The Role of Mitochondrial Dysfunction in Neurodegenerative Proteinopathies and Aging.

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THE ROLE OF MITOCHONDRIAL DYSFUNCTION IN NEURODEGENERATIVE PROTEINOPATHIES AND AGING

By
Alejandro Ocampo

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida
May 2012
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

THE ROLE OF MITOCHONDRIAL DYSFUNCTION IN NEURODEGENERATIVE
PROTEINOPATHIES AND AGING

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Age-related neurodegenerative proteinopathies, including polyglutamine (polyQ) diseases such as Huntington’s disease, are a group of disorders in which a single protein or a set of proteins misfold and aggregate resulting in a progressive and selective loss of anatomically or physiologically related neuronal systems. Despite evidence showing a clear relationship between mitochondrial dysfunction, aging and neurodegenerative proteinopathies, the extent of the mitochondrial respiratory chain deficits, the involvement of mitochondrial dysfunction and the mechanisms responsible for these processes are largely unknown. Using yeast models of cellular aging and polyQ disorders we show that mitochondrial dysfunction is an important contributor to the process of aging and age-related neurodegenerative diseases. Preserving mitochondrial function is essential for standard wild-type aging. Enhancement of mitochondrial biogenesis ameliorates polyQ cytotoxicity and is a required component of interventions that retard the aging process.
DEDICATION

To Vane and Roucky
ACKNOWLEDGEMENTS

First, I would like to thank my mentor and friend, Dr. Antonio Barrientos, for teaching me how to think as a scientist, work hard and stay focused. Thank you Toni. I would like to thank all current and past members of my lab for all the support, help, and for creating the optimal environment to conduct my research.

I also acknowledge my dissertation committee including Dr. Abigail Hackam, Dr. Yanbin Zhang, Dr. Feng Gong and Dr. Miguel Perez-Pinzon for reviewing and enriching my research with their questions and suggestions. I would like to thank my external examiner, Dr. Valter Longo, from whom I have learned all I know about yeast aging.

I thank all the students, faculty and administrative staff of the University of Miami Department of Biochemistry and Molecular Biology, especially Dr. Richard Myers, Dr. Kenneth Rudd, Dr. Louis Elsas. I also thank Dr. Gerald S. Shadel from Yale University, Dr. Kent Lai from the University of Utah and Dr. Susan Lindquist from the Whitehead Institute for Biomedical Research.

Above all, I thank my family, my parents, my parents-in-law, and the rest of my relatives for supporting me from far away. Finally I would like to thank my wife Vanesa and my dog Roucky for their unconditional support, for believing in me, and for sharing with me this journey. I love you and thank you.
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LIST OF ABBREVIATIONS

AA: antimycin A
AD: Alzheimer’s disease
ALS: amyotrophic lateral sclerosis
ATP: adenosine triphosphate
CFU: colony formation unit assay
CLS: chronological life span
COX: cytochrome c oxidase
CR: caloric restriction
DHE: dihydroethidium
ER: endoplasmic reticulum
Exp: exponential phase of growth
FCM: flow cytometry
GFP: green fluorescent protein
HD: Huntington’s disease
Htt: huntingtin
mtDNA: mitochondrial DNA
OLI: oligomycin
OXPHOS: mitochondrial oxidative phosphorylation
PD: Parkinson’s disease
PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI: propidium iodide
PolyQ: polyglutamine
RLS: replicative life span
ROS: reactive oxygen species
SDC: synthetic complete media containing glucose
SOD: superoxide dismutases
Sta: stationary phase of growth
TMRM: tetramethyl rhodamine methyl ester
TOR: target of rapamycin
CHAPTER 1
INTRODUCTION

1.1 MITOCHONDRIAL METABOLISM AND BIOGENESIS

Mitochondria are at the center of cellular life and death. Mitochondria, frequently referred as the “powerhouses of the cell”, are double membrane cellular organelles responsible for many fundamental processes in eukaryotic cells including energy production. Under aerobic conditions, most of the ATP used by eukaryotic cells is generated through mitochondrial oxidative phosphorylation (OXPHOS). OXPHOS couples oxygen reduction by the mitochondrial respiratory chain (MRC) to ATP synthesis.

The mitochondrial respiratory chain is a set of biochemically linked enzymes (complexes I, II, III, and IV) and two electron carriers (ubiquinone/coenzyme Q and cytochrome c) (Figure 1.1). The energy stored in nutrients, in the form of electrons, is transferred through the different complexes of the mitochondrial respiratory chain to molecular oxygen generating water. The transfer of electrons is coupled to the generation of a proton gradient across the mitochondrial inner membrane which is used by the F$_1$F$_0$-ATP synthase (complex V) to drive the synthesis of ATP (Figure 1.1). Subsequently, ATP is distributed through the cell. During the transfer of electrons through the different complexes of the MRC, some electrons may escape and prematurely react with oxygen molecules generating reactive oxygen species (ROS). ROS are physiological byproducts of the mitochondrial respiratory chain and their generation increases
when the MRC malfunctions. ROS can react and damage multiple macromolecules in the cell including DNA, protein and lipids.

Figure 1.1. Mitochondrial Respiratory Chain (MRC).

Mitochondria are also the site of essential pathways of intermediate metabolism, amino acid biosynthesis, fatty acid oxidation, and steroid metabolism (Figure 1.2). In addition to these biochemical functions, mitochondria plays a key role in many other essential processes including the maintenance of calcium homeostasis (Jacobson and Duchen, 2004), redox regulation (Droge, 2002) and programmed cell death or apoptosis (reviewed in (Danial and Korsmeyer, 2004)). Impaired mitochondrial function can result in catastrophic consequences, due first to the primary loss of ATP, but also due to the secondary alteration of these other "downstream" functions leading to cell death. Therefore, mitochondria have been proposed as a central player in aging and age-related diseases including neurodegenerative proteinopathies.
Mitochondria have their own DNA (mtDNA), which encode a handful of proteins, all of them subunits of MRC enzymes. The rest of mitochondrial proteins are encoded in the nuclear DNA. Therefore, mitochondrial respiratory chain biogenesis is an intricate process that requires the coordinated assembly of multiple subunits from dual origin. For this reason, expression of most nuclear genes encoding for different components or assembly factors of the different mitochondrial respiratory complexes required for respiration is co-regulated. In the yeast *Saccharomyces cerevisiae*, the transcriptional activator complex Hap2,3,4,5 globally activates transcription of nuclear genes involved in mitochondrial respiration (Forsburg and Guarente, 1989). Likewise, in vertebrates, the transcriptional co-activator PGC-1α regulates several metabolic processes including mitochondrial biogenesis and respiration (Cui et al., 2006).

![Figure 1.2. Mitochondrial functions. Respiration and beyond.](image-url)
1.2 NEURODEGENERATIVE PROTEINOPATHIES AND MITOCHONDRIAL DYSFUNCTION

Neurodegenerative proteinopathies are age-related neurological diseases characterized by the accumulation of misfolded proteins resulting in the progressive and selective loss of anatomically or physiologically related neuronal systems. Prototypical examples in this group of diseases include Alzheimer’s disease (AD), Parkinson’s disease (PD) and Huntington’s disease (HD). Age is the major risk factor for neurodegenerative proteinopathies and as a consequence, the number of patients is expected to increase dramatically in the years to come particularly in industrialized countries where the aged population is increasing. HD belongs to a group of neurodegenerative diseases known as polyglutamine (polyQ) disorders that will be part of the focus of this dissertation. PolyQ disorders are a group of diseases caused by a CAG codon repeat expansion in disease-specific genes resulting in the expression of misfolding/aggregation-prone proteins with expanded polyQ stretches. Eight polyQ disorders have been described until now in this group including Huntington’s disease (HD), spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA) and several spinocerebellar ataxias (SCA) (Shao and Diamond, 2007).

Huntington’s disease (HD). HD is a fatal autosomal dominant neurodegenerative disease characterized by a selective loss of neurons from the striatum and deep layers of the cortex (Vonsattel et al., 1985). HD affects approximately 1 per 10,000 people with typical symptoms including abnormal involuntary movements, lack of coordination, cognitive dysfunction and changes
in personality. Most of these diseases are dominantly inherited and their onset and severity correlate with the length of abnormal polyQ sequences (Gusella and MacDonald, 1998). In HD, the CAG expansion occurs in the exon 1 of the IT15 gene, which encodes for the protein huntingtin (htt) (Kauffman et al., 2003). The HD mutation results in the expansion of an N-terminal polyQ stretch above 36 repeats (Vonsattel et al., 1985).

The understanding of HD pathogenic mechanism/s has been delayed by the difficulty of establishing the normal htt function. Huntingtin is a 348 kDa protein restricted to vertebrates which shares very little homology to other known proteins and is localized in many subcellular compartments, including the cell body, nucleus, dendrites and nerve terminations of neurons (reviewed in (Li and Li, 2004)). Htt has been found associated with several organelles including the endoplasmic reticulum, Golgi apparatus and mitochondria (reviewed in (Harjes and Wanker, 2003)). The role of htt is likely to be at a number of cellular levels (Harjes and Wanker, 2003; Landles and Bates, 2004; Li and Li, 2004). For example, wild type htt forms part of the dynactin complex (Engelender et al., 1997) colocalizing with microtubules and interacting directly with tubulin (Gauthier et al., 2004; Hoffner et al., 2002) and it could play a role in vesicle trafficking and/or cytoskeletal anchoring. Htt has also been shown to play a role in clathrin-mediated endocytosis, neuronal transport and postsynaptic signaling (reviewed in (Harjes and Wanker, 2003; Li and Li, 2004)).

Neuronal degeneration in HD is probably the result of the combination of a gain-of-function mutation resulting from the mutant htt polyQ expansion, along
with a loss of function of the wild type protein. The essential role of htt has been demonstrated in knockout mice models. In these mice, the absence of htt causes cell degeneration, aberrant brain development and embryonic lethality at day 7.5 (Murphy et al., 2000). The conditional deletion of the gene in the forebrain also leads to neurodegeneration (Dragatsis et al., 2000) suggesting htt plays a role in cell survival.

Mutant htt polypeptides with expanded polyQ acquire an unusual conformation which produces cell toxicity and facilitates their aggregation into intracellular inclusion bodies (DiFiglia et al., 1997; Scherzinger et al., 1997). It is still controversial whether or not the aggregates contribute to the pathogenic mechanism of the disease. In the YAC128 mouse models of the disease which contain the full-length human htt with a 128Q tract both behavioral and neuropathological changes including neuronal loss are observed prior to htt inclusion formation, thus suggesting that inclusions are not the trigger in the initiation of neuronal loss (Slow et al., 2005; Slow et al., 2003). The aggregates located in the nucleus and in the cytoplasm could still be pathogenic in later stages and cause cell toxicity by sequestering other proteins, disturbing organelle integrity, impairing gene transcription directly or by sequestering transcription factors, and interfering with some enzymatic activities (Davies et al., 1997; Sanchez et al., 2003). In some instances, the aggregates may have a protective role by trapping mutant htt thereby reducing their toxicity (Arrasate et al., 2004; Saudou et al., 1998). The idea that the misfolded htt monomers and oligomers or small aggregates are toxic before assembling into large macromolecular
inclusions is the currently favored hypothesis ((Nagai et al., 2007) and reviewed in (Shao and Diamond, 2007)). Mutant htt is cleaved both in vivo and in vitro (Lunkes et al., 2002; Wellington et al., 2002) to form N-terminal fragments containing polyQ repeats. It is believed that these fragments, including approximately the first 150 residues of htt encoded in exon 1 of the HD gene, are actually the toxic forms of the protein. Taking this into account, many laboratories have modeled HD pathogenesis by expressing exon 1 fragments, which cause toxicity and promote protein misfolding and aggregation in vivo and in cell models (reviewed in (Landles and Bates, 2004; Li and Li, 2004)).

Impaired mitochondrial energy production can result in catastrophic consequences, due first to the primary loss of ATP, but also due to the secondary alteration of these other "downstream" functions leading to cell death as mentioned earlier. For this reason, mitochondrial dysfunction has been considered an attractive candidate for an "executioner's" role in neuronal degeneration (reviewed in (Schon and Manfredi, 2003)). However, whether the contribution of mitochondrial dysfunction to the pathogenesis of such neurodegenerative diseases is primary or secondary is not yet fully understood. Evidence of mitochondrial dysfunction associated with HD pathogenesis has accumulated over the last 30 years. Recent findings suggest that mutant htt may damage neurons by directly interfering with mitochondrial function. Specifically, mitochondrial calcium homeostasis defects have been detected in HD patients as a direct mutant polyQ effect (Panov et al., 2002). By electron microscopy, Panov et al. identified N-terminal mutant htt on neuronal mitochondrial membranes
(Panov et al., 2002). Moreover, by incubating normal mitochondria with a fusion protein containing an abnormally long polyQ repeat, they reproduced the mitochondrial calcium defect seen in human patients and transgenic animals (Panov et al., 2003; Panov et al., 2002). Determination of in situ mitochondrial respiratory function in intact stimulated HD striatal neurons from several mouse models confirmed the alteration in mitochondrial calcium handling (Oliveira et al., 2007). Indirect evidence from several sources also indicates that a defect in energy metabolism and consequent excitotoxicity could be involved in HD (Calabresi et al., 2001; Gu et al., 1996). Toxin models of HD have been induced by 3-nitropropionic acid or malonate, both inhibitors of mitochondrial respiratory chain complex II (Brouillet et al., 1995; Garcia et al., 2002; McGeer and McGeer, 1976; Ruan et al., 2004). In HD patients, the caudate and putamen have severe deficiencies in complexes II and III (Browne et al., 1997; Gu et al., 1996; Tabrizi et al., 1999). Expression of two complex II subunits is decreased in the striatum of HD patients compared with controls, and thus affecting the activity of the complex (Benchoua et al., 2006). Similar results were reported in cultured striatal neurons expressing 82Q. In this model, the over-expression of either Ip or Fp subunit restored complex II levels and blocked striatal cell death induced by 82Q (Benchoua et al., 2006). These data support a model of HD pathogenesis involving alterations in mitochondrial physiology and aerobic energy production.

Despite evidence in support of mitochondrial involvement in the pathogenesis of HD, the exact mechanism by which mutated htt could cause bioenergetic dysfunction is still unknown. Several hypotheses have been
proposed. 1- Misfolded/aggregated polyQ could directly interact with mitochondrial membranes altering their permeability and producing mitochondrial calcium homeostasis defects (Panov et al., 2003; Panov et al., 2002). 2- Misfolded/aggregated htt could also block the activity of several polyQ-containing critical transcription factors thought to play a role in regulating the expression of proteins involved in mitochondrial energy metabolism and in mitochondrion-initiated apoptosis such as cAMP-responsive element–binding (CREB) protein, Sp1 and p53 (McCampbell et al., 2000; Nucifora et al., 2001). Mutant htt has been shown to alter mitochondrial function by directly inhibiting expression of the transcriptional co-activator PGC-1α which regulates mitochondrial biogenesis and respiration (Cui et al., 2006; Weydt et al., 2006). Mutant htt represses PGC-1α gene transcription by interacting with the promoter and interfering with the CREB/TAF4-dependent transcriptional pathway critical for PGC-1α gene expression regulation (Cui et al., 2006), thus establishing a link between transcriptional deregulation and altered energy metabolism in HD pathogenesis.

