degraded by the proteasome.

Whereas UPS mainly removes normal proteins after performing their normal function as well as abnormal, misfolded, soluble proteins (Hochstrasser, 1995; Gomes *et al.*, 2006), autophagy not only degrades misfolded proteins but also protein aggregates, dysfunctional organelles and unnecessary cellular components (Yoshimori, 2004; Zheng *et al.*, 2009).

2.4 Autophagy – Mirco- and Macroautophagy

Beside UPS, autophagy is the second main mechanism that degrades cellular content (Reggiori and Klionsky, 2013). Autophagy, which already occurs under normal conditions (reviewed Rehman *et al.*, 2021), can be intensified by a plethora of stimuli and stress factors, such as starvation for nutrients or ER stress due to protein misfolding (Takeshige *et al.*, 1992; Matsuura *et al.*, 1997; Yorimitsu *et al.*, 2006; Yorimitsu and Klionsky, 2007) and is subcategorized into micro- and macroautophagy. Both types of autophagy can occur selectively and specifically or in-bulk and non-specifically (Schuck, 2020; Tekirdag and Cuervo, 2018).

While specific microautophagy happens on the interface between different organelles and the vacuole, specific macroautophagy leads to the formation of so-called autophagosomes that later fuse with the vacuole and begins with the formation of a phagophore in the cytosol (Klionsky *et al.*, 2011). About 40 *ATG* genes (Suzuki and Ohsumi, 2007; reviewed Rehman *et al.*, 2021), 19 of which are core proteins (Ohsumi, 2014), are involved in the entire process (Fig. 6).

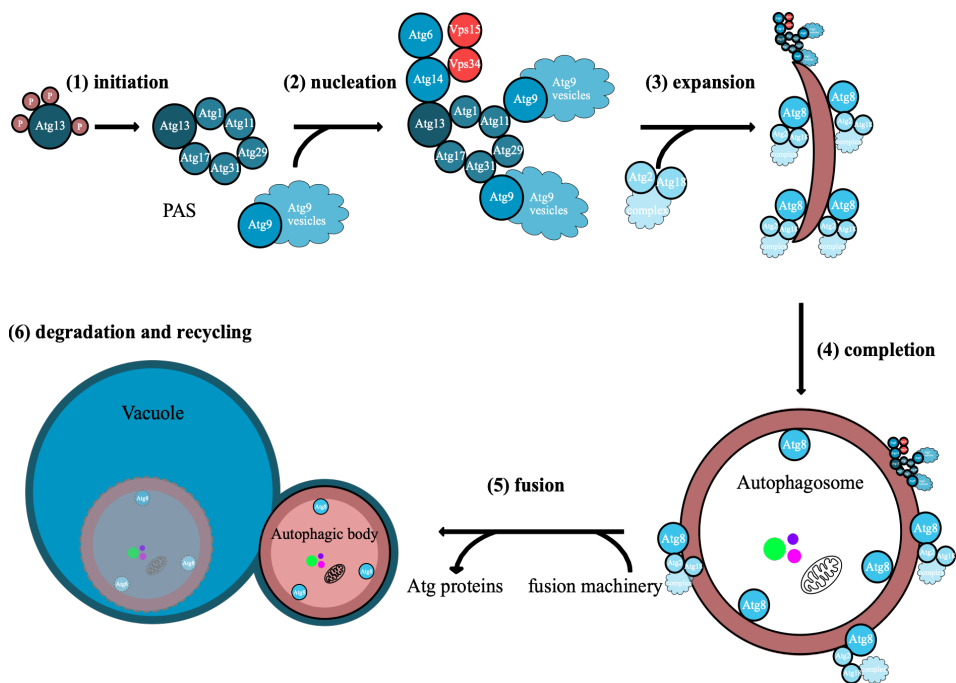


Fig. 6: Steps of macroautophagy in yeast (PAS = phagophore assembly site): **(1) initiation:** phosphorylation of Atg13 and the recruitment of six additional proteins lead to the formation of PAS. **(2) nucleation:** more components are recruited to PAS. **(3) expansion:** Atg8 is conjugated to phosphatidylethanolamine and the formation of the autophagosome is initiated. **(4) completion:** The autophagosome is completely formed and contains cytosolic cargo. **(5) fusion:** The autophagosome fuses with the lytic organelle (vacuole). **(6) degradation and recycling:** Inside the vacuole the autophagosome gets degraded and molecules recycled.

There are six major steps in macroautophagy and the first one initiates the formation of a phagophore assembly site (PAS) and involves 6 Atg proteins. The main player here is Atg13 that gets hypophosphorylated when autophagy is induced. Its hypophosphorylation enables its interaction with Atg1 and Atg17 (Fujioka *et al.*, 2014). Thereafter, Atg17 forms a heterotrimer with Atg31 and Atg29 that interacts with Atg11 (Mao *et al.*, 2013) and the second step, the nucleation, is triggered where other factors and further Atg proteins, such as Atg9 and Atg14 together with Atg6, are recruited to the PAS (Suzuki *et al.*, 2007). Then, the third step, the autophagosome formation and its expansion, is facilitated by other Atg proteins, especially by the Atg2-Atg18 complex as well as Atg8 (Nakatogawa *et al.*, 2007). Atg8 is conjugated to phosphatidylethanolamine by the E1- and E2-like proteins Atg7 and Atg3 in a ubiquitin-like manner (Geng and Klionsky, 2008). This is followed by the next process, the completion. Here, the cargo that will be degraded is surrounded by a double-membrane which leads to the formation of an autophagosome (Lipatova *et al.*, 2012). This autophagosome then fuses in the

fifth step with the vacuole, which causes the release of the Atg proteins, and is finally degraded during the last step within it (Farré and Subramani, 2016) leading to the liberation and recycling of macromolecules that can be reused. This process is a highly conserved degradation mechanism of all eukaryotic kingdoms (plants, fungi, and animals). Whereas plants and fungi use the vacuole as the lytic organelle, in animals, lysosomes fulfill this task (reviewed Rehman *et al.*, 2021).

Specific microautophagy, in contrast, does not start in the cytosol but on the interface between the vacuole and another organelle, for instance mitochondria (Deffieu *et al.*, 2009), peroxisomes (Dunn *et al.*, 2005), lipid droplets (LD; Singh *et al.*, 2009; van Zutphen *et al.*, 2014) and the nucleus (Krick *et al.*, 2008; see 2.4.1). Micromitophagy for instance allows the degradation of parts of mitochondria that dysfunction, experienced too much oxidative stress or accumulated too many mutations in the mtDNA, resulting in prolonged life span of yeast (Lemasters, 2005), and relies on all *ATG* core (Kanky *et al.*, 2011) and additionally on one mitophagy-specific *ATG* gene, namely *ATG32* (Kanky *et al.*, 2009; Okamoto *et al.*, 2009). Microlipophagy, also named LD autophagy, can be induced by for instance nitrogen starvation (Kumar *et al.*, 2020), growth into the stationary phase (Kumar *et al.*, 2020; Wang *et al.*, 2014), acute glucose reduction (Seo *et al.*, 2017), lipid stress (Vevea *et al.*, 2017), and ER stress (Garcia *et al.*, 2020).

Besides lipophagy, this study also focuses on both macro- and macroautophagic processes that occur at the nucleus, namely piecemeal microautophagy of the nucleus and perinuclear reticulophagy.

2.4.1 Piecemeal microautophagy of the nucleus (PMN)

The specific microautophagic process that happens at the interface between the vacuole and the nucleus, is termed piecemeal microautophagy of the nucleus (PMN; Roberts *et al.*, 2003; Kvam and Goldfarb, 2007; Fig. 7). PMN is a process that is triggered particularly when cells reach stationary phase due to glucose exhaustion. Parts of both the nucleus and the NVJs themselves get engulfed by the vacuole and specific PMN vesicles bud off into the vacuolar lumen that later are degraded by soluble hydrolases within the vacuole (Roberts *et al.*, 2003). PMN requires core Atg proteins to fully function, such as Atg1, 8, and 9. Even if PMN blebs are formed in cells lacking one core Atg protein, they are not engulfed by the vacuole (Krick *et al.*, 2008).

Whereas PMN is an example of a specific microautophagic process that does not involve the formation of an autophagosome and regulates protein turnover at the interface between the nucleus and the vacuole, reticulophagy as a type of macroautophagy relies on the formation of an autophagosome and occurs at different localizations.