3- Expression of full-length mutant htt impairs vesicular and mitochondrial trafficking in mammalian neurons in vitro and in vivo, probably by disrupting the microtubule and actin cytoskeletal networks (Trushina et al., 2004). These defects occurred early in development prior to the onset of measurable neurological or mitochondrial abnormalities (Trushina et al., 2004) suggesting a mechanism of neuronal dysfunction in HD in which mutant htt misfolding/aggregation leads to cytoskeleton disruption and eventually alters mitochondrial morphology and distribution, disturbing its function and promoting
neuronal death. In summary, although many different mechanisms have been involved in HD pathogenesis, the crucial initiation mechanism induced by mutant polyQ misfolding/aggregation is still unclear. A large body of evidence supports an early and critical involvement of defects in mitochondrial function and energy metabolism in the pathogenesis of the disease but whether this role is primary or secondary still remains undecided (reviewed in (Browne and Beal, 2006)).

1.3 AGING AND MITOCHONDRIAL DYSFUNCTION

Biological aging can be defined as the progressive decline in the ability of a cell or an organism to resist stress, damage or disease and is consider a major risk factor for many of the diseases affecting modern societies including cardiovascular conditions, cancer and neurodegenerative diseases. Due to its important value from a medical point of view, understanding the process of aging has become one of the biggest challenges of biomedical sciences in the last decades.

Among the multiple theories proposed to explain the process of aging, the free radical/mitochondrial theory of aging proposed by Harman (Harman, 1956, 1972) is one of the most widely accepted nowadays. According to this theory, the cumulative oxidative damage to cellular macromolecules such as DNA, protein and lipids produced by reactive oxygen species (ROS) during the course of life will ultimately lead to the aging process. In addition to its central role as power source in the cell, mitochondrial respiratory chain is also the major source of reactive oxygen species in the cell and therefore the vicious cycle between mitochondrial dysfunction and ROS production is considered to play a major role
during aging and disease (Figure 1.3) (Harman, 1972). Although eukaryotic cells have developed during the course of evolution, defense mechanisms that include enzymatic systems such as catalase, glutathione reductase, glutathione peroxidase and superoxide dismutases (SOD) to convert ROS into non-toxic forms, these cellular systems, which activity decreases during age, are overwhelm during the course of life leading to a net accumulation of oxidative damage.

In agreement with the free radical/mitochondrial theory of aging, altered mitochondrial function is a common feature in multiple aspects of aging (Lin and Beal, 2006) and reduced respiratory capacity and increased oxidative stress associated with age has been reported in several brain regions (Lin and Beal, 2006). Several signaling pathways to and from mitochondria serve to communicate functional status to the nucleus to impact cellular function by modulating mitochondrial biogenesis (Jazwinski, 2005; Schieke and Finkel,
Transcriptional profiling studies have shown that the expression of genes involved in mitochondrial energy metabolism declines with age in worm and flies (McCarroll et al., 2004), mouse and human (Zahn et al., 2006), suggesting that it may be a common marker for aging across species.

Despite the wide popularity of the free radical theory of aging, evidence from studies on diverse model organisms supporting and contradicting its postulates continues to accumulate. Over-expression of SOD in Drosophila melanogaster was shown to have beneficial effects and extend life span (Sun et al., 2002). Similarly, mice over-expressing a mitochondrial-targeted catalase, not only showed increase life span, but also presented a reduction in ROS levels and oxidative damage and age-associated pathologies (Schriner et al., 2005). On the other hand, mice expressing a mitochondrial polymerase deficient in proof-reading activity, accumulated mitochondrial mutations and displayed premature aging, but without increase in ROS generation (Trifunovic and Larsson, 2008). Moreover, deletion of the mitochondrial superoxide dismutase (SOD2) in Caenorhabditis elegans extended life span and increased in ROS generation (Van Raamsdonk and Hekimi, 2009). According to these new studies and on the contrary to the expected negative effect, low levels of mitochondrial ROS, may actually have beneficial effects acting as signal molecules that promote cellular states of higher stress resistance and extend life span, a concept know as mitohormesis (Ristow and Schmeisser, 2011). Mitochondrial ROS have also recently been showed as signaling mechanism that extends yeast chronological life span upon reduction of TOR signaling (Pan et al., 2011) and a mild increase
in ROS has been reported in long-lived flies and worms carrying mutations that cause partial disruption of the mitochondrial respiratory chain (Miwa et al., 2004; Rea, 2005). Overall, these studies suggest that although oxidative damage is an important contributor, the relation between aging and ROS remains complex.

Despite the fact that the average life span has increase significantly in the last century mainly due to the advances in preventive and therapeutic medicine, the maximal life span stays unchanged. Research efforts have been devoted to strategies for longevity extension, regarded as a retardation of biological aging. Although longevity extension strategies are not expected to eliminate aging-related diseases, they are expected to postpone their age of onset, thus contributing to the objective of extending health-span (Colman et al., 2009). Caloric restriction (CR) is the most robust environmental intervention known to slow aging and extend life span in yeast, worms, fruit flies, rodents and primates through conserved mechanisms (Colman et al., 2009; Wei et al., 2008). Studies to understand the molecular mechanisms of CR mediated longevity, allowed for the identification of several longevity genes. In yeast, the conserved Ras/cAMP/PKA, Tor and Sch9 signaling pathways integrate the nutrient and other environmental cues to regulate cell growth, division and life span (Bitterman et al., 2003; Wei et al., 2008). Deletion of RAS2, TOR1 and SCH9 enhances cellular protection against thermal and oxidative stresses and extends yeast chronological life span (Longo and Finch, 2003). Inhibition of these pathways converges on the activation of stress resistance transcription factors that will induce the expression of heat shock proteins and antioxidant defenses.
(e.g. catalase and superoxide dismutase - SOD2). Over-expression of cytoplasmic SOD1 (5% localizes to mitochondria) and mitochondrial SOD2 were among the first genes identified to extend chronological life span in yeast (Longo et al., 1996).

A significant portion of the CR effects on longevity seem to be mediated through the down-regulation of Tor, Sch9 (Akt1) and Ras signaling, thus increasing resistance to ROS and other stressors (Wei et al., 2008). Additionally, regulators of energy metabolism may play a key role in the mechanism of CR by inducing, metabolic reprogramming (Anderson and Weindruch, 2007). Growing evidence suggest that the longevity pathways play an important role in the regulation of mitochondrial biogenesis. Deletion of the TOR1 gene could extend chronological life span in S. cerevisiae primarily by increasing mitochondrial mass, respiration and ROS production (Bonawitz et al., 2007; Pan et al., 2011). The Ras/cAMP/PKA pathway senses excessive ROS to signal to the Hap2,3,4,5 transcriptional system and down-regulate mitochondrial biogenesis (Chevtzoff et al., 2009). Also in mammals, modulation of mitochondrial biogenesis and metabolism through the Tor, Akt1 and Ras pathways involves the transcriptional co-activator PGC-1α (Anderson and Prolla, 2009). PGC-1α transcriptional activity seems to be induced in the oxidative stress response and CR through a shared mechanism, suggesting that in mammals, regulation of mitochondrial function is a key element in both cellular survival and longevity (Anderson and Prolla, 2009).
1.4 YEAST MODELS OF NEURODEGENERATION AND AGING

The yeast *Saccharomyces cerevisiae*, also known as baker’s or budding yeast, is the most extensively studied eukaryotic organism (Figure 1.4). *S. cerevisiae* is an excellent single cell model organism because despite their simplicity yeast cells have similarities to higher eukaryotes (Botstein, 1991). Cellular activities conserved from yeast to humans include DNA replication, recombination and repair, RNA transcription and translation, intracellular trafficking, enzymatic activities of general metabolism, and mitochondria biogenesis (Foury and Kucej, 2002).

![Figure 1.4. Yeast Saccharomyces cerevisiae.](image)

*S. cerevisiae* is a facultative aerobe/anaerobe yeast that can produce ATP through two mechanisms. When glucose is present, glycolysis is activated to make ATP, while gluconeogenesis and mitochondrial respiration are repressed. When fermentable carbon sources are not available, the cell resets to oxidative phosphorylation for the production of ATP. Experimentally, *S. cerevisiae* metabolism can be manipulated simply by changes in culture conditions allowing
for a convenient selection of respiratory defective mutants (reviewed in (Barrientos, 2003)). These properties make the yeast models especially appropriated for the study of mitochondrial function alterations involved in neurodegeneration and aging. At a practical level, S. cerevisiae possesses many other characteristics that make it especially useful as a model system in the laboratory, such as its ability to reproduce quickly and grow under a wide variety of conditions. The availability of both haploid and diploid yeast, and the possibility to physically separate and identify all four haploid cells from a single meiotic event using tetrad analysis, have made yeast an invaluable organism for genetic manipulations. In addition, the creation of genetic knockouts requires simple technology. As a result of these advantages, S. cerevisiae was the first eukaryote organism to be fully sequenced in 1996 (Goffeau et al., 1996). Finally, a whole array of biological and bioinformatics tools is today available for researchers to work with yeast in different scientific areas, making it a perfect candidate as a model organism. S. cerevisiae not only serves as an important experimental organism for revealing gene function but it has also a great impact in establishing the molecular mechanisms of human diseases and aging (Barrientos, 2003; Fontana et al., 2010; Foury, 1997).

1.4.1 Yeast models of neurodegeneration

Although yeast cells lack many structural and functional hallmarks of neuronal cells, the basic mechanism and pathways involved in neurodegenerative proteinopathies, such as mitochondrial dysfunction, transcriptional dysregulation, trafficking defects and proteasomal impairment, are
highly conserve from yeast to humans. Therefore, in recent years, yeast models for multiple neurodegenerative disorders have been established. When modeling a human neurodegenerative disease in yeast, two main approaches are generally followed, depending on whether the protein or proteins potentially responsible for the disease have a yeast homologue. In the case that a yeast homologue exists, the yeast gene/s can be disrupted, mutated, replaced by the human gene or overexpressed to evaluate the phenotype of the loss or gain of function. This approach has been successfully used, for example, in studies of the yeast \textit{YHF1} and \textit{SOD1} which human homologs are involved in Friedreich’s ataxia (Puccio and Koenig, 2000) and amyotrophic lateral sclerosis (ALS) (Leitch et al., 2009). Instead, if the human gene has no clear or potential yeast homolog and the disease results at least in part from a “gain of function” of the protein involved, the strategy used to create a yeast model involves the heterologous overexpression of the wild type or mutant gene/s. In this line, yeast models of the most common neurodegenerative proteinopathies such as AD, HD and PD caused by gain of function of a particular protein have been generated (Figure 1.5) (Caine et al., 2007; Chen et al., 2005; Dixon et al., 2005; Giorgini et al., 2005; Krobisch and Lindquist, 2000; Meriin et al., 2002; Middendorp et al., 2004; Outeiro and Lindquist, 2003) These models, that are based on the overexpression of one or several proteins, recapitulate the crucial events preceding cell death that manifest during the course of the human disorder, including protein misfolding and aggregation, consequently they have been
highly useful in the elucidation of basic cellular mechanisms of toxicity triggered by human neurotoxic proteins.

**Figure 1.5. Yeast models of polyQ diseases and α-synucleinopathies.** Visualization of cells expressing 103Q domains or α-Synuclein fused to green fluorescence protein (GFP) (Ocampo and Barrientos, 2011a).

Yeast models for polyQ disorders, particularly HD, have been thoroughly investigated in mitotic cells (Tenreiro and Outeiro, 2010). Although there is no huntingtin homolog in yeast, heterologous expression of htt exon I fragments comprising the polyQ stretches faithfully recapitulates htt misfolding/aggregation and reproduces many of the cellular and molecular features of HD pathology in patients. As an example, yeast cells expressing mutant htt display aggregation and toxicity in a polyQ length-dependent manner (Krobitsch and Lindquist, 2000; Meriin et al., 2002). Additionally, yeast polyQ expression affects several pathways that are known to be affected in HD patients, including endocytosis (Meriin et al., 2003), transcription (Hughes et al., 2001), mitochondrial function (Solans et al., 2006), oxidative stress (Giorgini et al., 2005), ER stress (Duennwald and Lindquist, 2008) and cytoskeletal disturbances (Solans et al., 2006) that contribute to growth arrest and cell death (Braun et al., 2009).
Concerning the objective of this project, the yeast model of HD can be considered an excellent model to study the role of mitochondrial dysfunction in neurodegenerative proteinopathies. In the first paper published by our lab on this subject, it was shown that expression of the first 17aa of exon 1 of huntingtin with a mutant polyQ tract in yeast produces a decrease in respiration as a consequence of the alteration to mitochondrial respiratory chain complex II+III (Solans et al., 2006) in congruency with data obtained from postmortem brain tissue of HD patients and toxin models. Additionally, an increase in ROS production and actin cytoskeleton alterations, which additionally altered mitochondrial distribution, were also observed (Solans et al., 2006).

Studies performed using S. cerevisiae models of polyQ diseases allowed to establish, among other things, that aggregation prone proteins, such as yeast prions or proteins containing Q/N-rich regions are enhancers of polyQ toxicity (Meriin et al., 2002). In the other hand, expression of chaperones, specific proteins that help in protein folding, such as member of the Hsp40 or Hsp70 family can modulate mutant polyQ and aggregation (Krobitsch and Lindquist, 2000; Willingham et al., 2003). In addition, genetic and drug screens have allowed the identification of genes that enhance or reduce mutant polyQ toxicity, such as protein involve in the kynurenine pathway for tryptophan degradation, vesicular transport, active microtubule-mediated transport, vacuolar degradation and transcription (Willingham et al., 2003) (Giorgini et al., 2005)and the identification of small-molecule inhibitors of polyQ aggregation(Zhang et al., 2005). In conclusion, we can consider yeast as an excellent tool to study the role
neurodegenerative proteinopathies and the role of mitochondrial dysfunction in polyQ disease.

1.4.2 Yeast models of aging

The budding yeast *S. cerevisiae* is one of the most important model organisms used in aging research, according to the basic information on aging and longevity that has provided so far. In addition to the characteristics already mentioned earlier, the relatively short life span of yeast cells and the ease with which it can be measured make yeast especially attractive and convenient to study the aging process. In yeast, two models of cellular aging have been established; replicative life span (RLS) and a chronological life span (CLS) (Figure 1.6).

![Diagram of yeast replicative (RLS) and chronological aging (CLS)](Image)

Figure 1.6. Schematic for yeast replicative (RLS) and chronological aging (CLS) (Kaeberlein et al., 2007).
RLS is defined as the number of daughter cells produced by a single mother cell before senescence, and it has been proposed to resemble the aging process of mitotically active cells in multicellular organisms. Studies of yeast RLS have lead to the discovery of conserved longevity factors, including the sirtuins, conserved NAD-dependent histone deacetylases (Kaeberlein et al., 1999) as well as pharmacological and environmental interventions that extend life span such as resveratrol (Howitz et al., 2003) and caloric restriction (Lin et al., 2002).

On the other hand, CLS is measured as the capacity of stationary (G0) cultures to maintain viability over time (1 to several weeks) and it has been proposed as a model of aging of post-mitotic cells such as neurons (Fabrizio and Longo, 2007). In the research work presented in this dissertation, we have focused on the aging process affecting non-dividing cells where the expected dependence on mitochondrial respiration and concomitant ROS production highly resemble neuronal aging. It has been established that the lack of mitochondrial respiration severely affects CLS (Aerts et al., 2009) but not RLS (Woo and Poyton, 2009) where constant nutrient supply allow cells to survive in the absence of functional mitochondria. Therefore, we selected CLS as the most appropriate model to study the role of mitochondrial dysfunction on aging and age-related proteinopathies.

For standard CLS assays, the cells will be grown in synthetic media containing glucose and allowed to reach the stationary phase. Under these conditions, the cells initially ferment the glucose and produce ethanol, which accumulates in the extracellular environment. After glucose depletion, the cells
will undergo a phase known as diauxic shift where mitochondrial function and respiration will join fermentation as sources of energy for the rest of their survival. Subsequently, survival in stationary phase will be monitored at different times and a survival curve will be generated. The use of CLS as a model of aging have also allowed the identification of novel conserved longevity factors that have been shown to be relevant for the aging process of higher organisms (Fontana et al., 2010). Nutrient-sensing pathways have been demonstrated to be key elements in the process of aging and a clear balance between growth and stress resistance seems to modulate the life span of all organisms. In the presence of nutrients, nutrient sensor pathways will promote cellular growth at the cost of lower cellular defenses therefore limiting life span. On the other hand, caloric restriction will downregulate nutrient sensor pathways and increase stress resistance with the consequent life span extension establishing the basic postulates for all known life extension mechanism.

Reduced activity of two major sensor pathways, mentioned earlier in this introduction, seem to mediate most of the effect of dietary restriction and extend both types of yeast life span (Figure 1.7) (Wei et al., 2008). First, the conserved target of rapamycin (TOR) pathway has been showed to play a fundamental role in the aging process. Deletion or downregulation of the conserved TOR1 or SCH9 genes, can increase stress resistance through the upregulation of different stress resistance transcription factors and extend life span in several organism from yeast to mice (Fabrizio et al., 2001). The second conserved nutrient sensor pathway includes RAS2, adenilate cyclase (AC) and protein kinase A (PKA) that
once again required activation of the transcription factors (MSN2 and MSN4) that control cellular protection to extend life span (Wei et al., 2008).

1.5 CONCLUDING REMARKS AND OPEN QUESTIONS

Evidence for the role of mitochondrial dysfunction on aging and age-related disease such as neurodegenerative proteinopathies has accumulated during the last decades. Mitochondria have been suggested to play a fundamental role in these processes due to their functions in cellular aerobic energy and reactive oxygen species (ROS) generation as well as their involvement in cell death. However, currently, it is not completely clear whether
mitochondrial respiratory dysfunction and ROS accumulation play a primary or secondary role in aging and age-related diseases and whether they are the cause or the consequence of the aging and neurodegenerative processes. We have used several yeast models of neurodegenerative proteinopathies and aging to study the extent and relevance of mitochondrial dysfunction on polyQ-induced cytotoxicity and chronological life span. We have studied the mechanism by which mutant polyQ domains and aging affect normal mitochondrial function and evaluated the effect of an enhancement of mitochondrial biogenesis and respiratory function as potential therapeutic strategies to ameliorate the outcome of polyQ diseases and delay the process of aging.
CHAPTER 2
FROM THE BAKERY TO BRAIN BUSINESS: DEVELOPING INDUCIBLE YEAST MODELS OF HUMAN NEURODEGENERATIVE DISORDERS

2.1 SUMMARY

In the last decade, the budding yeast Saccharomyces cerevisiae has been used as a model system to study the mechanisms of the human aging process and of age-associated neurodegenerative disorders such as Parkinson’s, Huntington's, Alzheimer's, and amyotrophic lateral sclerosis. S. cerevisiae is a facultative aerobic, unicellular yeast, and despite their simplicity, yeast cells possess most of the same basic cellular machinery as neurons in the brain, including pathways required for protein homeostasis and energy metabolism. The power of yeast genetics and the use of high-throughput screening technologies have provided important clues concerning the pathophysiology of these disorders and the identification of candidate therapeutic targets and drugs. The yeast models are based on the expression of human disease proteins in yeast and recapitulate some of the cytotoxic features observed in patients. However, the currently available models mostly suffer from high-level protein expression that results in acute cytotoxicity, and from metabolic constraints when the models are based on extensively used, strong, galactose-inducible promoters. The models would increase their significance if they were based on continuous and tightly regulated gene expression systems for both activation and levels of expression. This would allow for more chronic cytotoxicity that better simulates the timing of events that occur during disease progression.
Additionally, the use of metabolism-independent inducers would allow for the study of cell toxicities under conditions where the cells are forced to exclusively respire, thus more reliably modeling the highly oxidative neuronal metabolism. Here we have constructed yeast models of Huntington’s disease based on the expression, under the control of different promoters, of the first exon of the huntingtin-containing polyglutamine tracts of both wild-type and mutant lengths. The different models are compared and evaluated.

### 2.2 Introductory Remarks

The unicellular yeast *Saccharomyces cerevisiae* has an extensive history of use in the areas of biotechnology and biomedicine. Known as baker's yeast or brewer's yeast, this organism has been used for centuries as leavening for bread and as a fermenter of alcoholic beverages (Samuel, 1996). More recently, *S. cerevisiae* has been used as a valuable organism for studying the principles of microbiology, characterizing biochemical pathways and understanding the biology of more complex eukaryotic organisms (Botstein, 1991). Cellular activities conserved from yeast to humans include DNA replication, recombination and repair, RNA transcription and translation, intracellular trafficking, enzymatic activities of general metabolism and mitochondrial biogenesis (reviewed in (Barrientos, 2003)).

Yeast has some properties that make it particularly suitable for biological studies, including rapid growth, the ability to be maintained in a haploid or diploid state, ease of mutant isolation, a well-defined genetic system, and a highly
versatile DNA transformation system. Unlike many other microorganisms, \textit{S. cerevisiae} is viable with numerous markers. Finally, being nonpathogenic, yeast can be handled with little precautions (Barrientos, 2003). \textit{S. cerevisiae} was the first eukaryotic organism whose DNA sequence was completely known (Goffeau et al., 1996). Over the last 10 years, this knowledge fueled the use of this yeast as a model organism for studying the pathophysiology of human diseases. Recently, yeast was used to model the human aging process and complex neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Parkinson’s disease (PD) and Huntington’s disease (HD) (reviewed in (Miller-Fleming et al., 2008)). In humans, these neurodegenerative disorders are characterized by the progressive, selective loss of neurons in different areas of the brain associated with the misfolding of disease-specific proteins. Although obviously yeast cells are less complex than human neurons, basic metabolic pathways involved in neurodegeneration are well conserved in \textit{S. cerevisiae}.

To be useful and significant, a yeast model of a particular disease must recapitulate the crucial events preceding cell death that are manifested during the course of the human disorder. Two basic strategies are usually followed in the construction of yeast models of human diseases depending on genetic and pathophysiological constraints. In most cases, human disorders results from a lost of function of the disease gene encoded protein. In these cases, when the human disease gene is conserved from yeast to human, functional complementation studies will determine whether the human disease gene product is able to partially or fully replace the function of the yeast gene product.
If complementation occurs, human disease gene mutant alleles are expressed in yeast and tested for functionality as for mutations in the Cu-Zn superoxide dismutase gene responsible for ALS (Gunther et al., 2004). If complementation does not occur, the disease mutations, frequently involving conserved protein residues, are alternatively introduced in the yeast protein and subsequently analyzed as reported for mutations in the adenine nucleotide translocator (ANT1) responsible for cases of external progressive ophthalmoplegia (Fontanesi et al., 2004).

In age-associated neurodegenerative disorders such as PD or HD, the human disease genes are restricted to vertebrates. In these diseases however, pathogenicity is believed to greatly result from a gain of function of the disease mutant protein. Mutant forms of the proteins huntingtin (h\(t\)) and \(\alpha\)-synuclein, responsible for HD and some familiar forms of PD, respectively, undergo misfolding and damage several cellular structures, which leads to cell death. Yeast models of these disorders are constructed by expressing the human gene in yeast, providing paradigms in which the effect of the function gained by the disease gene mutant allele on the cellular physiology and metabolism can be conveniently studied. Here we will focus on describing the construction of yeast models of this type of disorders.

A valuable yeast model of a gain-of-function neurodegenerative disease based on the heterologous expression of a human gene is defined by several critical properties. The expression of the gene must recapitulate essential cytotoxic events that appear during the course of the disease leading to a
particular cellular phenotype that can be conveniently studied. Importantly, gene expression must be tightly regulated for both activation and levels of expression. Regulation is achieved by placing gene expression under the control of the appropriate promoters. Although models constructed with constitutive promoters (expression always on) are reported in the literature, their use is not recommended. In these models, upon yeast transformation to construct the yeast model, the expression of a toxic protein can result in either cell death prior to the formation of a yeast colony or in the generation of suppressor mutations in a subset of cells making them competent to grow in the presence of toxic proteins, a situation that will adulterate the models. To achieve a regulated gene expression researchers have used several natural or artificially modified inducible promoters that are turned either on or off upon addition or subtraction of a particular inducer/repressor.

To date, most inducible yeast models of neurodegenerative diseases have been created by heterologous expression of human genes under the control of the strong \textit{GAL1} promoter, which is activated by galactose and repressed by glucose. Although these models have provided a significant amount of information, generally they suffer from a lack of regulation of expression level: expression is usually high, which makes toxicity far too acute as compared to the toxicity produced by the slow accumulation of mutant proteins observed in neurons from human patients. The expression of the toxic peptides can be so harmful for the cell that it can have a tendency to eliminate the plasmid expressing the mutated protein when episomal vectors are used. This problem
that can be solved by integrating the engineered gene into the yeast chromosomal DNA. We recognize that high expression of mutant proteins producing acute toxic effects can save time and be effective when screening for drugs or genetic suppressors of cytotoxicity, but they are probably not the ideal system for analyzing metabolic or physiological disturbances leading to cytotoxicity. Additionally, gene expression under the control of a galactose inducible promoter introduces a metabolic constraint. *S. cerevisiae* is a facultative aerobe/anaerobe. It can produce energy by two different mechanisms (fermentation or respiration) depending on the available carbon sources and oxygen accessibility. In all reported models, expression is induced upon transferring the cells to media containing 2% galactose, which is a fermentable substrate. The use of galactose-inducible promoters represents a significant disadvantage when the metabolic changes associated to switches in carbon source are relevant to the study. For example, the use of metabolism-independent inducers would allow for the study of cell toxicities under conditions where the cells are forced to exclusively respire, creating a better model of the highly oxidative neuronal metabolism. In addition, the use of metabolic-dependent inducers in the way mentioned above prevents studies in non-dividing postmitotic cells using the yeast stationary phase model of aging (Sinclair et al., 1998). In this post-mitotic state, energetic dependence on mitochondrial respiration and concomitant free radicals of oxygen ROS production highly resembles the situation in which neuronal cells age.
We have decided to create refined inducible yeast models of neurodegenerative disorders to increase their usefulness and relevance. We chose to start by creating refined models of Huntington’s disease (HD), a genetic disorder characterized by a selective neurodegeneration of the striatum and deep layers of the cerebral cortex (Vonsattel et al., 1985). HD is caused by the expansion of a polyglutamine (polyQ) tract in huntingtin (htt). Here we have constructed yeast models of HD based on the expression of the polyQ domains of normal and pathological length under the control of different promoters, including the CUP1 promoter, two different β-estradiol-inducible GAL1 promoter systems and the GAL1 promoter in a Δgal1 mutant background. The different models are evaluated and compared.

2.3 MATERIALS AND METHODS

2.3.1 Yeast strains, media and growth conditions.

All experiments were performed with strains isogenic to the haploid reference strain W303-1A (MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15). In the Δgal1 mutant, the GAL1 gene was replaced with the kanMX deletion cassette.

Cells were cultured in either standard rich media YPRAF (2% raffinose, 1% yeast extract and 2% bactopeptone), YPD (2% dextrose, 1% yeast extract and 2% bactopeptone) and YPG (2% glycerol, 1% yeast extract and 2% bactopeptone) or synthetic minimal medium (0.67% yeast nitrogen base
containing the required amino acids). Geneticin-resistant cells were grown on YPD plates containing 200 mg/L of geneticin.

Routinely, polyQ expression in yeast cells growing in YPRAF was induced for 6 and 24 h by supplementing the media with different concentrations of the appropriate inducers. Cells expressing polyQ constructs under the control of the \textit{CUP1} promoter were grown in the presence of 0 and 2 mM CuSO$_4$. Cells expressing polyQ-GFP under the control of the $\beta$-estradiol-inducible \textit{GAL1} promoter were grown in the presence of 0, 10 and 50 nM $\beta$-estradiol (Tokyo Chemical Industry, Tokyo, Japan). Finally, cells expressing polyQ-GFP under the control of the \textit{GAL1} promoter were grown in the presence of 0%, 0.01% and 0.1% galactose.

\textbf{2.3.2 Plasmid construction and transformation.}

As mentioned above, HD mutations result in an expansion of an N-terminal polyQ domain in htt (encoded in the first exon of the gene) from 6-35 glutamines to more than 36 repeats. Mutant htt is cleaved both \textit{in vivo} and \textit{in vitro} to form N-terminal fragments containing polyQ repeats. It is the general understanding that these fragments are actually the toxic forms of the protein. Although the exact identity of these cleavage fragments is not known, they appear to include approximately the first 150 residues of htt. For this reason, many laboratories, including ours, have modeled HD pathogenesis by expression of exon 1 fragments, which cause toxicity and promote protein misfolding/aggregation \textit{in vivo} and in cell models (reviewed in (Landles and Bates, 2004; Li and Li, 2004)). Two polyQ constructs (25Q and 103Q in the
plasmid pYES2) previously described (Meriin et al., 2002) were obtained from Dr. Michael Y. Sherman (Boston University, Boston, MA). In brief, exon 1 sequences containing the first 17 amino acids followed by 25 or 103 glutamines were fused in frame to green fluorescent protein (GFP) at the C-terminus of each construct, and a FLAG tag was attached to their N-terminus (resulting in 25Q or 103Q). In each case, a KpnI/XbaI fragment containing the polyQ domain fused to GFP was excised from these constructs and inserted into an integrative YIp351 plasmid (Hill et al., 1986) previously modified to express the proteins under the control of the GAL1 promoter (Solans et al., 2006). The polyQ constructs were also cloned in the plasmid YIplac128 as KpnI/XbaI downstream the CUP1 promoter (as HindIII/KpnI), obtained by PCR amplification using the W303-1A genomic DNA as the template. Additionally, the preparation of the β-estradiol-inducible GAL1 promoter systems required two independent constructs as explained below. In a first construct, the fusion cassette GAL4.ER.VP16 was expressed under the control of the alcohol dehydrogenase 1 (ADH1) promoter in the plasmid pRS313 obtained from Dr. D. Picard (Dep de Biologie Cellulaire, Université de Genève, Genève, Switzerland). In the second construct, the GAL4.ER.VP16 fragment was cloned as NotI/SphI downstream a mutant form of the translation and elongation factor 1 (TEF1) promoter (as EcoRI/NotI) in the plasmid YIplac204. The mutant promoter TEF1-7 retains 16% of TEF1 activity (Nevoigt et al., 2006) and was obtained as part of a collection of TEF1-based promoter replacement cassettes from Dr. G. Stephanopoulos (Massachusetts Institute of Technology, Cambridge, MA).
Standard procedures were used for the preparation and ligation of DNA fragments, and for transformation and recovery of plasmid DNA from *Escherichia coli* strain DH5α (Sambrook et al., 1989). Yeast were transformed by the method of Schiestl and Gietz (Schiestl and Gietz, 1989). The one-step gene insertion method (Rothstein, 1983) was used to integrate linear plasmids at the *LEU2* or *TRP1* locus of yeast nuclear DNA of W303-1A and W303Δgal1 strains. In the β-estradiol-inducible models, the plasmids containing the 103Q-GFP under the control of *GAL1* promoter and the GAL4.ER.VP16 cassette were co-transformed into the same cells.