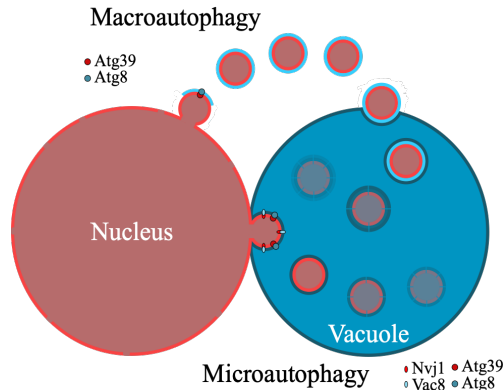


Fig. 7: Simplified representation of macro- and microautophagy in yeast on the example of the nucleus: Macronucleophagy (perinuclear reticulophagy) is dependent on Atg39 and Atg8. Pieces of the nucleus as well as of the pER are packaged into a double-membrane vesicle which is delivered to the vacuole for degradation. Micronucleophagy (Piecemeal microautophagy of the nucleus) depends not only on Atg39 and Atg8 but also on Nvj1 and Vac8. Parts of both the nucleus and the pER directly bud off into the vacuolar lumen where they get degraded.

2.4.2 Reticulophagy – a macroautophagic process

Besides PMN, a specific microautophagic process in which parts of the ER and nuclear membranes are directly engulfed into the vacuole and degraded there, macroautophagic pathways also exist, referred to as ERphagy or reticulophagy. These pathways also maintain ER membrane homeostasis and do not necessarily occur at the NVJs or in their vicinity. As a macroautophagic process, they rely on the formation of an autophagosome (Fig. 7). The general autophagy receptor Atg8 not only plays a role in PMN (Krick *et al.*, 2008) but is also involved in macroautophagic processes, such as reticulophagy where it interacts with Atg39 or Atg40, two ER receptors that are involved in two distinct types of ERphagy (Mochida *et al.*, 2015). The ER is divided into three subdomains, the perinuclear ER (pER/nER) that surrounds the nucleus, the cortical ER (cER) that is close to the plasma membrane as well as the tubular ER (tER) that connects both. While Atg39 localizes to the pER and enables its degradation, Atg40 is found in both the cER and tER and facilitates their degradation. Consequently, in yeast there

are both an Atg39-dependent perinuclear reticulophagy, also termed nucleophagy, and an Atg40-dependent cortical/cytoplasmic reticulophagy (Nakatogawa and Mochida, 2015). Although in this case both micro- and macroautophagy degrade parts of both the nucleus and the ER, the evolution of both distinct processes is beneficial for the organism since it allows for the removal and recycling of different target materials. In this way, some cellular components can be selectively excluded from certain autophagic processes, while others can be preferentially degraded by other means. Since specific microautophagy does not require the synthesis of a separate vesicle it can often be initiated more quickly than macroautophagy, allowing the cell to recycling cellular material more rapidly if intrinsic or extrinsic factors trigger such a scenario. Macroautophagy, on the other hand, generally facilitates the removal and breakdown of larger cellular components, as an autophagosome is synthesized that can vary in its size.

3. Mitochondrion – the powerhouse of the cell

Usually, eukaryotic cells harbor mitochondria with a double-membrane structure (Fig. 8), relics of an ancient α -proteobacterial endosymbiont (Gray *et al.*, 1999). Some cells however, such as human red blood cells are depleted of mitochondria due to their specialized role in the human body as fast oxygen transporters. Yet, the lack of mitochondria leads to a shortening of their lifespan (Shijin *et al.*, 2024). The only known eukaryote that lost its mitochondrion completely is the unicellular flagellate *Monocercomonoides exilis* (Karnkowska *et al.*, 2015; Treitli *et al.*, 2021) that was isolated from the intestine of the long-tailed chinchilla (*Chinchilla lanigera*; Hampl *et al.*, 2005).

In most eukaryotic cells, mitochondria fulfill crucial tasks and are thus essential for survival. For instance, they are involved in energy conservation (Van der Bliek *et al.*, 2017), calcium homeostasis (Oxenoid *et al.*, 2016), iron-sulfur cluster assembly (Freibert *et al.*, 2017), and others. To overcome the latter issue, *Monocercomonoides exilis* acquired a prokaryotic alternative and uses the so-called sulfur mobilization system (Vacek *et al.*, 2018).

Not surprisingly, yeast – as a eukaryote – also relies on its well-functioning mitochondria that perform diverse important tasks. Inter alia, yeast mitochondria are involved in metabolite flux across the mitochondrial membrane (Palmieri *et al.*, 2006, 2014; Palmieri and Pierri, 2010), amino acid metabolism (García-Campusano *et al.*, 2009; Falco *et al.*, 1985; Cullin *et al.*, 1996; Kispal *et al.*, 1996; Velasco *et al.*, 1993), iron-sulfur cluster biogenesis (Beinert *et al.*, 1997;

Braymer and Lill, 2017; Kispal *et al.*, 2005), heme biosynthesis (Hoffman *et al.*, 2003), lipid homeostasis (Zinser *et al.*, 1991; Osman *et al.*, 2011; Birner *et al.*, 2001), and programmed cell death (Guaragnella *et al.*, 2012; Eisenberg *et al.*, 2007; Pereira *et al.*, 2008).

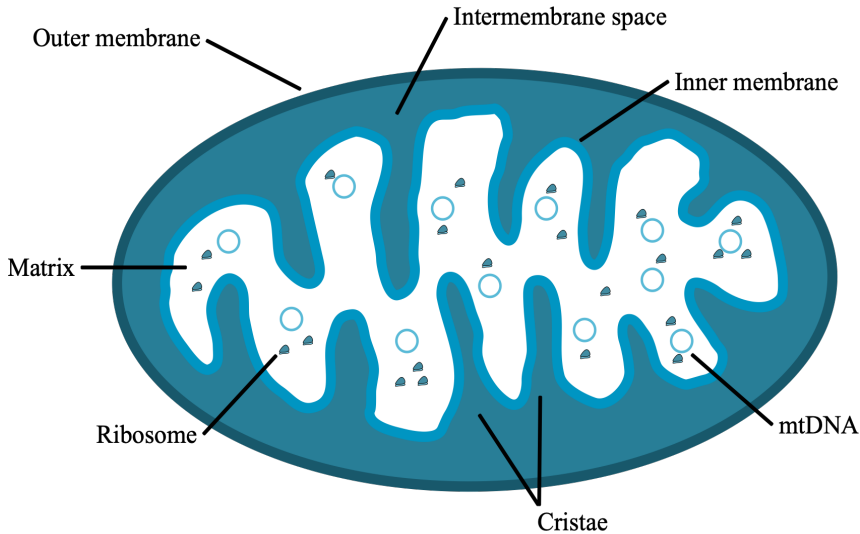


Fig. 8: Schematic representation of an eukaryotic mitochondrion.

In conclusion, beyond their primary role in energy generation, mitochondria are complex, semi-autonomous organelles that are central to cellular life and orchestrate a variety of crucial cellular processes. Due to their origin and role in all these processes, mitochondria differ from other organelles and possess special features.

3.1 Special features of mitochondria

Due to their special origin as an α -proteobacterial endosymbiont (Gray *et al.*, 1999) mitochondria show biochemical and structural similarities to prokaryotic cells (John and Whatley, 1975). For instance, although most of their genes were relocated to the nucleus, they usually still possess prokaryotic chromosomes that harbor different amounts of genes, depending on the species (Burger *et al.*, 2013). One example of mitochondria that do not contain any chromosomes are those of mature human sperm cells (Lee *et al.*, 2023). Whereas in humans, there are 37 genes encoded by the mitochondrial DNA (mtDNA), in yeast, there

are 34 genes, whereof eight are protein-coding, one of these is a ribosomal protein-coding gene, 24 code for tRNAs and two for rRNAs (reviewed in Malina *et al.*, 2018).

Interestingly, despite their bacterial descent and the fact that they are parts of eukaryotes, their ribosomes – the so-called mitoribosomes – display unique species-specific characteristics and are neither like eukaryotic nor prokaryotic ribosomes (Ott *et al.*, 2016; Greber and Ban, 2016). For instance, only about half of the proteins of yeast ribosomes have bacterial homologs (Smits *et al.*, 2007). Their structural properties also differ from mammalian ones as they also need to transcribe one soluble ribosomal protein (Var1; Amunts *et al.*, 2014; Desai *et al.*, 2017; reviewed in Malina *et al.*, 2018). The rest of the proteins encoded on yeast mtDNA are membrane proteins and part of the mitochondrial oxidative phosphorylation complex (Cytb, Cox1, Cox2, Cox3, Atp6, Atp8, and Atp9; reviewed in Malina *et al.*, 2018: s. 3.2). For mitochondrial transcription, the specific, bacteriophage-like RNA polymerase Rpo41 is needed (Masters *et al.*, 1987), which is also involved in the replication of mtDNA (Greenleaf *et al.*, 1986) by priming the mitochondrial DNA polymerase Mip1 (Sanchez-Sandoval *et al.*, 2015).

3.2 Mitochondrial oxidative phosphorylation (OXPHOS)

The mitochondrial oxidative phosphorylation (OXPHOS) system in yeast is composed of both the mitochondrial respiratory chain (MRC) and the ATP synthase, which is also named complex V (CV) and localized to the inner mitochondrial membrane (IMM; Reviewed in Eldeeb *et al.*, 2024). Whereas MRC generates an electrochemical gradient across the IMM, CV finally uses this energy to synthesize ATP, the most important energy source in cells to facilitate many biochemical processes (Fig. 9).

MRC in yeast consists of three NADH dehydrogenases (Nde1, Nde2, and Ndi1), three major enzymatic complexes (complex II - IV (CII, CIII, and CIV)) as well as two electron carriers (coenzyme Q (CoQ) and cytochrome *c* (Cyt*c*)). While higher eukaryotes also possess a complex I, the three NADH dehydrogenases in yeast partially cover for it.

Nde1 and Nde2 are directly anchored via an N-terminal TM domain into the IMM (Luttik *et al.*, 1998), Ndi1 however only associates to the IMM peripherally (Feng *et al.*, 2012). Since the active center of Nde1 and Nde2 are localized to the intermembrane space they catalyze the reaction of NADH to NAD⁺ in this space. Ndi1 on the other hand catalyzes the same reaction but in the mitochondrial matrix due to its localization. Both reactions lead to a transfer of electrons to CoQ. Moreover, the oxidation of succinate to fumarate at CII transfers electrons

via the cofactor FAD to CoQ. The pool of reduced CoQ diffuses through the IMM to CIII where it transfers its electrons to this complex. CIII pumps protons across the IMM to the intermembrane space to generate a proton gradient. The electrons are further transferred to the soluble Cyt_c that is localized in the intermembrane space and brings the electrons to CIV. Similar to CIII, CIV also pumps protons to across the IMM. Here, oxygen serves as an electron acceptor. At CV, the ATP synthase, protons flow back to the mitochondrial matrix and the energy liberated by this process is used to generate ATP.

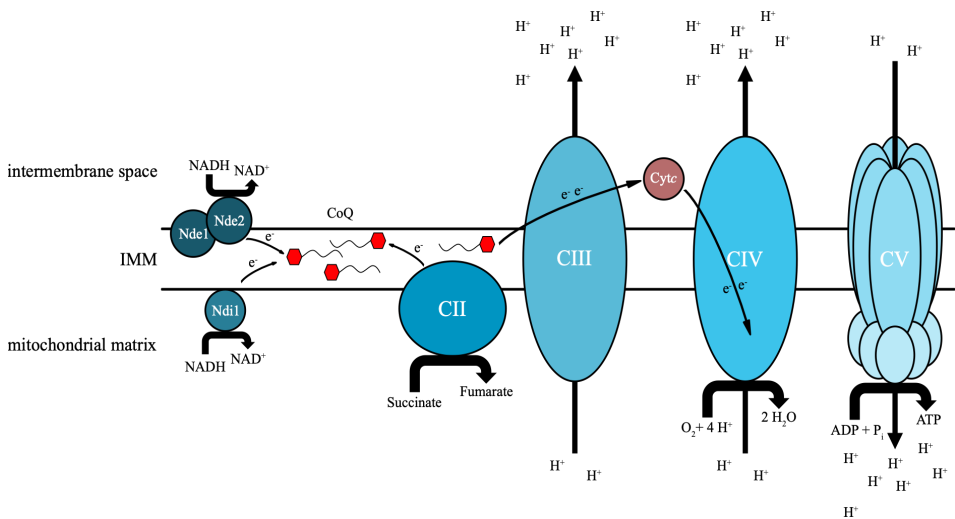


Fig. 9: Simplified representation of OXPHOS in yeast, which is composed of the mitochondrial respiratory chain as well as the ATP synthase (complex V). (IMM = inner mitochondrial membrane). Nde1, Nde2, and Ndi1 catalyze the reaction of NADH to NAD⁺ which leads to a liberation of electrons within the IMM that get captured by CoQ. CoQ also receives electrons from CII that catalyzes the reaction from succinate to fumarate. CoQ brings the electrons to CIII where they are transported further to Cyt_c. During this process, protons are pumped from the matrix to the intermembrane space. Cyt_c brings the electrons to CIV. This complex also pumps protons to the intermembrane space and transfers the electrons to oxygen, forming water. Finally, at CV the protons flow back to the matrix and ATP is generated.

Usually, the MRC complexes form so-called respiratory supercomplexes (SC) to optimize mitochondrial functionality. MRC complexes are composed of an obligatory CIII dimer with one or two CIV monomers (III₂IV₁ SC or III₂IV₂ SC) where the CIII dimer is either flanked by one CIV monomer on one side or by two CIV monomers on both sites (Schagger and Pfeiffer, 2000; Fig. 10).

The SCs are mainly stabilized via hydrogen and pi-pi bonds formed by Cor1 from CIII and Cox5 from CIV (Harley *et al.*, 2019; Rathore *et al.*, 2019). Due to the tight positioning of CIV to CIII, electrons can be shuttled much faster since Cyt_c has a shorter travel distance (Berndtsson *et al.*, 2020), which in turn renders MRC more efficient. Generally, diffusion of

Cytc is thought to be the rate-limiting factor within MRC (Stuchebrukhov *et al.*, 1861). Minimizing its travel distance seems therefore beneficial. Yet, this conclusion still remains controversial.

The formation of two different SCs allows yeast to adapt to different environmental stimuli. For instance, in exponentially growing yeast when respiration is not that important yet III₂IV₁ SC is predominant whereas cells in stationary phase show an increase of III₂IV₂ SC since glucose is depleted and they rely more on respiration (Schagger and Pfeiffer, 2000). Attaching two CIV to the CIII dimer guarantees an increased capacity of electron transfer. Since, however, this attachment is very flexible it can be detached easily if environmental stimuli change, e.g.; glucose is available again. Thus, the efficiency of MRC and consequently the production of ATP can be regulated finely tuned and according to the actual energy requirements.

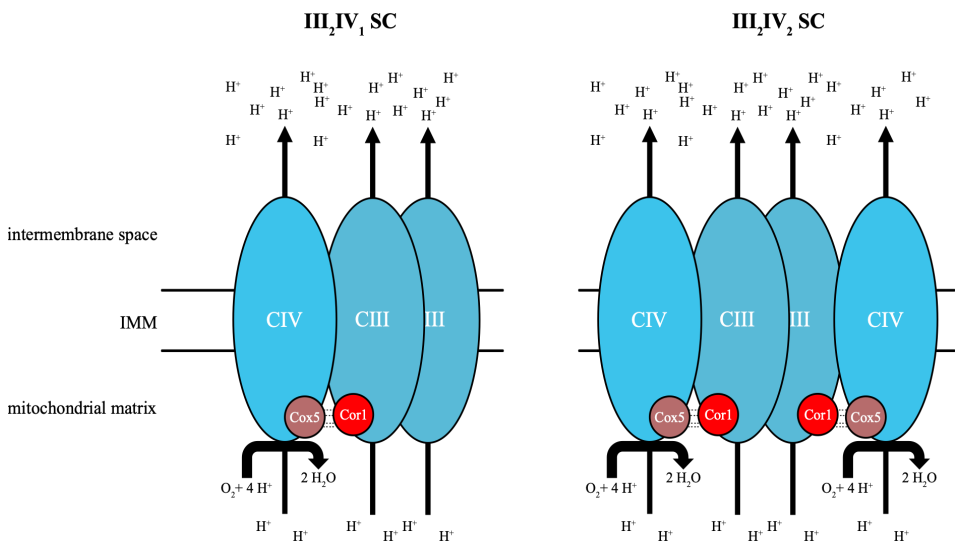


Fig. 10: Simplified representation of the respiratory supercomplexes (SCs) in yeast, which is either composed of one CIII dimer and one CIV monomer (III₂IV₁ SC) or two CIV monomers (III₂IV₂ SC). The SCs are mainly held together through hydrogen and pi-pi bonds (dashed lines) formed by Cox5 and Cor1. (IMM = inner mitochondrial membrane).