### 2.3.3 Fluorescence microscopy.

Wide-field fluorescence microscopy was performed to detect polyQ-GFP expression. We used an Olympus fluorescence BX61 microscope equipped with Nomarski differential interference contrast (DIC) optics (Olympus, Center Valley, PA, USA), a Uplan Apo 100X objective (NA 1.35), a Roper CoolSnap HQ camera (Photometrics, Roper Scientific, Pleasanton, CA, USA), Lambda10-2 excitation and emission filter wheels and a 175 W Xenon remote source with liquid light guide. Images were acquired using SlideBook 4.01 (Intelligent Imaging Innovations, Denver, CO, USA).

### 2.4 RESULTS AND DISCUSSION

We have tested the ability of four different promoters or expression systems to regulate gene expression in yeast models of neurodegenerative disorders by monitoring the expression of the first 17 amino acids (exon 1) of the
human protein huntingtin containing either 25Q or 103Q fused to GFP. GFP monitoring has allowed us to easily visualize protein expression, follow accumulation, and, for 103Q, macro-aggregation. The fitness of the models has been initially evaluated for the tightness of the system (the residual expression or leakage of the system when the inducer is not present) and its strength and regulation by using different concentrations of the inducer.

2.4.1 Copper inducible models.

The *CUP1* gene codes for metallothionein, a protein that binds copper and mediates resistance to high concentrations of copper and cadmium (Butt et al., 1984). The *CUP1* promoter (*CUP1*pr) is induced by the presence of copper in the growth media. The *CUP1*pr-polyQ-GFP constructs were integrated in single copy into the yeast *LEU2* locus (Figure 2.1A). Cells were grown in 2% YPRAF and gene expression was induced with 0 mM and 2 mM CuSO₄. Albeit some interclone variability exists, we easily selected clones in which *CUP1*pr tightly regulated polyQ-GFP expression. Very low protein levels were expressed in the absence of copper (0 mM) following growth for 6 h (Figure 2.1B) or 24h (data not shown). By contrast, in the presence of 2 mM CuSO₄, protein expression was significantly increased after 6 h of induction (Figure 2.1B) and slowly continued at least up to 24 h (data not shown). While 25Q-GFP was completely diffused in the cytoplasm, 103Q-GFP was localized diffused in the cytoplasm and forming small aggregates as expected (Figure 2.1B). However, by using low copper concentrations, the amount of protein expressed was low and the cells did not express significant toxicity after 24 h of induction (data not shown).
Figure 2.1. Copper inducible model. (A) Representation of the polyQ-GFP expression cassette under the control of the *CUP1* promoter (*CUP1pr*). (B) Visualization of 103Q-GFP expression under the control of *CUP1pr*. Cells were grown for 6 h in YPRAF media in the presence or absence of 2 mM CuSO₄. After induction cells were mounted on slides and visualized under an Olympus fluorescence BX61 microscope, as described in Materials and Methods section. The bar represents 5 μm.

Longer incubation times can result in the generation of spontaneous suppressor mutations that suppress potential mutant polyQ toxicities (Ocampo and Barrientos, unpublished observations). On the other hand, increasing the amount of inducer can produce deleterious effects given the high reactivity of copper, a redox transition metal able to activate oxygen through Fenton’s reactions, thus producing ROS (Sutton and Winterbourn, 1989). Copper should be used with caution particularly when modeling diseases in which mitochondrial oxidative phosphorylation disturbances and ROS production could be involved in
their pathogenic mechanisms, such as in the neurodegenerative disorders considered here (Solans et al., 2006).

2.4.2 β-estradiol inducible models.

Hormone (β-estradiol) inducible systems for gene expression in yeast were devised by Dr. D. Picard (Louvion et al., 1993). They are based on the constitutive expression of a transactivator fusion protein (GAL4.ER.VP16) formed by a GAL4 DNA binding domain, a β-estradiol receptor domain and a virus protein 16 transcriptional activator, which can activate transcription of a gene placed under the control of a galactose inducible promoter (GAL1pr). In the absence of the hormone, the fusion protein is repressed by the yeast chaperones from the heat shock protein 90 (Hsp90) family (Louvion et al., 1993). Upon media supplementation with β-estradiol, the fusion protein is released from its repression, binds to GAL1pr through the GAL4 DNA binding domain, and the VP16 recruits the transcriptional machinery to start transcription.

Although β-estradiol inducible systems have been extensively used in biotechnological projects focused on protein production, they have not been used until now to generate yeast models of human diseases. Here we explored the potential application of these systems to create yeast models of neurodegenerative disorders. Our β-estradiol-inducible HD models were created by transforming wild type yeast cells with two constructs. In the first construct, polyQ-GFP was cloned downstream GAL1pr and integrated in a single copy into the yeast LEU2 locus. The transformants were subsequently transformed with a second construct expressing GAL4.ER.VP16 under the control of either the
strong constitutive $ADH1$ promoter ($ADH1pr$) (Figure 2.2A) or the weaker constitutive $TEF1-7$ promoter ($TEF1-7pr$) (Figure 2.2C) in a multicopy or integrative vector, respectively.

Figure 2.2. β-Estradiol inducible models. (A and C) Representations of the polyQ-GFP expression cassettes under the control of the β-estradiol inducible promoter. Expression of the transactivator GAL4.ER.VP16 was placed under the control of either the $ADH1$ promoter ($ADH1pr$, in panel A) or $TEF1-7$ promoter ($TEF1-7pr$, in panel C) as explained in the text. (B and D) Visualization of polyQ-GFP expression under the control of the β-estradiol inducible promoters in systems using either $ADH1pr$ (in panel B) or $TEF1-7pr$ (in panel D) to activate expression of the transactivator GAL4.ER.VP16. Cells were grown for 6 h in YPRAF media in the presence of 0, 10 and 50 nM β-estradiol. Processing and visualization was performed as in Figure 2.1B. The bar represents 5 μm.

Cells were grown in 2% YPRAF media, and polyQ expression was induced by supplementing the media with 0, 10 and 50 nM β-estradiol. After 6 h of induction, gene expression was monitored by fluorescence microscopy. In the absence of β-estradiol, significant levels of expression were detected in cells containing the GAL4.ER.VP16 under the control of $ADH1pr$ indicative of
significant leakage of the system under these conditions (Figure 2.2B). Upon addition of 10 and 50 nM β-estradiol, polyQ expression was significantly higher showing a good response to increasing concentrations of the inducer. Mutant polyQ was detected diffused in the cytoplasm as well as forming large macro-aggregates indicative of high levels of expression after only 6 h of induction (Figure 2.2B). When GAL4.ER.VP16 expression was placed under the control of the weak TEF1-7pr, the system was significantly tightly regulated because no leakage was detected in the absence of β-estradiol after 6 or 24 h (Figure 2.2D). In the presence of 10 and 50 nM β-estradiol, a significant protein expression was observed. The increase was roughly proportional to the amount of hormone present in the media as visualized by the number, size and fluorescence intensity of the polyQ aggregates detected inside the cells (Figure 2.2D).

However, we have observed that the GAL4.ER.VP16 construct alone produces some degree of toxicity, reducing the growth fitness of yeast cells (data not shown), presumably by trapping general transcription factors required at RNA polymerase II promoters (Berger et al., 1992). The VP16 toxic effect was significantly higher when its expression was under the control of the strong ADH1pr than when its expression was under the control of the weak TEF1-7pr. This toxic effect, which could not be completely eliminated even using very low β-estradiol concentrations, will interfere and potentially produce misleading interpretations when analyzing mutant polyQ-induced cytotoxicity. At this point we cannot discard that by using promoters even weaker than TEF1-7pr, VP16
toxicity could be totally eliminated, a possibility that is currently being tested in our laboratory.

2.4.3 Gratuitous galactose-inducible promoters.

To generate a gratuitous galactose-inducible system we have taken advantage of our originally reported GAL1-promoter-based models (Solans et al., 2006) and safely mutate the galactose metabolic pathway. Gratuitous induction of the GAL promoters can be achieved by mutations of the chromosomal GAL1 gene (Hovland et al., 1989). The GAL1 product, galactokinase, is the enzyme required for the first step in the galactose metabolism and catalyzes the conversion of galactose into galactose-1-phosphate. In a Δgal1 strain, galactose metabolism is deactivated early in the pathway avoiding accumulation of any potentially harmful metabolite. Hence, galactose serves as a gratuitous inducer. To prepare these models, we have created a W303Δgal1 strain by replacing the full GAL1 gene with a kanamycin resistance cassette using standard molecular biology techniques. The W303Δgal1 strain failed to grow in media containing galactose but grew at a similar rate than the parental wild type strain W303-1A in media containing fermentable (glucose or raffinose) or respiratory (ethanol-glycerol or lactate) substrates (data not shown). The W303Δgal1 strain was subsequently transformed with the integrative galactose-inducible expression plasmids YIp351-25Q-GFP and YIp351-103Q-GFP to integrate the constructs into the chromosomal LEU2 locus.

Transformants were grown in 2% YPRAF (Figure 2.3B) for 8 h in the presence of 0.01% and 0.1% galactose. Protein expression was not detected in
the absence of galactose (data not shown) and induced when galactose was added to the media even at concentrations as low as 0.01%. In cells expressing 25Q-GFP the protein was diffused in the cytoplasm, and no cytotoxicity was observed (data not shown). In cells expressing 103Q-GFP, the protein was diffused in the cytoplasm with a fraction forming visible aggregates (Figure 2.3B) and produced cytotoxicity (data not shown). The levels of expression (Figure 2.3B) and 103Q-GFP toxicity (data not shown) correlated to the amount of galactose in the media. Thus, by adding 0.1% galactose the levels of protein expressed as well as the number and size of the aggregates were further increased (Figure 2.3B).

Figure 2.3. Gratuitous galactose-inducible model. (A) Representation of the polyQ-GFP expression cassette under the control of the GAL1 promoter. (B) Visualization of polyQ-GFP expression under the control of the GAL1 promoter. Cells were grown for 6 h in YPRAF media in the presence of 0.01% and 0.1% galactose. Processing and visualization was performed as in Figure 2.1B. (C) Comparison of the polyQ-GFP expression under the control of the GAL1 promoter in W303-1A and W303Δgal1 cells. Cells were grown for 24 h in YPRAF media in the presence of 0.1% galactose. Processing and visualization was performed as in Figure 2.1B. The bar represents 5 μm.
Deletion of the *gal1* gene prevents the metabolism of the galactose present in the media as explained above, thus maintaining constant levels of protein expression induced by small amounts of galactose. We have compared 103Q-GFP expression regulated by the *GAL1* inducible promoter in W303Δgal1 cells and W303-1A cells growing in either fermentable YPRAF (Figure 2.3C) or respiratory YPG media (Ocampo and Barrientos, unpublished data). After induction times shorter than 10 h, polyQ protein cellular levels and homogeneity through the cell population were similar in both W303Δgal1 and W303-1A cells (data not shown). However, after 24 h of induction with 0.1% galactose, protein levels in W303Δgal1 cells were higher than in W303-1A cells and significantly homogenous in the whole cell population. For example, all W303Δgal1 cells expressing the mutant 103Q-GFP protein contained large macroaggregates. In W303-1A cultures, the small amount of galactose present in the media was mostly metabolized before the 24 h of incubation, resulting in variable levels of protein expression across the cell population with a significant proportion of cells in which the protein was not detected. In W303-1A cells expressing 103Q-GFP, the amount as well as the number and sizes of macroaggregates were significantly smaller (Figure 2.3C) than in W303Δgal1 mutant cells.

We conclude that induction of polyQ-GFP expression under the control of *GAL1*pr in W303-1A cells by supplementing media containing fermentable 2% raffinose or respiratory 2% glycerol with small amounts of galactose is limited, but can be used for short term studies. However, more versatile and solid models are obtained in the absence of galactose metabolism as explained above.
2.3.4 Concluding remarks.

All the yeast models of polyQ disorders presented here reproduced at least the basic hallmarks of neuronal degeneration observed in human patients represented by protein aggregation and cytotoxicity as described. However, not all of them can be used safely and cannot be equally recommended. The different expression systems tested to create the models have been evaluated considering their tightness, regulation of levels of expression, self-toxicity and metabolic independence.

We would not recommend the use of models based on copper-induced polyQ expression given the strong possibility that the inducer will catalyze ROS production. The β-estradiol inducible models are strongly regulated and hold promise if we were able to eliminate VP16 toxicity by using very weak constitutive promoters for the expression of the GAL4.ER.VP16 transactivator.

Our current data suggest that the highly regulatable, non-toxic, gratuitous induction system based on GAL1 gene disruption to safely avoid galactose metabolism is the best choice for stable and metabolically independent polyQ expression in S. cerevisiae. In this system, cells can be grown in media containing either fermentable (i.e. raffinose) or respiratory (i.e. lactate or ethanol-glycerol) substrates; the polyQ expression, under the control of a Gal inducible promoter, is regulated by addition of low concentrations of galactose to the media. The GAL1 disruption system allows for maintaining a stable and galactose concentration throughout cell growth, thus resulting in permanent induction of galactose-inducible promoters. In addition, by using this system,
nondividing yeast cells can also be induced to produce the recombinant protein of interest.

Finally, it is our hope that the data presented in this report for the yeast models of HD will be useful when modeling other diseases as well.
CHAPTER 3
SUPPRESSION OF POLYGLUTAMINE-INDUCED CYTOTOXICITY IN SACCHAROMYCES CEREVISIAE BY ENHANCEMENT OF MITOCHONDRIAL BIOGENESIS

3.1 SUMMARY

Alterations in mitochondrial metabolism have been associated with age-associated neurodegenerative disorders. This is seen in diseases caused by misfolding of proteins with expanded polyglutamine (polyQ) tracts, such as Huntington's disease. Although evidence of mitochondrial impairment has been extensively documented in patients and disease models, the mechanisms involved and their relevance to the initiation of polyQ cytotoxicity and development of clinical manifestations remain controversial. We report that in yeast models of polyQ cytotoxicity, wild-type and mutant polyQ domains might associate early with the outer mitochondrial membrane. The association of mutant domains with mitochondrial membranes could contribute to induce significant changes in mitochondrial physiology, ultimately compromising the cell's ability to respire. The respiratory defect can be fully prevented by enhancing mitochondrial biogenesis by overexpression of Hap4p, the catalytic subunit of the transcriptional activator Hap2/3/4/5p complex, the master regulator of the expression of many nuclear genes encoding mitochondrial proteins in yeast. Protecting cellular respiratory capacity in this way ameliorates the effect of expanded polyQ on cellular fitness. We conclude that mitochondrial dysfunction is an important contributor to polyQ cytotoxicity. Our results suggest that
therapeutic approaches enhancing mitochondrial biogenesis could reduce polyQ toxicity and delay the development of clinical symptoms in patients.

### 3.2 INTRODUCTORY REMARKS

Polyglutamine (polyQ) diseases are a group of genetic disorders characterized by neurodegeneration of specific brain regions. They are caused by an unstable trinucleotide repeat expansion in disease-specific genes resulting in an expanded polyQ tract in the encoded protein. Most polyQ diseases are dominantly inherited, and their onset and severity correlate with the length of abnormal polyQ sequences (Shao and Diamond, 2007). Huntington’s disease (HD) is the most prevalent polyQ disease, affecting 1 per $10^5$ people. HD is a fatal autosomal dominant disease characterized by selective loss of neurons in the striatum and deep layers of the cortex (Vonsattel et al., 1985). In HD, the CAG expansion occurs in exon 1 of the IT15 gene, which encodes the protein huntingtin (htt) (Group, 1993) and results in the extension of an N-terminal polyQ stretch to beyond 36 repeats (Vonsattel et al., 1985).