Another convenience of the generation of SC can be the reduced production of reactive oxygen species (ROS), a toxic byproduct of respiration. Under normal conditions, CIII is the main source of ROS production due to the leaky Q cycle, the transfer of electrons from CoQ to Cytc (Mazat *et al.*, 2020). During this cycle electrons can be erroneously transferred to oxygen instead of Cytc which causes the production of ROS (Mazat *et al.*, 2020; Brand, 2016). Positioning CIV near CIII can decrease ROS production since the final reaction of CIV is the

reduction of oxygen to water, which thereby limits the availability of free oxygen in the close vicinity of CIII.

OXPPOS is an aerobic process in which mitochondria convert energy from nutrient oxidation into ATP. It occurs in the IMM, where a series of respiratory complexes transfer electrons to create a proton gradient that finally fuels the ATP synthase. One of many crucial steps in the OXPPOS system is the transport of electrons within the IMM, which is enabled by CoQ. In all eukaryotes, CoQ is synthesized in the IMM (Hauß *et al.*, 2005; Lenaz, 2001; Kagan and Quinn, 2000).

3.3 Coenzyme Q synthesis

Coenzyme Q (CoQ₁₀ in humans; CoQ₈ in *E. coli*; CoQ₆ in yeast (Turunen *et al.*, 2004); here: CoQ) is a lipidic molecule that is embedded in the membrane of the IMM where it serves as both an electron carrier and an antioxidant. Although CoQ is synthesized exclusively in the mitochondria, it also localizes in membranes of other organelles, where it also serves as an antioxidant (Hauß *et al.*, 2005; Lenaz, 2001; Kagan and Quinn, 2000).

In yeast, as in all eukaryotes, CoQ is also synthesized in mitochondria and involves several Coq proteins whose genes are encoded in the nucleus on the nDNA (Tran and Clarke, 2007; Fig. 11). The first step of CoQ synthesis is the initiation. During this process Coq1 and Coq2 generate 4-hydroxy-3-hexaprenyl benzoate (HHB). These two proteins are not part of the Coq complex (González-Mariscal *et al.*, 2014). Potentially, Coq4 initiates as an assembly factor the formation of the actual Coq complex and binds to HHB (Xie *et al.*, 2012; He *et al.*, 2014). Coq4 is further needed to stabilize the presence of Coq3 in the complex (Marbois *et al.*, 2009). After this initial step further Coq proteins are recruited to the pre-complex, which allows the conversion of HHB to 5-demethoxyubiquinone (DMQ; González-Mariscal *et al.*, 2014). Under inactive conditions, Coq7 is phosphorylated which prevents the interaction with this complex due to steric hindrance. In order to join the complex, Coq7 needs to be dephosphorylated by the phosphatase Ptc7 (Martin-Montalvo *et al.*, 2013). Due to this fact, Coq7 seems to be the major regulator of CoQ synthesis (Xie *et al.*, 2011; Martin-Montalvo *et al.*, 2011) and finally, catalyzes the reaction from DMQ to CoQ. Whereas DMQ levels increases in yeast cells lacking Coq7 (Padilla *et al.*, 2009), CoQ levels are drastically increased when the three phosphorylation sites of Coq7 are mutated to alanine, which renders Coq7 constitutively active (Martin-

Montalvo *et al.*, 2011). Lastly, Coq10 binds to CoQ and shuttles it to its proper localization (Barros *et al.*, 2005).

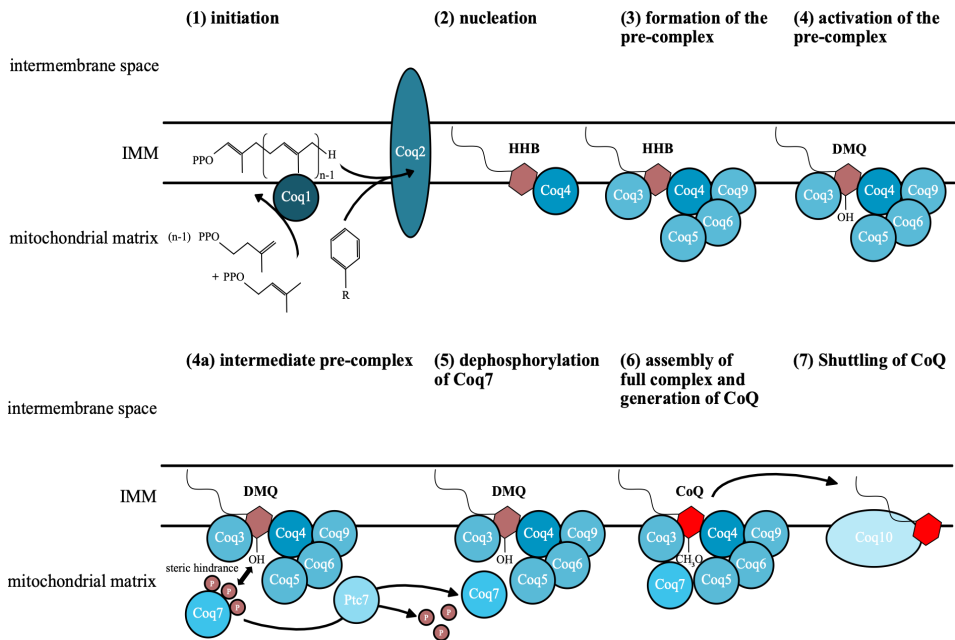


Fig. 11: Simplified representation of coenzyme Q (CoQ) synthesis. (IMM = inner mitochondrial membrane; HHB = 4-hydroxy-3-hexaprenyl benzoate; DMQ = 5-demethoxyubiquinone; CoQ = coenzyme Q). (1) initiation: Coq1 and Coq2 generate HHB. (2) nucleation: Coq4 enables the nucleation of the COQ complex. (3) formation of the pre-complex: Other Coq proteins are recruited to the complex. (4) activation of the pre-complex: HHB is catalyzed to DMQ, exposing a OH group. (4a) intermediate pre-complex: Due to steric hindrance Coq7p cannot interact with the Coq complex. (5) dephosphorylation of Coq7: Ptc7 dephosphorylates Coq7 that now can interact with the Coq complex. (6) assembly of full Coq complex and generation of CoQ: DMQ is catalyzed to CoQ. (7) shuttling of CoQ: Coq10 enables the shuttling of CoQ to its desired localization.

Findings of another study suggest that the putative oxidoreductase Coq11 is also involved in the synthesis of CoQ as part of the Coq complex (Allan *et al.*, 2015). Furthermore, the kinase Coq8 seems to be a key regulator of the CoQ synthesis since it potentially phosphorylates different Coq proteins (Coq3, Coq5, and Coq7) to control their activities (He *et al.*, 2014). For instance, the phosphorylation of Coq7, presumably caused by Coq8, leads to its inactivation (He *et al.*, 2014). In this context, Coq8 seems to be the counterpart to Ptc7.

Overall, the CoQ synthesis is a fundamental, highly conserved metabolic pathway across all life (reviewed in Staiano *et al.*, 2023) and yeast Coq proteins can even be replaced by human homologs. Deficiencies of CoQ lead to mitochondrial dysfunctions and are linked to diseases in humans, such as myopathy, nephropathy, anemia and others (Doimo *et al.*, 2014).

3.4 Mitochondria during cellular growth

Under glucose-rich conditions when yeast grows exponentially, the expression of many genes coding for proteins involved in the TCA cycle, the mitochondrial respiratory chain (MRC) as well as ATP production and mitochondria biogenesis are repressed since mitochondria are not essential yet to produce ATP. This repression is guaranteed by the Mig1 complex that represses the expression of *HAP4* encoding Hap4, a protein involved in the glucose-repressed Hap2/3/4/5 complex, also known as HAP complex (Carrillo-Garmendia *et al.*, 2022; Lundin *et al.*, 1994). Once glucose gets depleted and yeast enters the diauxic shift *HAP4* repression ends due to the dissociation of the Mig1 complex, and its expression begins. The HAP complex can consequently be formed and acts as a transcriptional activator that facilitates the expression of the previously repressed genes (Capps *et al.*, 2022; reviewed in Bolotin-Fukuhara, 2017) and amongst others, mitochondrial biosynthesis and remodeling start (Fig. 12).

A deletion of *HAP4* leads to a growth arrest of yeast cells in glycerol when mitochondria are needed (Di Noia *et al.*, 2023). Further phenotypes of a *HAP4* deleted mutant are increased mitophagy, absence of respiratory growth, decreased cell size as well as decreased cellular fitness (reviewed in Bolotin-Fukuhara, 2017). In contrast, overexpression of *HAP4* seems to be beneficial for yeast since it prolongs lifespan (Lin *et al.*, 2002; Piper *et al.*, 2006).

However, in this context, it is important to note that the HAP complex positively regulates a plethora of genes that are not directly linked to mitochondria and their biogenesis but to other cellular processes, such as adaption to oxidative stress, biosynthesis of amino acids as well as rearrangements of cellular morphology in general. In addition, the HAP complex negatively regulates genes involved in the synthesis and processing of rRNA and sterol biosynthesis (Buschlen *et al.*, 2003 Repetto and Tzagoloff, 1989; Dorsman and Grivell, 1990; Oechsner *et al.*, 1992; Rosenkrantz *et al.*, 1994; Daignan-Fornier *et al.*, 1994).

All in all, the evolutionary conserved HAP complex is a master heteromeric transcription factor in yeast that coordinates nuclear gene expression not only with mitochondrial function but also with other cellular processes during diauxic shift. Its activation is fundamental when yeast transitions from fermentation to respiration, which causes an extensive cellular remodeling in general, and mitochondria are needed.

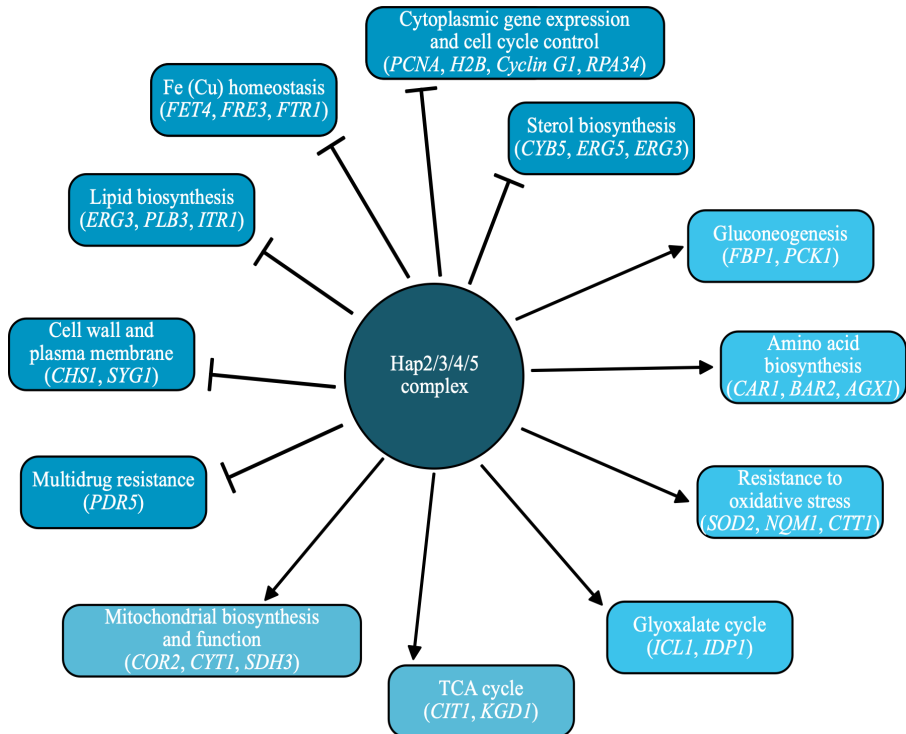


Fig. 12: Representation of Hap2/3/4/5 complex regulated genes (Buschlen *et al.*, 2003; Repetto and Tzagoloff, 1989; Dorsman and Grivell, 1990; Oechsner *et al.*, 1992; Rosenkrantz *et al.*, 1994; Daignan-Fornier *et al.*, 1994).

3.5 Involvement of mitochondria in aging

In the 60s, studies in male houseflies already showed that mitochondrial functionality declines in the process of aging, which might also contribute to aging and age-induced organ changes, such as the capability of flying (Rockstein and Brandt, 1963). Much later, a similar correlation between age-associated decline of mitochondrial functionality and changes of organ efficiency was shown as aged mitochondria contributed to insulin resistance in the elderly (Petersen *et al.*, 2003). Age-dependently, mitochondrial DNA (mtDNA) acquires mutations but whether this phenomenon directly contributes to age-induced changes remains a debate since mtDNA exists in multiple numbers of copies, and other factors seem more plausible (Sun *et al.*, 2016). Besides mutations caused by aging, aging is also accompanied by a decline in mitochondrial enzymatic activity as well as the overall respiratory capacity and an increase of the production of reactive oxygen species (ROS; Sun *et al.*, 2016). In this context however, it remains difficult to distinguish between the cause and the consequence.

Interestingly, a modest disruption of mitochondrial function in *C. elegans* as well as mice leads to an increase of lifespan (Dillin *et al.*, 2002; Lee *et al.*, 2003; Liu *et al.*, 2005). What seems contradictory and contra-intuitive at first glance is understandable upon closer inspection. All these slight disruptions seem to trigger the mitochondrial unfolded protein response (UPR^{mt}; Durieux *et al.*, 2011), which in turn leads to a nuclear transcriptional response and the expression of genes of mitochondrial chaperons (Zhao *et al.*, 2002) as well as proteins involved in ROS defense (Nargund *et al.*, 2015; Schulz and Haynes, 2015). This phenomenon is also known as mitohormesis and describes the fact that mild mitochondrial stress triggers cellular changes that makes the cell less susceptible to future stress (Yun and Finkel, 2014; Cheng *et al.*, 2023). Mitohormesis stimulates not only UPR^{mt} but also mitophagy (reviewed in Da *et al.*, 2024). For instance, mild oxidative stress leads to the promotion of fission effects, which facilitates the removal of damaged mitochondria through mitophagy and enhances the overall mitochondrial membrane potential (Palmeira *et al.*, 2019). Impaired mitophagy in humans on the other hand leads to the age-associated disease Parkinson (Durcan and Fon, 2015).

Also in yeast, mitochondrial integrity is of great importance for cellular survival. Removing prohibitins (prohibitin 1 and prohibitin 2), the general mitochondrial sentinels, in yeast impacts mitochondrial functionality negatively which reduces cellular survival (Jazwinski, 1996; Nijtmans *et al.*, 2002). Prohibitins are highly conserved proteins that assemble into large, ring-shaped macromolecules, the PHB complex, within the IMM (Tatsuta *et al.*, 2005). The PHB complex is involved in the regulation of the OXPHOS system by physical interaction with it. Whereas the absence of prohibitin 1 leads to an instability of mitochondrial-encoded subunits of the OXPHOS system, its overexpression stabilizes newly synthesized subunits encoded by the mtDNA (Steglich *et al.*, 1999; Nijtmans *et al.*, 2000; Bayot *et al.*, 2010). Furthermore, the PHB complex forms a supercomplex with the *m*-AAA protease, which enables the removal of unassembled subunits of the OXPHOS system (Bayot *et al.*, 2010) and acts as a receptor for Atg8, thereby regulating mitophagy (García-Chávez *et al.*, 2024).

All these observations therefore lead to the conclusion that mitochondrial functionality indeed influences aging processes to some degree. While mitochondria exhibiting decreased structural impairment due to increased UPR^{mt}, mitophagy and overall proteolytic capacity, have a positive impact on cellular survival, those that show structural and functional disturbances lead to a reduction of cellular fitness and longevity.

4. Membrane contact sites – bringing organelles together

Membrane contact sites (MCSs) are areas of juxtaposition between membranes of two or more organelles (Helle *et al.*, 2013; Levine and Loewen, 2006) and are named homotypic when they occur between identical organelles or heterotypic when different organelles are involved (Scorrano *et al.*, 2019). All eukaryotes harbor MCSs, which are involved in a plethora of different cellular processes, including transfer of calcium, lipids, and metabolites as well as organelle biogenesis and cellular signaling (Osman *et al.*, 2011; Wang and Benning, 2012; Horvath and Daum, 2013; Helle *et al.*, 2013; English and Voeltz, 2013; Prinz, 2014). Almost every organelle, if not all, can form MCSs with every other organelle (Kakimoto *et al.*, 2018; Shai *et al.*, 2018; Valm *et al.*, 2017).