The CAG expansion is a dominant gain-of-function mutation. Mutant htt polypeptides with expanded polyQ tracts acquire an unusual conformation that produces cell toxicity and facilitates their oligomerization and aggregation into intracellular inclusion bodies (DiFiglia et al., 1997; Scherzinger et al., 1997). It is still controversial whether or not these large aggregates contribute to the pathogenic mechanism of the disease. However, it has been shown undoubtedly that inclusions do not trigger the initiation of neuronal loss in mouse models of
HD (Slow et al., 2003). Thus, it is believed that the misfolded htt monomers and oligomers are toxic before their incorporation into large macromolecular inclusions (Nagai et al., 2007). Mutant htt is cleaved both in vivo and in vitro (Wellington et al., 2002) to form N-terminal fragments containing polyQ repeats. These fragments, including approximately the first 150 residues of htt encoded in exon 1 of the HD gene, are considered the toxic forms of the protein. For this reason, HD pathogenesis has been extensively modeled by expressing exon 1 fragments, which causes toxicity and promotes protein misfolding and aggregation in yeast, mammalian cultured cells and animal models (Krobitsch and Lindquist, 2000; Landles and Bates, 2004; Meriin et al., 2002).

To date, a significant number of cellular pathways have been reported to be impaired by polyQ expansion proteins. Among them, polyQ toxicity involves transcriptional dysregulation by inactivation of transcriptional factors (Cui et al., 2006), deficient protein turnover owing to general alterations in the ubiquitin proteasome system (Bence et al., 2001; Bennett et al., 2007), and to early specific defects in endoplasmic reticulum (ER)-associated degradation (Duennwald and Lindquist, 2008), vesicular and organelle trafficking alterations (Panov et al., 2002; Trushina et al., 2004), perturbations in the kynurenine pathway (Giorgini et al., 2005), and disturbances in energy metabolism and mitochondrial function (Cui et al., 2006). In this study, we have used yeast models of polyQ expansion to focus on examining the mechanism by which polyQ-induced mitochondrial dysfunction occurs and its significance as a contributor to polyQ toxicity.
Mitochondrial dysfunction associated with the pathogenesis of polyQ diseases has been extensively documented. Several studies using postmortem brain tissue from HD patients have reported energy metabolism impairment, decreased mitochondrial oxygen consumption, reduced glucose metabolism, elevated lactate concentrations and a significant decrease in mitochondrial respiratory chain complex II and III activities (Gu et al., 1996). Toxin models of HD have been induced by 3-nitropropionic acid or malonate, both of which are inhibitors of mitochondrial respiratory chain complex II (Brouillet et al., 2005; Ruan et al., 2004). Expanded htt polyQ domains may affect several mitochondrial functions. Mitochondrial integrity and dynamics were reported altered in HeLa cells and C. elegans models (Wang et al., 2009), and mitochondrial axonal trafficking was found impaired in mouse striatal neurons in vivo and in vitro (Trushina et al., 2004). Recent findings suggest that HD mutant htt may damage neurons by inducing mitochondrial depolarization and altering mitochondrial calcium homeostasis in patients and in mouse models (Panov et al., 2002). These alterations could result from a direct binding of htt to mitochondria (Choo et al., 2004; Panov et al., 2002). In addition, mutant htt has been shown to alter mitochondrial function indirectly by inhibiting expression of the transcriptional co-activator PGC-1α, which regulates mitochondrial biogenesis and respiration (Cui et al., 2006). Mutant htt represses PGC-1α gene transcription by interacting with the promoter and interfering with the CREB/TAF4-dependent transcriptional pathway critical for PGC-1α gene expression regulation (Cui et al., 2006). In this
way, altered PGC-1α function links transcription dysregulation and mitochondrial dysfunction in HD.

The yeast *Saccharomyces cerevisiae* has been frequently used to model polyQ toxicity by expressing N-terminal fragments of huntingtin (htt exon I) which recapitulates major hallmarks of neuronal pathology (Krobitsch and Lindquist, 2000; Meriin et al., 2002; Willingham et al., 2003). In the yeast model, we recently reported that polyQ toxicity involves actin cytoskeleton alterations and increased free radical generation leading to altered mitochondrial distribution, early decrease in mitochondrial respiratory chain complexes II and III, and progressive decline in respiratory capacity (Solans et al., 2006).

Here, we have created additional models by the expression of wild-type and mutant polyQ tracts of variable lengths. We show that polyQ domains might interact with the mitochondrial outer membrane, which contributes to induce biophysical and functional mitochondrial alterations and cellular growth defects that increase with the length of polyQ tracts. Further, we found that increasing mitochondrial biogenesis and respiratory capacity by overexpressing Hap4p, the catalytic subunit of the Hap2,3,4,5p complex, ameliorates polyQ cytotoxicity. Our results, therefore, suggest that polyQ domains directly affect mitochondrial function and that preventing these alterations by bolstering mitochondrial biogenesis is a promising target for therapeutic interventions aiming to retard polyQ-associated neurodegeneration.
3.3 MATERIALS AND METHODS

3.3.1 Yeast strains and media.

The *S. cerevisiae* strains used were the wild-type W303-1A (MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1) and the previously reported isogenic 25Q and 103Q strains (Solans et al., 2006). Strains expressing 2Q, 46Q and 72Q and strains overexpressing HAP4 were constructed as explained below. The compositions of the growth media have been described elsewhere (Myers et al., 1985). Prior to induction of polyQ expression with 2% galactose, all cultures were grown on media containing non-fermentable carbon sources (YPEG or WOEG). All our experimentation was done by using freshly transformed cells.

3.3.2 Cloning of 2Q-GFP, 46Q-GFP and 72Q-GFP in integrative plasmids.

Two polyQ constructs (46Q and 72Q in the plasmid p306-Gal) were previously reported (Duennwald et al., 2006). A *KpnI/XbaI* fragment containing either the 46Q or 72Q domain fused to GFP was excised. A 2Q-GFP was created by overlapping PCR using 25Q fragment as template. The fragments were inserted in the integrative YIp351 plasmid previously modified to express the proteins under the control of the *GAL1* promoter.

3.3.3 Mitochondrial preparation.

Mitochondria with intact outer membrane were prepared by the method of Herrmann *et al.* (Herrmann et al., 1994) from cells pregrown in medium containing ethanol and glycerol and transferred to medium containing 2% galactose for 30 min to 20 h to induce polyQ expression.
3.3.4 Endogenous cell respiration.

Endogenous cell respiration was assayed polarographically using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK) at 30°C as described previously (Barrientos et al., 2002). Cells were grown in galactose medium for 15 h, re-inoculated in fresh galactose medium at the same confluence (OD\textsubscript{600} = 0.4) and grown for 1 additional hour prior to measuring maximal cellular respiration. The specific activities reported were corrected for KCN-insensitive respiration.

3.3.5 In vivo mitochondrial protein synthesis.

Mitochondrial gene products were labeled with [35S]-methionine (7 mCi/mmole, Amersham, Piscataway, NJ, USA) in whole cells at 30°C in the presence of cycloheximide (Barrientos et al., 2002). Equivalent amounts of total cellular or mitochondrial proteins were separated by SDS-PAGE on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and exposed to Kodak X-OMAT X-ray film (Eastman Kodak, Rochester, NY, USA).

3.3.6 Fluorescence microscopy.

Wide-field fluorescence microscopy was performed to detect polyQ-GFP expression. We used an Olympus fluorescence BX61 microscope (Olympus, Tokyo, Japan) equipped with Nomarski differential interference contrast (DIC) optics, an Uplan Apo 100× objective (NA 1.35), a Roper CoolSnap HQ camera (Roper Scientific, Trenton, NJ, USA), and Sutter Lambda10-2 excitation and emission filter wheels (Sutter Instrument Co., Novato, CA, USA) and a 175-W
xenon remote source with liquid light guide. Images were acquired using SlideBook 4.01 (Intelligent Imaging Innovations, Denver, CO, USA).

### 3.3.7 Measurement of mitochondrial membrane potential ($\Psi_m$).

$\Psi_m$ was estimated using tetramethyl rhodamine methyl ester (TMRM; Molecular Probes, Eugene, OR, USA), a cell-permeant, cationic, fluorescent dye that is readily sequestered by viable mitochondria. A 30 min incubation of $10^6$ yeast cells/ml in 5 $\mu$M TMRM at 24°C allowed sufficient uptake of TMRM into the matrix to self-quench as reported (Nicholls and Ward, 2000). Cells were then washed 2X in water. Flow cytometry analysis was performed on a Becton Dickinson FACS Aria II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Excitation was performed at 532 nm, which does not excite GFP, thus avoiding signal interference; emission was detected using a 25-nm bandpass filter centered at 575 nm (Becton Dickinson). Dissipation of $\Psi_m$ causes TMRM to leak out of mitochondria into the cytosol where TMRM became unquenched, producing an increase in fluorescence (Nicholls and Ward, 2000). Cells incubated in the presence of the ionophore carbonyl cyanide $m$-chloro phenyl hydrazone (CCCP), which dissipates the membrane potential in a concentration-dependent manner, and cells treated with oligomycin, an $F_1F_0$-ATPase inhibitor that causes mitochondrial hyperpolarization, were used as controls.

### 3.3.8 Kinetics of mitochondrial volume (swelling and shrinking).

Wild-type, 25 and 103Q mitochondria with intact outer membrane prepared by the method of Herrmann (Herrmann et al., 1997) were used to analyze the kinetics of mitochondrial volume. Four hundred $\mu$g mitochondrial
protein was subjected to strong hypotonic conditions (20 mM HEPES 7.2, 1 mM EDTA, 0.2% BSA and 0.025 M sorbitol to induce a 24-fold decrease in osmolarity) followed by partial restoration of tonicity (to 0.18 M) by adding concentrated sucrose. Kinetics of mitochondrial volume was monitored by following the change of apparent absorbance at 540 nm (Hunter and Smith, 1967). Mitochondrial swelling was further assessed by increasing the permeability of the mitochondrial membrane in isotonic conditions (20 mM HEPES 7.2, 1 mM EDTA, 0.2% BSA and 0.6M sorbitol). Opening of the yeast mitochondrial unspecific channel was promoted with ATP in a medium supplemented with 0.5 mM P_i and 2 mM NADH. In another experiment, 400 μg of mitochondrial protein was added to the reaction isotonic medium supplemented with 2 mM NADH, and the mitochondrial membranes were permeabilized with the pore-forming antibiotic alamethicin (2 mM) as described previously (Ma et al., 2004). A Δcrd1 cardiolipin mutant strain (Ma et al., 2004) was used as a control.

3.3.9 Miscellaneous procedures.

Standard procedures were used for the preparation and ligation of DNA fragments, for transformation and recovery of plasmid DNA from *E.coli*, and for yeast transformation. Western blots were treated with antibodies against the appropriate proteins, followed by a second reaction with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO, USA). The SuperSignal West Pico and Femto substrate kit (Pierce, Rockford, IL, USA) were used for the final detection.
3.3.10 Statistical analysis.

All experiments were done at least in triplicate. Data are presented as means ± SD of absolute values or percentage of control. Values were compared by Student t test. \( P < 0.05 \) was considered significant.

3.4 RESULTS

3.4.1 Mutant polyQ length correlates with the extent of cell growth defects and cellular respiration decline in a yeast model of polyglutamine diseases.

To study the relevance of mitochondrial metabolism alterations in the mechanism of polyQ-induced cytotoxicity, we constructed yeast strains carrying integrative plasmids to allow for the inducible expression of polyQ domains of pathogenic and nonpathogenic length.

We previously reported W303-1A cell lines expressing htt exon 1 fragments containing either 25Q or 103Q fused to green fluorescent protein (GFP) under the control of a Gal1 promoter (Solans et al., 2006). For the present study, similar constructs were prepared containing polyQ tracts of 2Q, 46Q and 72Q. The plasmids were linearized and integrated into the LEU-2 gene in the W303-1A strain carrying the prion-like protein Rnq1, required for expression of mutant polyQ toxicity in yeast (Meriin et al., 2002). All polyQ domains were expressed equivalently under the control of the Gal1 promoter (exact nucleotide sequence in all cases) in the presence of 2% galactose. Expression and aggregation of the polyQ-GFP domains were followed by fluorescence microscopy. Following induction in the presence of galactose, the polyQ domains fused to GFP accumulate in the cytoplasm but not in the vacuole (Figure 3.1A).
After 3 h of induction, the 2Q, 25Q and 46Q domains appeared diffuse in the cytoplasm, whereas we observed some spherical cytoplasmic aggregates in cells expressing 72Q and more abundant spherical and amorphous aggregates in cells expressing 103Q (Figure 3.1A). While 2Q and 25Q never form aggregates, 46Q cells start forming small spherical aggregates only after 6 h of induction in galactose (not shown).

The models were further validated by assessing polyQ-induced cytotoxicity with growth tests either in solid or liquid medium. In contrast to 2Q, 25Q and 46Q, expression of 72Q and 103Q from integrative plasmids was toxic to dividing wild-type cells (Figures 3.1B and 3.1C). Since the Gal1 promoter is repressed in the presence of glucose in yeast, our experimental cultures were always pregrown in medium containing glycerol and ethanol before induction with galactose. No difference in colony size was observed on glucose-containing medium without polyQ expression, thus indicating that accumulation of 72Q and 103Q was responsible for the growth defect in each case. Although cells expressing 72Q and 103Q have a significant growth arrest (Figures 3.1B and 3.1C) the cultures maintain similar viability than W303 and 25Q cells after 24 h of polyQ induction as assessed by the Live/Dead yeast viability kit (Molecular Probes, Eugene, OR, USA) (Supplemental Fig. S3.1).

We previously reported that cells expressing 103Q manifest a progressive decline in the capacity of the cells to respire (Solans et al., 2006). Now, we asked whether polyQ length-dependent cytotoxicity correlated with the extent of mitochondrial respiratory chain dysfunction. Polarographic measurement of
endogenous cell respiration showed that after 16 h of induction, cells expressing 2Q and 25Q had respiratory capacity slightly increased compared to that of wild-type cells (Figure 3.1D). The reasons for this increased respiration remain unexplained. Instead, cells expressing larger polyQ domains of pathological length displayed a reduced ability to respire compared to cells expressing 2Q, which declined directly according to the length of the polyQ tract (Figure 3.1D).

Figure 3.1. PolyQ-induced growth arrest correlates with polyQ tract length and mitochondrial toxicity. (A) Visualization of polyQ-GFP fusion protein expression and aggregation. The indicated strains were induced during 3 h in complete medium containing 2% galactose, mounted on slides, and visualized by fluorescence microscopy. (B) Growth curve on liquid medium containing 2% galactose. Density of the cultures was estimated by measuring absorbance at 600 nm. Bars (hidden behind the symbol marks) indicate means ± SD from 3 independent sets of measurements. (C) The same strains as in B were grown overnight in liquid YPEG. Ten-fold serial dilutions of the cultures were plated on solid YPD or YPGal medium and incubated at 30°C. Pictures were taken after 2 d of incubation. (D) KCN-sensitive endogenous cell respiration measured polarographically in cells grown in the presence of galactose for 16 h. Bars indicate means ± SD from at least 3 independent sets of measurements. *, P<0.05; **, P<0.01.
3.4.2 PolyQ domains localize to the mitochondrial outer membrane.