In yeast, mitochondria for instance form MCSs with peroxisomes (PerMit; Shai *et al.*, 2018), the vacuole (vacuole and mitochondria patches = vCLAMP; Bisinski *et al.*, 2022), the nucleus (Eisenberg-Bord *et al.*, 2021), and the ER (ER-Mitochondria encounter structure = ERMES; Kornmann *et al.*, 2009). Alongside the MCSs with the mitochondria, the ER also forms them with LDs (LiDER; Barbosa and Siniossoglou, 2017; Castro *et al.*, 2022; Hugenroth and Bohnert, 2019), the plasma membrane (West *et al.*, 2011), and the vacuole (NVJ; Pan *et al.*, 2000). Moreover, MCSs are formed between LDs and the plasma membrane (pCLIP; Castro *et al.*, 2022), peroxisomes (Novikoff *et al.*, 1980; Schrader, 2001) as well as the vacuole (vCLIP; Álvarez-Guerra *et al.*, 2024; Diep *et al.*, 2024) and between peroxisomes and Golgi (GoPo; Castro *et al.*, 2022), the plasma membrane (Hulmes *et al.*, 2020) as well as the vacuole (perVale; reviewed in Joshi, 2021; Bellu *et al.*, 2001). Peroxisomes and LDs also form homotypic MCSs (Schrader *et al.*, 2000; Eisenberg-Bord *et al.*, 2018).

The individual organelles in the cell are therefore not isolated from each other, but on the contrary, in constant communication with each other via MCSs. In addition to diffusion and active transport through the cytosol as well as vesicle trafficking, MCSs represent a third pathway for the exchange of signals and metabolites within eukaryotic cells. Although this study mainly examines the two MCSs NVJ and vCLAMP, ERMES will also be described in more detail below due to its special interaction with vCLAMP.

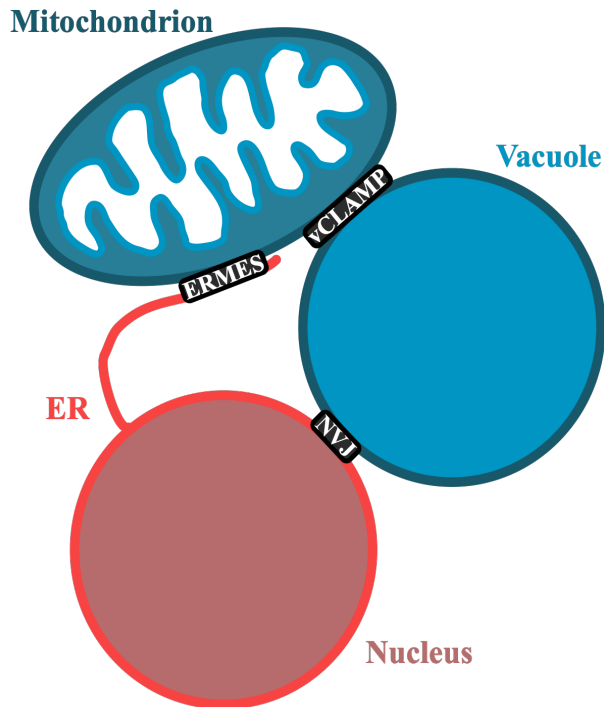


Fig. 13: Simplified representation of three membrane contact sites (MCSs) in yeast: The nucleus vacuole junction (NVJ) connects the nucleus via the ER to the vacuole, the MCS ER-Mitochondria encounter structure (ERMES) bridges the mitochondrion to the ER, and the MSC vacuole and mitochondria patches (vCLAMP) enables the direct contact of the mitochondrion to the vacuole.

4.1 The nucleus vacuole junction – bridging the gap

The NVJ is both the first discovered and one of the best characterized MCS in yeast and connects the perinuclear to the vacuolar membrane (Pan *et al.*, 2000). The two main tether proteins, Nvj1 and Vac8, hold the membranes of these two organelles together, thus enabling the formation of this MCS (Pan *et al.*, 2000; Jeong *et al.*, 2017). At the NVJs essential cellular processes are mediated, such as PMN (Roberts *et al.*, 2003; Kvam and Goldfarb, 2007; s. 2.4.1) and LD () as well as general lipid biosynthesis (Levine and Munro, 2001; Kohlwein *et al.* 2001; Kvam and Goldfarb, 2004; Kvam *et al.*, 2005).

During aging and especially upon glucose exhaustion, NVJs undergo significant, structural remodeling to adapt to changing cellular needs. Initially only small foci, the NVJs gradually grow into elongated patches (Pan *et al.*, 2000; Levine and Munro, 2001). Other stimuli that lead to the enlargement of NVJs are proteostatic stresses at the nuclear envelop. General ER stress for instance results in the expansion of NVJs and an increased frequency of PMN to

remove damaged material (Hariri *et al.*, 2018). Furthermore, when yeast experiences problems assembling nuclear pore complexes, NVJs become crucial to remove misassembled subunits of these complexes via PMN (Lord and Wentz, 2020).

NVJs thus serve as critical, flexible hubs for balancing metabolic demands. By expanding during both nutrient and proteotoxic stress, these junctions facilitate PMN to recycle cellular materials and maintain proteostatic health.

4.2 ERMES and vCLAMP – two mitochondrial MCSs

Although mitochondria are semi-autonomous organelles, they depend on other organelles for the supply of cellular material. While most of their genes were transferred to the nucleus and thus their proteins are synthesized in the cytosol, most of their lipids are synthesized in the ER (Tatsuta *et al.*, 2014). Consequently, both their proteins and their lipids need to be transported into that organelle. Since mitochondria are not part of the endomembrane system through which lipids can be shuttled via vesicles, they rely on an alternative pathway to obtain their lipids. In order to receive lipids from the ER, mitochondria come into close physical contact with the ER at the so-called mitochondria-associated membranes (MAMs). MAMs most likely mediate non-vesicle lipid exchange between both organelles. In yeast, the MCS ERMES was found to enable lipid transfer to the mitochondria and back to the ER again (Kornmann *et al.*, 2009). Interestingly, yeast lacking ERMES only display a mild lipid phenotype (Nguyen *et al.*, 2012). Later, the MCS between the mitochondria and the vacuole (vCLAMP) was discovered, which can compensate for the loss of ERMES to some extent by shuttling lipids from the ER through the vacuole to the mitochondria and vice versa (Elbaz-Alon *et al.*, 2014; Hönscher *et al.*, 2014; Klecker and Westermann, 2014), highlighting a complex interorganellar crosstalk. Consequently, both ERMES and vCLAMP facilitate lipid transfer from the ER to the mitochondria, thus guaranteeing lipid homeostasis within the mitochondria. Although mitochondria do not synthesize most of their lipids themselves, they are nevertheless supplied with them by the formation of these two MCSs.

4.2.1 ERMES – connecting the mitochondria to the ER

ERMES is involved not only in lipid transfer, but also in mitochondrial fission, mitophagy as well as in the attenuation of ER stress (Böckler and Westermann, 2014; Belgareh-Touzé *et al.*,

2017; Kawano *et al.*, 2018; Kakimoto-Takeda *et al.*, 2022; Renne and Ernst, 2024). Interestingly, ERMES also seems to influence CoQ synthesis by assembling the Coq complex in its vicinity (Eisenberg-Bord *et al.*, 2019). Loss of ERMES destabilizes the Coq complex and leads to a reduction of CoQ levels within the mitochondria (Eisenberg-Bord *et al.*, 2019). Main tethers of this MCS are Mdm34 and Mdm10 in the OMM and the integral ER membrane protein Mmm1 as well as the soluble cytosolic protein Mdm12 (Kornmann *et al.*, 2009). Due to its diverse functions, ERMES ultimately ensures mitochondrial homeostasis, allowing the mitochondria to operate efficiently. Loss of ERMES can be compensated by the direct contact of the mitochondria to the vacuole via vCLAMP.