Mitochondrial dysfunction in our yeast models could result, at least in part, from a direct interaction of polyQ domains with mitochondrial membranes as previously shown for human cells (Choo et al., 2004; Panov et al., 2002). To analyze this possibility, we fractionated polyQ-expressing induced cells and isolated high-quality mitochondria with intact outer membrane. Because the method is largely based on differential centrifugation, care was taken to limit the induction of polyQ expression to times shorter than those required for accumulation of misfolded protein aggregates. Mitochondria were prepared from the different strains after induction time ranging from 30 min to 4 h. Subsequently, mitochondria were stained with Mitotracker red (Molecular Probes, Eugene, OR, USA) and monitored under fluorescence microscopy for traces of cofractionated green polyQ microaggregates. Mitochondrial fractions prepared from 103Q cells induced for 4 h contained a significant amount of microaggregates, which were detected when the preparations were exposed for 15 s to generate the images (Supplemental Figure S3.2). Mitochondria prepared from 25Q cells induced for 4 h were polyQ-aggregate free as expected. Similarly, we did not detect polyQ aggregates in mitochondrial fractions prepared from 46Q and 72Q cells after 1 h of induction (not shown), nor in the case of 103Q cells when mitochondria were prepared following 30 min of induction (Supplemental Figure S3.2).

To analyze whether soluble, non-aggregated polyQ domains localize to mitochondria, 25Q and 103Q cells were fractionated into postmitochondrial
supernatant containing cytoplasmic soluble proteins, and into mitochondrial fractions following 4 h and 30 min of induction respectively. Western blot analyses using an anti-GFP antibody enabled us to detect most wild-type and mutant polyQ-GFP in the postmitochondrial supernatant and an additional portion in the mitochondrial fractions (Figure 3.2A). The purity of the two fractions was assessed using antibodies against the cytoplasmic marker 3-phosphoglycerate kinase subunit 1 (Pgk1p) and the mitochondrial marker porin (Figure 3.2A). The association of polyQ domains with mitochondria was further probed by testing polyQ sensitivity to proteinase K digestion in both isolated mitochondria and mitoplasts prepared by hypotonic mitochondrial swelling. As shown in Figure 3.2B, 25Q and 103Q are extensively sensitive to proteinase K digestion in both mitochondria and mitoplasts. Several markers were used to control the experiment: Sco1p, an inner membrane protein that faces the intermembrane space (IMS), was digested in mitoplasts but not in mitochondria; as expected, the hypotonic conditions used to disrupt the outer membrane resulted in the loss of IMS soluble cytochrome \( b_2 \), and the integral outer membrane protein porin remained largely unaffected by the protease.

These results suggest that 25Q and 103Q domains associate with the outer mitochondrial membrane (Figure 3.2C). Our results do not discern whether polyQ domains integrate into the membrane, as we have depicted in Figure 3.2C, or they peripherally interact with the outer surface of the membrane, a possibility not included in the figure for simplification purposes.
Figure 3.2. PolyQ domains associate with the mitochondrial outer membrane. (A) Western blot analyses of cytoplasmic and mitochondrial fractions obtained from cells expressing 25Q-GFP and 103Q-GFP after 4 h and 30 min of induction, respectively. PolyQ-GFP fusion proteins were detected using an anti-GFP monoclonal antibody. Cytosolic marker 3-phosphoglycerate kinase subunit 1 (Pgk1p) and the mitochondrial marker porin were used as loading controls and to determine the purity of the fractions. (B) Mitochondrial localization and topology of polyQ-GFP fusion proteins by proteinase protection assays. Four aliquots of 40 μg of mitochondrial protein from cells expressing either 25Q-GFP or 103Q-GFP were pelleted and resuspended in buffer containing either 20 mM HEPES or 0.6 M sorbitol/20 mM HEPES. One aliquot in each buffer was supplemented with final concentration of 12.5 μg/ml proteinase K (PK) and incubated on ice for 60 min. Reaction was stopped with 2 mM phenylmethylsulfonyl fluoride. Mitochondria (Mt) and mitoplasts (Mp) were recovered by centrifugation at 40,000 rpm for 15 min at 4°C. Pelleted fraction was resuspended in gel buffer and loaded on a 10% Tris-Tricine gel. Western blots were probed with antibodies against GFP, cytochrome b2, Sco1p and porin. (C) Cartoon depicting the submitochondrial localization and topology of polyQ domains and mitochondrial proteins used as references. (D) Western blots analyses of cytoplasmic and mitochondrial fractions obtained from 46Q and 72Q cells induced during 60 min, performed as in panel B. A mitochondrial fraction obtained from 103Q cells induced during 30 min as in panel A was also included.
The cosedimentation of polyQ domains with mitochondrial membranes was further confirmed by the analyses of cytoplasmic and mitochondrial fractions obtained from 46Q and 72Q cells induced for 1 h (Figure 3.2D).

3.4.3 Dissipated ΔΨ\textsubscript{m} and defective energetic coupling in 103Q mitochondria.

To understand the effect of mitochondrial outer membrane-associated polyQ domains on mitochondrial bioenergetics in our yeast models, we measured the mitochondrial ΔΨ\textsubscript{m} by cytofluorometry using the cationic dye TMRM. In the assay conditions, explained in Materials and Methods, TMRM fluorescence quenching is proportional to ΔΨ\textsubscript{m} (Nicholls and Ward, 2000). In controls, the ionophore CCCP dissipated the ΔΨ\textsubscript{m} in wild-type cells in a concentration-dependent manner while the $F_{1}$-$F_{0}$ ATPase inhibitor oligomycin caused membrane hyperpolarization, measured as an increase and decrease in TMRM fluorescence, respectively (Figure 3.3A). In this assay, wild-type cells expressing an empty vector or the 25Q construct induced in galactose medium for 16 h had similar ΔΨ\textsubscript{m}, while 103Q cells had significantly dissipated ΔΨ\textsubscript{m} (Figure 3.3A). A subsequent time course ΔΨ\textsubscript{m} measurement following galactose induction in wild-type and 103Q cells showed that in the latter, the decline in ΔΨ\textsubscript{m} was detected as early as after 6 h of induction and was already significant after 8 h (Figure 3.3B). These results correlate with the time course of mitochondrial respiration decline and growth arrest that we previously reported (Solans et al., 2006).
Figure 3.3. Mutant polyQ domains alter the properties of the mitochondrial membranes and their biogenesis. (A) Cytofluorometric analysis of $\Delta \Psi_m$ using TMRM as a fluorescent probe in W303-1A, 25Q and 103Q cells induced for 16 h in liquid medium containing 2% galactose. Ten thousand cells of each indicated strain were measured in each experiment. Average fluorescence is expressed as percentage of wild-type cells. Wild-type cells treated with 20 $\mu$M oligomycin or the indicated concentrations of CCCP were used as controls. (B) Time course of $\Delta \Psi_m$ in wild-type and 103Q cells. (C) Defective shrinkage of hypotonically swollen 103Q mitochondria. Mitochondria prepared as for Figure 3.2 following 16 h of induction of polyQ expression were suspended in hypotonic medium. Shrinkage of swollen mitochondria was induced by the addition of concentrated sucrose to 0.18 M. (D) Defective ATP-induced swelling in 103Q mitochondria. Four hundred micrograms of mitochondrial protein was added to the reaction isotonic medium supplemented with 0.5 mM K$_3$PO$_4$ (pH 7.4) and 2 mM NADH. Arrow indicates the addition of 2 mM ATP. (E) Defective alamethicin-induced swelling of mitochondria from cells expressing 103Q. Four hundred micrograms of mitochondrial protein was added to the reaction isotonic medium supplemented with 2 mM NADH. Arrow indicates the addition of 2 mM alamethicin. (C-E) Absorbance was monitored at 540 nm. Figures shown are typical of three experiments. (F) In vivo mitochondrial protein synthesis in wild-type W303-1A, 25Q-GFP and 103Q-GFP cells induced for the indicated times. Two independent 103Q clones (103Q-1 and 103Q-2) were included. Cells were labeled with [35S]-methionine at 30°C for 15 min in the presence of cycloheximide, separated by PAGE, transferred to a nitrocellulose membrane and exposed to X-ray film. The mitochondrial translation products are identified on the left, based on their migration pattern. (G) Quantification of the signals in F. Images were digitalized, and densitometry of the bands corresponding to Cox1p, Cox2p and Cyt b was performed using the histogram function of the Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Values corresponding to the two 103Q clones, essentially identical, were pooled. Bars indicate means ± SD from at least three independent sets of measurements.
Dissipation of $\Delta \Psi_m$ is expected to lead to decreased energetic coupling during mitochondrial respiration. We tested this possibility in mitochondria isolated from cells induced during 10 h to express either the 25Q or 103Q constructs. As shown in Table 3.1, NADH-oxidizing mitochondria from the 103Q strain showed that state 3 respiration decreased to $\sim$40% of wild-type values. In addition, the ADP/oxygen ratio (which measures the relationship between ATP synthesis and oxygen consumption) and respiratory control ratios (RCR, which measures the coupling between electron flux and ATP synthesis) were significantly decreased compared to the wild-type and 25Q strains (Table 3.1).

**Table 3.1. Coupling parameters in NADH-oxidizing mitochondria.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>State 3 (mmol O$_2$/min/mg protein)</th>
<th>RCR</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1A</td>
<td>0.583 ± 0.032</td>
<td>2.673 ± 0.049</td>
<td>2.006 ± 0.051</td>
</tr>
<tr>
<td>25Q</td>
<td>0.565 ± 0.014</td>
<td>2.761 ± 0.138</td>
<td>2.129 ± 0.081</td>
</tr>
<tr>
<td>103Q</td>
<td>0.240 ± 0.013*</td>
<td>2.254 ± 0.176#</td>
<td>1.847 ± 0.035¥</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD. Respiration rates in the presence of NADH were measured polarographically in mitochondria isolated from the mentioned strains after 10 h of induction with galactose. Phosphorylation and respiratory control ratio (RCR) were estimated from ADP-stimulated respiration. RCR is calculated as the ratio of oxygen consumption rate in the presence (state 3) and absence (state 4) of ADP. Experiments were repeated in triplicate. *$P<0.001$, #$P=0.016$, ¥$P=0.009$.

3.4.4 Altered osmotic properties in isolated 103Q mitochondria.

The possible interaction of polyQ domains with mitochondrial membranes could alter their capacity to respond to osmotic variations. Changes in mitochondrial volume play a basic role in the regulation of mitochondrial metabolism, affecting a number of parameters, from respiratory rates and the redox state of cytochromes to the ADP/ATP ratio and coupling between electron transfer and oxidative phosphorylation (Halestrap, 1989). Beyond physiologically
adaptive low-amplitude mitochondrial swelling and shrinking cycles, high-amplitude swelling leads to irreversible damage of mitochondrial membranes (Halestrap, 1989) and alters the capacity of mammalian mitochondria to buffer some ions such as calcium. In yeast, respiration and ATP induce the opening of the mitochondrial unspecific channel (equivalent to mammalian permeability transition pore or MPTp). Therefore, mitochondrial respiratory defects result in alterations of MPTp opening.

To determine the effect of 103Q expression on swelling and shrinking, wild-type, 25Q, and 103Q mitochondria were subjected to strong hypotonic conditions followed by partial restoration of tonicity (to 0.18 M) by adding concentrated sucrose. Mitochondrial volume kinetics were monitored by following the change of apparent absorbance at 540 nm (Hunter and Smith, 1967). Wild-type, 25Q, and 103Q mitochondria all exhibited immediate shrinkage upon the addition of sucrose (Figure 3.3C). However, the shrinkage extent of 103Q mitochondria was significantly decreased (Figure 3.3C) compared to wild-type and 25Q cells. Swelling of 103Q mitochondria was further assessed by increasing the permeability of the mitochondrial membrane in isotonic conditions. To achieve that, we either promoted the opening of the yeast mitochondrial unspecific channel with ATP or permeabilized mitochondria with the pore-forming antibiotic alamethicin (Figures 3.3D and 3.3E). Mitochondria from 103Q exhibited defective swelling in response to both ATP (Figure 3.3D) and to alamethicin (Figure 3.3E), as defined by a change in optical density smaller than in wild-type
mitochondria. These data indicate that the stretching capacity of mitochondrial membranes from 103Q cells is reduced.

We concluded that 103Q expression compromises mitochondrial membrane stability in a way equivalent to the observations made in human cells and mouse models of polyQ disorders (Panov et al., 2002), as explained in the discussion section.

The phenotypes that we observed in 103Q mitochondria are similar to those of yeast mutants in cardiolipin (Δcrd1; used as a control in the experiments presented in Figures 3.3C to 3.3E) or other mitochondrial phospholipids (Ma et al., 2004). The defective biophysical properties of 103Q mitochondrial membranes could be related to alterations in mitochondrial membrane composition. However, analysis of mitochondrial phospholipids by 1-D and 2-D thin-layer-chromatography failed to detect any obvious qualitative and quantitative alterations in cells expressing 103Q (not shown).

3.4.5 Reduced mitochondrial protein synthesis efficiency in 103Q cells.

Because mitochondrial protein synthesis is highly dependent on the integrity of the mitochondrial membranes and their energization state (McKee and Poyton, 1984), a decline could be expected in the efficiency of mitochondrial translation in 103Q cells. In vivo mitochondrial protein synthesis was performed in cells induced for increasing times up to 20 h. We observed a progressive decline in overall mitochondrial protein synthesis in 103Q cells compared with wild-type and 25Q cells (Figure 3.3F) with a reduction of more than 50% after 10 h of induction on the synthesis of all polypeptides, including Cox1p, Cox2p and
cytochrome \( b \), which quantification is presented in Figure 3.3G. Reduced mitochondrial translation would probably affect mitochondrial biogenesis in medium/late stages of polyQ toxicity and would be expected to affect enzymes with faster turnover earlier.