4.2.2 vCLAMP – direct contact between vacuole and mitochondria

Areas where the mitochondria and vacuole literally embrace are named vCLAMP and represent another type of MCS. Depending on the main tethers involved, two different vCLAMPs can be distinguished: Ypt7-Vps39-Tom40 (González Montoro *et al.*, 2018) or Vps13-Mcp1 vCLAMP (Elbaz-Alon *et al.*, 2014; Hönscher *et al.*, 2014). Whereas the latter one was shown to compensate for the lack of ERMES (Eisenberg-Bord *et al.*, 2019), the other one is thought to have a similar effect (González Montoro *et al.*, 2018). Nevertheless, both of these two types of vCLAMPs are crucial for lipid and ion exchange, coordinate metabolism, and facilitate mitophagy, especially under stress conditions (Mao *et al.*, 2021; Elbaz-Alon *et al.*, 2014; Hönscher *et al.*, 2014; Du Toit, 2014; González Montoro *et al.*, 2018).

Just as vCLAMP can compensate for a loss of ERMES, ERMES can also compensate for the lack of vCLAMP as the absence of one leads to the expansion of the other. Elimination of both, however, is lethal (Elbaz-Alon *et al.*, 2014). This crosstalk not only adds another layer to the complexity of communication between organelles, as the mitochondria communicate with the ER either directly or indirectly via the vacuole, but also allows flexibility in responding to different intrinsic and extrinsic factors.

5. Conclusions and future perspectives

The goal of this study was to investigate intraorganellar connectivity during stress and aging, with aging representing a special form of stress that leads to the gradual decline of cellular processes. To perform this study, the model organism and eukaryote Baker's yeast

(*Saccharomyces cerevisiae*) was used. Despite its simple structure and the fact that yeast is a single-celled organism, it nevertheless serves as a suitable organism for studying eukaryotic features, since most cellular processes are also conserved in higher, more complex and multicellular organisms, such as humans. Many eukaryotic pathways, such as the DNA damage response (reviewed in Vanderwaeren *et al.*, 2022) as well as autophagy (Tsukada and Ohsumi, 1993), were first unveiled in yeast. Obviously, especially in respect to aging research, its short lifespan of only several weeks is also an advantageous aspect, since many different analyses from diverse angles can be carried out in a much shorter time compared to organisms with longer life expectancies.

In **paper I**, we addressed the question how two different nutritional regimes influenced both cell cycle exit and regrowth capacity. Due to nutrient exhaustion, yeast cells exit cell cycle and enter into a quiescent state in which they no longer divide but continue to live. This reversible state is characterized by stress tolerance, longevity and an extensive cellular remodeling. If environmental conditions change, yeast cells can exit this state and resume their growth (Sagot and Laporte, 2019). To be able to exit from quiescence, yeast stores glucose in form of trehalose or glycogen in the vacuole and neutral lipids in lipid droplets (LD; Shi *et al.*, 2010; Mohammad *et al.*, 2020). Different nutritional regimes lead to distinct forms of cell cycle exit and entry into stationary phase (Gasch *et al.*, 2000; Klosinska *et al.*, 2011; Conway *et al.*, 2012). Here, we showed that phosphate scarcity led to higher levels of neutral lipids in form of sterol esters and to the formation of more LDs compared to glucose exhaustion, which supported regrowth. Under nutritional stress, LDs biogenesis is generally mediated at the NVJs (Hariri *et al.*, 2018). In this study, we showed that phosphate exhaustion led to both a rapid expansion of NVJs and an increased level of NVJ tethers, which also supported LD biogenesis. A way of degrading LD and thus mobilization of stored lipids is lipophagy that was induced by glucose but not by phosphate exhaustion, as we were able to demonstrate in this study. In contrast, sterol ester synthesis was critical for survival upon phosphate but not glucose depletion. In sum, we could show that sterol metabolism differentially contributes to maintenance and exit of quiescence. Our results contribute to the understanding how different diets shape subcellular structures and how this in turn affects non-dividing yet metabolically active cells in their quiescent state and their preparation for possible regrowth. Depending on the nature of the scarced nutrient, cells develop both unique adaptation mechanisms to pause their division, and distinct prerequisites for resuming growth, underscoring the complexity of varying alimentations.

In **paper II**, the effects of aging on the structural organization of the endoplasmic reticulum (ER) and its microdomains were analyzed. To address these research questions, artificial ER membrane thickness reporters (^{GFP}WALPs) were used, based on a TMD of varying length (19 – 29 amine acids), achieved by different numbers of Ala-Leu dipeptides. These reporters also contain both a specific signal sequence (SS^{Suc2}) to facilitate ER membrane targeting and a second signal sequence (KKXX) for retention within the ER. In young cells, while reporters with short TMDs (WALP19 and WALP21) were evenly distributed throughout both the nER and the cER patches, those with long TMDs (WALP27 and WALP29) that decorate regions of increased thickness, indicative of lipid raft-like microdomains were concentrated in distinct foci at the nER. In aged cells, when yeast transitions from fermentation to respiration, reporters with short TMDs were particularly enriched at the interface between the nucleus and vacuole, reflecting the NVJs, and those with long TMDs were gradually excluded from the nER. Instead, they progressively accumulated in foci at the cER. Age-dependently, the frequency of those foci increased, meaning that aging leads to the formation of more ER microdomains.

To further study the ER membrane organization at the NVJs, cells were also equipped with Nvj1^{mCherry}, one of main tether of NVJs (Pan *et al.*, 2000). We could show, that the TMD length determines NVJ localization, independently on protein-protein or protein-lipid interaction. While, after diauxic shift, reporters with short TMD indeed accumulated at NVJs, those with long TMDs were completely excluded from them, and those with intermediate TMDs (WALP23 and WALP25) formed specific foci at the rim of the NVJs. During diauxic shift, the rim of the NVJs is linked to LD biosynthesis (Hariri *et al.*, 2018), which depends on the protein Mdm1 (Hariri *et al.*, 2019). Although some LDs colocalized with some WALP25 decorated, NVJ adjacent foci, *MDM1* deletion did not impact the formation of those foci, indicating that those foci form independently on LD biosynthesis.

Another process that is mediated at the NVJs is PMN (Roberts *et al.*, 2003; Kvam and Goldfarb, 2007; s. 2.4.1) and leads to the autophagic degradation of parts of both the nucleus and the ER and associated proteins. In line with our previous results, we could show that reporters that are excluded from NVJs are also excluded from PMN. Besides the microautophagic PMN, parts of the nuclear and ER membrane can also be degraded via specific macroautophagy, involving the autophagy receptor Atg39 (Mochida *et al.*, 2015) and termed nucleophagy. We could show, that WALP21 but not WALP29 is degraded via nucleophagy, indicating that autophagic processes in general cannot degrade lipid raft-like ER microdomains. Overall, we were able to show that partitioning of proteins into lipid raft-like ER microdomains prevents their

autophagic removal. Interestingly, this partitioning also led to an increased protein stability, as we could also demonstrate.

Since autophagy is not the only pathway that degrades ER proteins, we also analyzed the contribution of ERAD to the removal of ER membrane proteins. To this end, ERAD was genetically inactivated via deletion of the two genes encoding Ubc6 and Ubc7, on which ERAD relies depends (Xu *et al.*, 2009; Christianson and Ye, 2014; Lips *et al.*, 2020; Lemberg and Strisovsky, 2021). Here, we could show that in contrast to autophagy, ERAD removes proteins TMD-independently since all reporters were degraded.

With the second study, we provide first insights into age-dependent changes of the remodeling of the ER membrane. ER microdomains appear in the nER of young cells, but disappear when cells age and transition from fermentation to respiration. Instead, they gradually appear in the cER, thus adapting to changing anabolic demands. The ER, as the main hub for lipid and protein biosynthesis, undergoes an extensive remodeling to enable not only itself but also the entire cell to adapt to a new environment and altered requirements. The progressive appearance of ER microdomains in the cER and thus in proximity to the plasma membrane could be linked to the fact that aged cells thicken their cell wall and therefore have an increased need for proteins involved in its synthesis. These proteins are frequently associated with plasma membrane lipid rafts and their synthesis has been shown to begin in the ER membrane (Mouyna *et al.*, 2000; Bagnat *et al.*, 2000). Beyond their function in sorting proteins, MCSs have been shown to represent raft-like microdomains, such as MAMs and ER-PM MCSs (Garofalo *et al.*, 2016; Hayashi and Fujimoto, 2010; Pani *et al.*, 2008), which might get more important during aging and thus causes the rise of ER microdomains. Here, we showed that the NVJ is actually an MCS of reduced thickness.

In summary, this study demonstrated the influence of aging on the organization of the ER and how the TMD of ER proteins affects their fates, providing first insights in this respect. Nevertheless, the exact functions of the successively emerging ER microdomains, especially in the cER, remains the subject for future investigations.

Project III investigated the interplay between the proteolytic capacity of the vacuole and mitochondrial activity and abundance, as well as its influence on cell survival. Here, we could show that an increased proteolytic capacity of the vacuole led to mitochondria that functioned more efficiently and in turns to cells that displayed a prolonged lifespan. These effects were dependent on the formation of the MCS between the vacuole and the mitochondria since

deleting main components of this MCS resulted in the abolishment of the effects caused by an increased proteolytic capacity of the vacuole.

To interfere with the proteolytic capacity of the vacuole, we mostly analyzed strains that either lacked or overexpressed one of the three main proteases. Pep4 is the most important vacuolar protease yeast and not only autoactivates itself but also processes Prb1 and Prc1, the two other main vacuolar proteases in yeast, thereby activating them (Wolff *et al.*, 1996; Zubenko *et al.*, 1983; van den Hazel *et al.*, 1992). Analyzing the proteome of *PEP4* deleted strains revealed an extensive deregulation of proteins involved in monosaccharide metabolic pathways and mitochondrial processes, such as the citric acid cycle, the MRC and the ATP synthesis. Furthermore, the mitochondrial transmembrane potential was reduced and cells displayed a shorter survival time. In contrast, deletions of *PRB1* and *PRC1* did not affect the mitochondrial transmembrane potential or cell survival.

Moreover, *PEP4* deletion led to the increased production of ROS, increased glucose consumption, and decrease of ATP synthesis. Pep4 overexpression, on the other hand, increased the mitochondrial transmembrane potential, ATP synthesis, oxygen consumption and the amount of the mitochondrial protein Cit1, but decreased ROS production. Pep4 overexpression also led to an increase of CoQ as well as *COQ1*, *COQ7* and *COQ9* mRNA levels. Overall, Pep4 overexpression improved mitochondrial functionality, which also led to a prolongation of cell survival. The effects of Pep4 overexpression depended on CoQ synthesis, the HAP complex, and the mitochondrial DNA polymerase Mip1. Interestingly, Pep4 overexpression also increased the direct contact between mitochondria and the vacuole as the abundancy of vCLAMP increased. The formation of vCLAMP was essential for the effects caused by Pep4 overexpression. Prc1 overexpression showed mild beneficial effects on mitochondria and cell survival, while Prb1 overexpression only impacted cell survival to some extent.

In summary, *PEP4* deletion, thus reduced proteolytic capacity of the vacuole, results in impaired mitochondria and reduces cell survival, while Pep4 overexpression, thus increased proteolytic capacity of the vacuole, enhances mitochondrial functionality and prolongs cell survival. However, the direct link between Pep4 overexpression and mitochondrial functionality remains unclear. Either due to the increased formation of vCLAMPs or through Pep4 leaving the vacuole and acting directly on mitochondria, mitochondria could be more protected from potentially harmful material, such as oxidized proteins caused by ROS production. Since mitochondrial functionality is associated with normal aging (Sun *et al.*,

2017), improving and protecting them could result in prolonged cell survival due to a reduced decline in mitochondrial quality and activity.

All in all, despite its important contribution to our knowledge about aging processes and their impact on intracellular connectivity, this thesis raises new and interesting research questions, the answer to which are currently shrouded in mystery, but may soon be revealed.

Abbreviations

cER	cortical ER
CoQ	coenzyme Q
Cytc	cytochrome <i>c</i>
CII	complex II
CIII	complex III
CIV	complex IV
CV	complex V (ATP synthase)
ECM	extracellular matrix
ER	endoplasmic reticulum
ERAD	ER associated degradation
ERMES	ER-mitochondria encounter structure
GET	guided entry of tail-anchored proteins
GoPo	Golgi-peroxisome contact site
IMM	inner mitochondrial membrane
INM	inner nuclear membrane
LD	lipid droplet
LiDER	lipid droplet-ER contact site
MAM	mitochondria-associated membrane
MCS	membrane contact sites
MRC	mitochondrial respiratory chain
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
nER	nuclear ER
NVJ	nucleus vacuole junction
OXPHOS	oxidative phosphorylation
PAS	phagophore assembly site
pCLIP	plasma membrane-LD contact site
pER	(peri)nuclear ER
PerMit	peroxisome-mitochondria contact site
perVale	peroxisome-vacuole contact site
PMN	piecemeal microautophagy of the nucleus
RIT	regions of increased thickness
ROS	reactive oxygen species
rRNA	ribosomal RNA
RRT	regions of reduced thickness
SC	supercomplex
SND	SRP-independent
SR	SRP receptor
SRP	signal recognition particle
TA	tail-anchored
tER	tubular ER
TM	transmembrane
TMD	transmembrane domain
tRNA	transfer-RNA
UPR	unfolded protein response
UPR ^{mt}	mitochondrial unfolded protein response
UPS	ubiquitin-proteasome system
vCLAMP	vacuole and mitochondria patch
vCLIP	vacuole-LD contact site

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Und nun zu meinen Geschwistern, eine wichtige und zugleich verlässige Stütze in meinem Leben. Zuerst ist da meine große, dennoch kleinere, Schwester **Sarah**. Danke, dass du mir den pinken Bären namens Eppi damals ganz freiwillig gabst, der mich bestimmt schon auf diesen Weg im Babyalter vorbereitet hat und der immer bei mir ist, auch hier in Schweden; mittlerweile schon etwas erblast und mit einem zerkratzten Auge. Vielen Dank, dass du mich hier besuchen kamst, um mein Leben hier in Stockholm zu erleben und um auch mal mehr oder weniger gemütlich in einer Hängematte in der freien schwedischen Natur zu nächtigen. Vielen Dank, dass du mir immer beiseite stehst und das nun schon knapp 35 Jahre. Danke für deine endlose Hilfe, deine Positivität und Zuversicht, deine Stärke! Dann ist da tatsächlich meine kleinere Schwester **Samira**. Auch dir möchte ich natürlich danken, dass du immer bei mir bist

– auf 35 Jahre kommst du zwar noch nicht ganz, aber immerhin dein Leben lang – auch wenn es leider normalerweise von der Ferne aus ist. Momentan trennt uns die Ostsee, vor einigen Jahren war es allerdings noch der gesamte Atlantische Ozean. Ich bin trotzdem über alles glücklich und dankbar, dass ich dich habe und du nie über meine Arbeit sprechen magst. Vielen Dank, dass du mich eher von der Realität ablenkst und mich teilweise in die Kindheit zurückkatapultierst. Danke für dein offenes Herz, deine Freude und deinen Frohsinn, deine Heiterkeit! **Samuel**, mein Bruder, auch dir dank ich selbstverständlich. Ich bin glücklich, dass ich auch dich und deine Unterstützung stets habe. Vielen Dank, dass du mir immer so tatkräftig hilfst und danke, dass du nicht nur mir, sondern jedem aus der Familie immer so beistehst. Auf dich ist immer Verlass. Deine Ausdauer und Hilfe sind echt einmalig. Danke für deine Warmherzigkeit, Großzügigkeit und Selbstlosigkeit! Und danke, dass wir dank dir nun ein tolles Haus in Mecklenburg-Vorpommern haben. Und auch dir, mein Bruder **Silas**, wer hätte es gedacht, danke ich sehr. Vielen Dank für die stundenlangen Telefonate, die wir regelmäßig haben und dein offenes Ohr. Vielen Dank, dass du mir hilfst, meine „schlechten“ Gefühle zu kontrollieren. Danke für deine emotionale Unterstützung, deine Empathie und deinen Humor. Euch allen vier danke ich natürlich im gleichen und unendlichen Maße. Ich bin unfassbar stolz auf euch. Wir sind das beste Team: Die ProKids. Unsere Unterschiede machen uns stark, unser Zusammenhalt macht uns unbesiegtbar. Möge die Zeit unsere Bande niemals schwächen, sondern sie zu einem noch unzerstörbareren Anker schmieden. Tragt stets die Gewissheit in euch, dass ihr für mich das Zweitwertvollste seid, was diese Welt zu bieten hat.

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„Glücklich allein ist die Seele, die liebt“ (Johann Wolfgang von Goethe)