3.4.6 Over-expression of HAP4 significantly suppresses polyQ-induced mitochondrial and cellular toxicities.

If polyQ toxicity is significantly mediated by mitochondrial alterations, including respiratory chain dysfunction, we could predict that an increase in the amount of mitochondrial mass and mitochondrial respiratory chain enzymes should suppress, at least partially, the toxic effect. To increase mitochondrial biogenesis in 103Q cells, we over-expressed the HAP4 gene. Hap4p is the catalytic subunit of the Hap2,3,4,5p transcriptional activator complex (Forsburg and Guarente, 1989) that globally activates transcription of nuclear genes involved in mitochondrial respiration during transition from fermentation to respiration. We and others have previously shown that overexpression of HAP4 results in increased mitochondrial biogenesis and respiratory capacities in cells grown in both fermentable and non-fermentable carbon sources (Fontanesi et al., 2008; Lascaris et al., 2003). Our expression system, which relays on the expression of HAP4 under the control of its endogenous promoter from an episomal plasmid results in a 5-fold increase in HAP4 expression (Fontanesi et al., 2008).
Figure 3.4. Overexpression of HAP4 restores mitochondrial function and partially alleviates toxicity in cells expressing 103Q. (A) Ten-fold serial dilution growth test of the indicated strains pregrown overnight in liquid minimum medium containing ethanol and glycerol (WOEG), spotted on solid YPD or YPGal medium and incubated at 30°C. Pictures were taken after 2 days of incubation. 103Q/HAP4 # 1 and # 2 are two independent HAP4 transformants of 103Q cells. (B) Cells pregrown on WOEG media and subsequently inoculated on liquid media containing 2% galactose. Density of the cultures was estimated by measuring absorbance at 600 nm. (C) Visualization of 103Q-GFP fusion proteins expression and aggregation in 103Q cells containing an empty vector or expressing HAP4. Following 4 and 8 h of induction on complete media containing 2% galactose, cells were mounted on slides and visualized by fluorescence microscopy as in Figure 1A. (D) KCN-sensitive endogenous cell respiration measured polarographically in cells induced for 16 h. Wild type W303-1A and 103Q cells either carrying an empty vector or overexpressing HAP4 were included. (E) Cytofluorometric analysis of mitochondrial membrane potential using TMRM as a fluorescent probe as explained in Fig. 3.3A after 16 h of induction on complete medium containing 2% galactose. Average fluorescence is expressed as percentage of wild-type cells. (F) In vivo mitochondrial protein synthesis performed after 16 h of induction in whole cells in the presence of cycloheximide as in Figure 3.3F. (G) Quantification of the signals in F performed as in Figure 3.3G. Bars (hidden behind symbols in B) indicate means ± SD from at least 3 independent sets of measurements.
We tested the effect of *HAP4* over-expression on 103Q toxicity by assessing growth in solid and liquid galactose-containing media. We observed a significantly increased growth rate in *HAP4* overexpressing cells (Figures 3.4A and 3.4B). The partial suppression of cytotoxicity was not due to a decrease in polyQ expression and accumulation (Supplemental Fig. S4.3) or the formation of 103Q amorphous macroaggregates (Figure 3.4C). Rather, *HAP4* overexpression suppressed polyQ-induced mitochondrial toxicities. After 16 h of induction, cellular respiration was recovered to levels close to wild-type (Figure 3.4D) while $\Delta \Psi_m$ dissipation was partially prevented (Figure 3.4E) and the rate of mitochondrial protein synthesis was significantly increased (Figure 3.4F).

We subsequently tested whether *HAP4* overexpression was able to suppress the significantly milder 72Q-induced toxicity. In this strain, the respiratory capacity slightly declines to 80% of wild type after 16 h of induction (Figure 3.1D), and is restored to wild-type levels by *HAP4* overexpression (Supplemental Figure S3.4B). However, *HAP4* did not affect cytotoxicity in 72Q cells (Supplemental Figure S3.4A). In yeast cells, 80% of respiratory capacity is enough to support full growth in fermentable galactose-containing medium. Therefore, we assume that in 72Q cells, the observed decline in respiration is insufficient to contribute to the cell growth defect in galactose-containing medium. For this reason, Hap4p overexpression, although restoring respiratory capacity in 72Q cells, did not alleviate their mild growth defect. These results confirm that pathways other than mitochondrial dysfunction concurrently contribute to polyQ-induced toxicity. Instead, in cases of more acute toxicity, as documented for
103Q cells, increasing mitochondrial biogenesis by HAP4 overexpression partially but effectively suppresses polyQ-induced cytotoxicity.

3.5 DISCUSSION

Disturbances in mitochondrial metabolism and aerobic energy production have been associated with neurodegenerative disorders. Specifically, disruption of mitochondrial function has been documented in Huntington’s disease pathophysiology in patients, as well as in cellular and animal models of the disease. Here, we have used inducible yeast models of polyQ diseases based on expression of htt exon 1 with wild-type and expanded polyQ domains to investigate the mechanisms leading to decline of mitochondrial function and its contribution to the progress of polyQ toxicity.

Despite evidence in support of mitochondrial involvement in the pathogenesis of HD and other polyQ diseases, the exact underlying mechanism remains unknown. Several hypotheses have been proposed, including: 1) direct effects of polyQ domains on mitochondrial membranes (Choo et al., 2004; Panov et al., 2002), inducing mitochondrial permeability transition pore opening and Ca\(^{2+}\)-dependent depolarization in isolated mammalian mitochondria (Choo et al., 2004; Panov et al., 2003), 2) transcriptional dysregulation involving transcriptional factors and coactivators that control mitochondrial biogenesis (Cui et al., 2006; McCampbell et al., 2000), 3) defective expression and import of nuclear-encoded mitochondrial proteins (Benchoua et al., 2006), and 4) disruption of microtubule and actin cytoskeletal networks secondarily impairing
vesicular and mitochondrial trafficking in mammalian neurons in vitro and in vivo (Trushina et al., 2004).

Previous studies allowed us to propose that also in the yeast model a combination of direct and indirect mechanisms could account for polyQ-induced mitochondrial toxicities (Solans et al., 2006). Here, we have further explored the possibility that soluble, nonaggregated polyQ domains could directly affect mitochondrial function. Our results suggest that both, wild-type and expanded polyQ domains might interact with the outer mitochondrial membrane very early after expression is induced. However, only expanded polyQ would eventually contribute to disrupt the mitochondrial membrane biophysical properties and mitochondrial physiology, subsequently contributing to cytotoxicity. Expanded polyQ domains are known to misfold and serve as a seed for oligomerization. We envision that polyQ oligomers interacting with the outer mitochondrial membrane could be one of the causes of mitochondrial dysfunction. Supporting this possibility, polyQ-induced mitochondrial toxicities increase with the length of the polyQ tract and correlate with the decrease in growth rate. Increasing toxicity with increased polyQ expansion is a hallmark of neuronal polyQ pathology.

As a concurrent mechanism, misfolded/aggregated mutant polyQ could alter other structures in the cell, such as the cytoskeleton (Meriin et al., 2003; Solans et al., 2006), which were proposed to indirectly lead to disturbances in mitochondrial distribution, increase in reactive oxygen species (ROS) production and decline in mitochondrial function in our yeast models (Solans et al., 2006). We cannot discard that primary polyQ-induced alterations in other pathways
could also contribute to inducing mitochondrial toxicities. For example, endoplasmic reticulum (ER)-associated degradation and ER stress have been recently identified as early and specific events in polyQ toxicity (Duennwald and Lindquist, 2008). ER stress-induced ROS generation is known to produce mitochondrial dysfunction and further ROS generation, leading to cell death (Haynes et al., 2004).

In our models, mitochondrial membrane potential and the mitochondrial ability to regulate its volume by opening of mitochondrial unspecific channels is compromised by expanded polyQ. In HD, mutant htt has been proposed to damage neurons by inducing mitochondrial depolarization and altering mitochondrial calcium homeostasis in patients and in mouse models (Panov et al., 2002). In transgenic mice expressing full-length mutant huntingtin, these mitochondrial defects preceded the onset of pathological or behavioral abnormalities (Panov et al., 2002). Although previous studies had reported hyperpolarized mitochondria in apoptotic yeast 103Q cultures following 12 and 24 h of incubation (Sokolov et al., 2006), our models suggest that mitochondrial membrane potential alterations occur early and are not the result of apoptotic cell death. The apparent discrepancy could be accounted for by a higher level of 103Q expression in the models reported by Sokolov et al. (Sokolov et al., 2006), thus inducing a more acute toxicity. Our results show that 103Q cells have a significantly dissipated ΔΨm. In addition, similar to 25Q cultures, 72Q and 103Q cultures were found virtually free of dead cells at least up to 16 h of incubation.
We further show that in the yeast models, overall mitochondrial protein synthesis is progressively affected. The protein synthesis defect is not specific to mitochondria since cytoplasmic protein synthesis was also found significantly reduced upon mutant polyQ expression (Supplemental Figure S3.5). Because ER stress is an early event in polyQ toxicity (Duennwald and Lindquist, 2008), the reduction in cytosolic protein synthesis could be a consequence of the unfolded protein response pathway that modulates gene expression and down-regulates protein translation in an attempt to restore homeostasis (DuRose et al., 2009). A global mitochondrial and cytosolic protein synthesis defect would be expected to result in generalized respiratory chain enzymatic decline in late stages of toxicity. We previously reported a significant respiratory complex II and III decline in early stages of toxicity (Solans et al., 2006). While complex II is formed by four nuclear DNA-encoded subunits, complex III contains eleven subunits, only one of which, cytochrome b, is encoded in the mitochondrial DNA. Because these two enzymes contain iron-sulfur clusters, which are highly susceptible to oxidation, we had speculated that these two enzymes could have a particularly faster turnover in the presence of mutant polyQ, which we and others had shown to induce an early increase in ROS generation (Giorgini et al., 2005; Solans et al., 2006).

Searching for suppressors of polyQ toxicity has proven valuable for the understanding of the pathways involved and their contribution to cellular degeneration and death. In addition, they provide a substrate for development of therapeutic interventions in patients. The different suppression mechanisms
reported to date can be classified into two categories: those avoiding the accumulation of misfolded proteins, and those suppressing the deleterious effects of misfolded polyQ domains on specific cellular pathways. Toxicity can be reduced by enhancing the clearance pathways for the removal of cytoplasmic aggregate-prone proteins by increasing autophagy (Sarkar et al., 2009), activating the ubiquitin proteasome system (Bauer and Nukina, 2009) or repairing the endoplasmic reticulum-associated degradation (ERAD) pathway (Duennwald and Lindquist, 2008). Toxicity is also ameliorated by modulating the chaperone systems involved in protein refolding, aggregation, and disaggregation, thus shifting the balance towards the non-toxic species (Krobitsch and Lindquist, 2000; Vacher et al., 2005). Suppression of polyQ toxicity is also achieved by protecting the cells against some events downstream from polyQ misfolding and oligomerization. Among them, suppression of cytoskeletal instability (Kaminosono et al., 2008), restoration of impaired gene transcription (Cui et al., 2006), suppression of the kynurenine pathway to limit the accumulation of toxic metabolites that increase the generation of reactive oxygen species (Giorgini et al., 2005), and protection of mitochondrial integrity and function (Benchoua et al., 2006; Cui et al., 2006) have been documented to be effective in several disease models.

These downstream polyQ misfolding pathways seem to interconnect with each other, thus further increasing cellular toxicity. A connection between cytoskeletal disturbances and mitochondrial lesions was established in cultured neurons and in mouse models of polyQ toxicity by the observation that mutant
htt-induced disruption of axonal transport pathways leads to loss of mitochondrial motility and eventual mitochondrial dysfunction (Trushina et al., 2004). A link between transcriptional dysregulation and mitochondrial function impairment was recently proposed by the discovery that in mouse models, mutant huntingtin causes disruption of mitochondrial function by inhibiting expression of PGC-1α, a transcriptional coactivator that regulates several metabolic processes, including mitochondrial biogenesis and respiration (Cui et al., 2006). PGC-1α overexpression in these models significantly ameliorated mutant htt-induced toxicity (Cui et al., 2006). Recently, PGC-1α has been proposed to be a modifier of onset age in HD (Taherzadeh-Fard et al., 2009).

As a suppression mechanism that highlights the importance of mitochondrial dysfunction as a contributor to polyQ-induced toxicity, here, we report that over-expression of Hap4p, the catalytic subunit of the transcriptional activator Hap2,3,4,5p complex, which is known to increase mitochondrial biogenesis (Fontanesi et al., 2008; Lascaris et al., 2003), ameliorates 103Q toxicity. The suppression mechanism consists on preventing the severe decline in mitochondrial respiration and in other mitochondrial physiological parameters, including ΔΨm, that significantly affect cell growth. Hap4p overexpression did not alleviate the polyQ toxic effect on cytosolic protein synthesis globally (Supplemental Figure S3.5) but did significantly restore mitochondrial protein synthesis. The Hap2,3,4,5p complex regulates the coexpression of most nuclear-encoded subunits of mitochondrial respiratory chain enzymes and transcription/translation factors required for mitochondrial DNA expression to
facilitate the transition from fermentation to respiration during the diauxic shift. We envision that by increasing mitochondrial biogenesis, the cell becomes equipped with a larger buffering system against polyQ-induced mitochondrial injury. Furthermore, preserving aerobic energy production is expected to have a positive impact in mutant polyQ clearance pathways and refolding chaperone systems, which require ATP for functioning. Finally, we have to take into consideration that the suppression by Hap4p is only partial, which could indicate that pathways other than mitochondrial dysfunction are targets of mutant polyQ domains (e.g. cytoskeletal disturbances (Solans et al., 2006), ER stress (Duennwald and Lindquist, 2008) and decreased cytosolic protein synthesis) and concurrently contribute to polyQ-induced toxicity. Alternatively, mitochondrial disturbances could be, at least to some extent, secondary to upstream polyQ-induced toxicities as mentioned earlier.

We conclude that in our yeast models, mitochondrial dysfunction is one of the important contributors to polyQ-induced cytotoxicity. Strategies for increasing mitochondrial biogenesis, either alone or as part of multitarget therapeutic interventions, might prove valuable to combat polyQ expansion diseases by delaying the development of clinical symptoms in patients.

As a final remark, mitochondrial dysfunction has been associated with several neurodegenerative disorders other than HD, and other polyQ diseases, including Parkinson’s disease (PD) and Alzheimer’s disease. In yeast, PD has been modeled by overexpression of α-synuclein, a protein component of Lewy bodies (abnormal aggregates found in PD neurons) (Willingham et al., 2003).
preliminary analysis of mitochondrial function in the yeast PD model established in our laboratory has revealed a profound respiratory deficiency early following induction of α-synuclein expression (unpublished results). Further characterization of this model will allow us to discern whether the mechanisms underlying mitochondrial toxicities are similar in cells overexpressing α-synuclein and mutant polyQ domains.

3.6 SUPPLEMENTAL DATA

Supplemental Figure S3.1. LIVE / DEAD yeast viability test for cells expressing 25Q and 103Q. Wild type W303-1A cells expressing 25Q-GFP and 103Q-GFP were pre-grown on respiratory media containing 2% ethanol glycerol (YPEG) and subsequently inoculated on media containing 2% galactose (YPGal) to induce expression of the proteins. After 8, 16, 20 and 24 h of induction, LIVE/DEAD Yeast Viability Kit (Molecular Probes) was used to determine cell viability following the recommendations of the manufacturer. Dead cells display a diffuse yellow-green fluorescence upon staining with FUN-1, while live (metabolically active) cells contain cylindrical, red-fluorescent structures in their vacuoles. (A) Cells were mounted on slides and visualized under a fluorescence microscope using a 100X oil immersion objective. To simplify the figure, only 103Q cell images are shown. The bar represents 5μm. Some dead cells are indicated by white arrows (B) The graph represents the quantification of the viability status of two hundred W303-1A, 25Q-GFP and 103Q-GFP cells explored as shown in (A).
Supplemental Figure S3.2. Preparation of polyQ-aggregate free mitochondrial fractions. Wild type W303-1A cells expressing 25Q-GFP and 103Q-GFP were incubated for 240 or 30 min in the presence of galactose to induce polyQ expression, respectively. Mitochondria were prepared from these strains by the method of Herrmann to preserve the intactness of the outer membrane. Mitochondrial suspensions were subsequently stained with Mito-tracker Red and observed under fluorescent microscopy. Pictures were taken with the indicated exposition times. The bar represents 5 μm.

Supplemental Figure S3.3. PolyQ expression in HAP4 overexpressing cells. (A) Western blots analyses of cytoplasmic fractions obtained from 25Q cells, overexpressing or not HAP4, incubated for 16 h in the presence of galactose to induce polyQ expression. Western blots were probed with antibodies against GFP and against the cytosolic marker 3-phosphoglycerate kinase subunit 1 (Pgk1p). (B) Quantification of the signals in panel (A) performed by densitometry as in Figure 3.3G.
Supplemental Figure S3.4. Overexpression of HAP4 restores mitochondrial function but does not alleviate toxicity in cells expressing 72Q. (A) Ten fold serial dilutions growth test of the indicated strains pre-grown over-night in liquid WOEG media, spotted on solid YPD or YPGal media and incubated at 30°C. Pictures were taken after 2 days of incubation. 72Q/HAP4 #1, #2 and #3 are three independent HAP4 transformants of wild type cells expressing 72Q. (B) KCN-sensitive endogenous cell respiration measured polarographically in cells grown in the presence of galactose for 16 h. Wild type W303-1A and 72Q cells either carrying an empty vector or overexpressing HAP4 were included. The bars indicate the mean ± SD from at least three independent sets of measurements.

Supplemental Figure S3.5. Cytoplasmic protein synthesis in cells expressing 103Q. In vivo cytoplasmic protein synthesis in wild-type W303-1A (WT), 103Q cells and 103Q cells overexpressing HAP4 induced for 16 h. Cells were labeled with [35S]-methionine at 30°C for 5 and 10 min, separated by PAGE, transferred to a nitrocellulose membrane and exposed to X-ray film.
4.1 SUMMARY

Yeast chronological life span (CLS) is defined as the capacity of stationary cultures to maintain viability over time, thus mimicking the situation of post-mitotic cells in multicellular organisms. Cellular viability is typically determined by using the colony formation unit assay (CFU). CFU counting is simple but laborious and does not accommodate large-scale experiments. Importantly, viability is determined on the basis of the cell’s ability to divide and form a colony, probably not the ideal parameter when studying post-mitotic cellular ageing. This study describes the optimization and validation of a method based on the flow cytometric monitoring of propidium iodide (PI) uptake for assessing yeast cell death during CLS. The optimized protocol is quick, reliable, reproducible and can accommodate high-throughput studies. The method was validated by determining CLS of several strains used in yeast ageing research and by evaluating the effect of genetic disturbances known to extend or reduce yeast chronological life span.

4.2 INTRODUCTORY REMARKS

Biological ageing is characterized by the progressive decline in the ability of a cell or an organism to resist stress, damage or disease. Management of age-associated diseases to extend health-span is one of the most important
challenges for biomedicine in the 21st Century. Ageing is the greatest risk factor for the development of age-related diseases including neurodegenerative conditions. For this reason, understanding the fundamental molecular and metabolic alterations that occur during ageing of post-mitotic cells such as neurons is crucial not only to comprehend the ageing process but also for understanding the etiology of age-related neurodegenerative diseases.

In the last decades, research model organisms of diverse complexity have been exploited to gain insight into the molecular mechanism underlying the ageing process. Among them, the unicellular yeast *Saccharomyces cerevisiae* has become an invaluable tool in ageing research given its simplicity, the ease to perform genetic and molecular manipulations and importantly, its relatively short life span, ranging from several days to weeks (Fabrizio and Longo, 2003; Kaeberlein et al., 2007; Sinclair, 2002).

Yeast has two life spans, replicative and chronological, which have been studied as two different models of cellular ageing. Yeast replicative life span (RLS) is defined as the number of mitotic cycles completed by a mother cell before senescence. RLS has been proposed as a model that may mimic the ageing process of mitotically active cells in multicellular organisms (Steinkraus et al., 2008). On the other hand, yeast chronological life span (CLS), the focus of this report, is defined as the length of time that a non-dividing cell retains viability during stationary phase. Yeast CLS may be a suitable model for the ageing process of post-mitotic cells, such as neurons, in multicellular organisms (Fabrizio and Longo, 2007).
Using the yeast models of ageing, research efforts have been devoted to strategies for longevity extension, regarded as a retardation of biological ageing. These studies have provided insight into the mechanisms regulating the ageing process which are conserved from yeast to human (Fontana et al., 2010). The results of these studies are highly relevant for human health. Although longevity extension strategies will not eliminate ageing-related diseases, they are expected to postpone their age of onset, thus contributing to the objective of extending health-span (Colman et al., 2009). For example, caloric restriction (CR) is the most robust environmental intervention known to slow ageing and extend life span in yeast (both RLS and CLS), worms, fruit flies, rodents and primates through conserved mechanisms (Colman et al., 2009; Wei et al., 2008). Studies to understand the molecular mechanisms of CR mediated longevity, allowed for the identification of several longevity genes. In yeast, the Ras/cAMP/PKA, Tor (target of rapamycin) and Sch9 signaling pathways, conserved up to human, integrate the nutrient and other environmental cues to regulate cell growth, division and life span (Bitterman et al., 2003; Wei et al., 2008). Deletion of RAS2, TOR1 or SCH9 enhances cellular protection against thermal and oxidative stresses (Longo and Finch, 2003), induce a remodeling of mitochondrial metabolism (Bonawitz et al., 2007; Pan and Shadel, 2009) and extends yeast CLS. Inhibition of these pathways converges on the activation of stress resistance transcription factors that will induce the expression of heat shock proteins and antioxidant defenses (e.g. cytosolic and peroxisomal catalases – encoded by CTT1 and CTA1 respectively) and cytosolic and mitochondrial
superoxide dismutases – encoded by *SOD1* and *SOD2* (Mesquita et al., 2010; Wei et al., 2008). Over-expression of cytoplasmic Sod1 (5% localizes to mitochondria) and mitochondrial Sod2 were among the first genes identified to extend CLS in yeast (Longo et al., 1996).

With an increasing number of researchers using the yeast chronological life span model to gain insight into the ageing process of non-dividing cells, it could seem of apparent contradiction that the assessment of cell viability in stationary phase cultures has long revolved around the ability of the cells to divide. Typically, yeast CLS has been measured by growing cells in liquid media and determining cellular viability over time. Cells are inoculated and allowed to grow, usually in synthetic complete media, until they reach post-diauxic stationary phase. Once the cells have stopped cell division, determination of cellular viability in a population at different time points is usually achieved by scoring colony formation units (CFU) from a small sample of at best a few hundred cells, on nutrient agar plates (Parrella and Longo, 2008). While this method has proven to be useful, it is laborious and time consuming and will not be suitable for high-throughput studies involving a significant number of strains or growth conditions. In addition, this method does not discriminate situations where cells are still alive but have their ability to reproduce partially or completely compromised. Attempting to eliminate some of the shortcomings of the CFU assay, it was recently reported a new quantitative method to determine CLS that relies on monitoring outgrowth of ageing cells by measuring the optical density at 600 nm of liquid cultures using specific instrumentation (Murakami et al., 2008).
This method is automated and can be used for large high-throughput studies. However, like in the classical CFU method, cellular viability over time is determined exclusively by monitoring the capacity of the ageing cells to reproduce.

The aim of the present work was to explore the use of a vital staining with propidium iodide (PI) to identify dead cells and flow cytometry (FCM) to provide a quick, reliable, reproducible and precise quantification of multiple large samples (10 to 50 thousand cells) of chronologically ageing yeast populations. The method, frequently used in microbiology, relies on the exclusion of PI by viable cells in which the membrane forms a selectively permeable barrier between the cellular content and the environment. Dead cells will lose this membrane permeability and therefore become PI-stained, thus facilitating their identification by flow cytometry. We validated this method by determining CLS of several yeast genetic backgrounds used in ageing research and by evaluating the effect of genetic interventions known to reduce or extend yeast life span. We made parallel CLS assessments using the classical CFU assay and the results obtained by using both methods were compared. We conclude that the PI-FCM method for CLS analysis described here is quick, reliable and reproducible, with a capacity for high-throughput studies. It can be used alone or to complement CFU assessments. Therefore, the PI-FCM method is recommended to be routinely implemented in yeast ageing research laboratories.
4.3 MATERIALS AND METHODS

4.3.1 Yeast strains.

The *S. cerevisiae* strains used were the wild-types W303-1A (MATα ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1), BY4741 (MATα his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0) and DBY2006 (MATα his3-Δ200 leu2-3,-112 ura3-52 trp1-Δ1 ade2-1). The W303-1A rho0 lacking mitochondrial DNA and W303-1A cyc3Δ were available in the laboratory and have been previously described (Barrientos et al., 2003; Zambrano et al., 2007). The W303-1A tor1Δ and ras2Δ were created by one-step gene replacement by insertion of a kanamycin cassette. A fragment containing the kanamycin cassette and the flanking regions of TOR1 and RAS2 genes was amplified by PCR from the tor1Δ strain in the DBY2006 background obtained from Dr. Gerald Shadel (Bonawitz et al., 2007) and the ras2Δ strain in the yeast BY4741 from the knockout collection (Open Biosystems) using the following primers: TOR1 5´-GAG GGT CCT TGG GAT GCT ACT AAA GCC-3´ and 5´- GGG TAC TTG GAC GGA ATA GTG AG-3´; RAS2 5´-AAC GTA ATA GAT GAA ATG ACT T-3´ and 5´-TGT GAT ACC AAG ACC TTT TCG CGA C-3´. Successful disruption of the TOR1 and RAS2 genes was verified by PCR.

4.3.2 Growth conditions for chronological life span assessments.

All chronological life span determinations were performed in cells grown in liquid synthetic complete media containing glucose (SDC) supplemented with standard amount of amino acids and nucleotide bases as previously described (Sherman, 1991) including a four fold excess of the supplements tryptophan, leucine, histidine, methionine, adenine and uracil to avoid possible artifacts due
to the auxotrophic deficiencies of the strains. First, yeast strains from frozen stock (-80°C) were patched onto YPD agar plates (2% glucose) and incubated at 30°C. The following day, cells were inoculated into 10 ml of SDC media and grown overnight. After 24 hours, cells were inoculated into 50 ml of SDC media in 250-ml flasks to an optical density at 600 nm (OD$_{600}$) of 0.250. Cultures were grown with shaking (250 rpm) at 30°C. Maximum cell density is normally reached after 48 hours of growth in SDC, therefore 3 days after inoculation was considered as Day 0 of chronological life span. Subsequently, cellular viability was determined at days 0, 2, 4, 7, 9 and 11 by either PI-FCM or CFU assay as described below.

4.3.3 Propidium iodide staining and flow cytometry analysis.

Cell viability was analyzed using Propidium Iodide (PI) (Molecular Probes, Eugene, OR, USA). A sample containing 10$^6$ yeast cells per ml in phosphate-buffered saline (PBS) was incubated for 30 min at 30°C in the presence of 2μM PI. Flow cytometry analysis was performed on a Becton Dickinson (BD) LSRFortessa™ cell analyzer. Excitation was performed using a yellow/green laser at 561 nm; emission was detected using a 20 nm bandpass filter centered at 660 nm (Becton Dickinson, NJ, USA). For each yeast population, three samples of ten thousand cells were analyzed.

4.3.4 Colony formation unit assay.

To perform the colony formation unit (CFU) assay, cell number was estimated by optical density (OD) for each population and serial dilutions of different cultures were plated onto 3 or 4 YPD plates at an approximate
concentration of 100 cells per plate. Plates were incubated at 30°C for 48 hours and CFU were counted.

4.3.5 Fluorescence microscopy.

Wide-field fluorescence microscopy was performed to visualize PI-stained cells. We used an Olympus fluorescence BX61 microscope equipped with Nomarski differential interference contrast (DIC) optics, a Uplan Apo 100× objective (NA 1.35), a Roper CoolSnap HQ camera, and Sutter Lambda10-2 excitation and emission filter wheels, and a 175 W Xenon remote source with liquid light guide. Briefly, PI-stained cells were mounted on to slides and examined using a CY3 filter. Images were acquired using SlideBook 4.01 (Intelligent Imageing Innovations, Denver, CO, USA).

4.3.6 Statistical analysis.

All experiments were done at least in triplicate and some repeated up to eight times. The number either of PI’ cells or CFU at day 0 (72 hours after inoculation) was considered to be the initial survival (100%) and was used to calculated percentage of survival at the different time points. Data are presented as means ± SD.

The mean and maximum survival time was calculated based on the survival integral (SI) for each experiment. SI is defined as the area under the survival curve and can be estimated by the formula:

\[SI = \sum_{n=2}^{n} \left( \frac{s_{n-1} - s_{n}}{2} \right) (age_{n} - age_{n-1})\]

where \(age_{n}\) is the age-point (e.g. 2, 4, 7, 9, 11 days) and \(s_{n}\) is the percent of yeast that has survived at that age-point (Murakami and Kaeberlein, 2009). The
survival curves for the two methods (PI-FCM and CFU), and for two different yeast strains were compared as following: the survival integral (SI) of each survival curve was calculated by the formula above and then the SIs for the two methods or two different yeast strains were compared using two sample $t$-test.

4.4 RESULTS

4.4.1 Propidium iodide staining of yeast cells during the stationary phase of growth.

Propidium iodide (PI) is a fluorescence dye that has been extensively used to study cellular viability (Achilles et al., 2006; Darzynkiewicz et al., 1994; Deere et al., 1998; Hutter, 1993; Prudencio et al., 1998) as well as to measure DNA content in cell cycle research (Sabatinos and Forsburg, 2009). Propidium iodide can be used in a wide range of cell types from bacteria to mammalian cells including unicellular eukaryotic organisms such as the yeast Saccharomyces cerevisiae. Typically, methods involving PI staining in yeast have been used in studies aiming to assess cell viability and cell cycle progression in rapidly dividing cells during the logarithmic phase of growth (Achilles et al., 2006; Deere et al., 1998; Prudencio et al., 1998). PI staining in combination with Anexin V staining and FCM analysis has been also reported to be useful to discriminate necrotic from apoptotic cells at early time points during the stationary phase (Buttner et al., 2008; Buttner et al., 2007; Herker et al., 2004). However, it has not been systematically used to determine yeast chronological ageing. Such a method would allow for the assessment of yeast chronological life span using a cell viability scoring method independent of cellular reproduction.
However, there could be a chance that PI staining of stationary phase cells might not be effective and produce artifactual results. Actually, following the growth of a yeast cell population, once the carbon source has been exhausted and cells enter in post-diauxic stationary phase, significant changes occur in the cells including modifications in cellular metabolism (Fabrizio and Longo, 2003), energy storage (Enjalbert et al., 2000; Samokhvalov et al., 2004), protein synthesis as well as plasma membrane and cell wall composition (Smith et al., 2000; Werner-Washburne et al., 1993). Since PI staining discriminates live and dead cells based on membrane permeability the first question we asked was whether PI staining could be used to determine cell viability after the cells have entered stationary phase and changes in the cell wall have occurred. To evaluate the capacity of PI to monitor cell viability during stationary phase we allowed yeast cells to reach stationary phase and used PI to assess cell death. W303-1A cells were inoculated in synthetic complete media containing glucose (SDC) (as described in Material and Methods). Following 72 h of growth, when the cells have already reached stationary phase, three samples were prepared. In a control sample, cells were killed by heat shock treatment of 5 minutes at 95ºC. In another sample, stationary phase cells were not further treated. In a third sample, aliquots of the first two samples were combined in equal amounts. As a proof of principle, cell viability in the three samples was assessed by PI staining. Cells were initially monitored under a fluorescence microscope and subsequently analyzed by flow cytometry.
Figure 4.1. Propidium iodide (PI) staining of yeast cells during the stationary phase of growth. W303-1A cells were inoculated in synthetic complete media containing 2% glucose (SDC) and allowed to reach stationary phase following 72 h of growth. At that point a sample of cells (dead cells) was killed by heat shock (5 min at 95°C). Samples of untreated cells (live cells), dead cells and a 1:1 mixture of both samples were prepared. Subsequently, the PI exclusion method was used to discriminate live vs dead cells and samples from the different population were both (A) visualized using fluorescence microscopy and (B) analyzed by flow cytometry using a yellow/green laser at 561 nm for excitation and a 20 nm bandpass filter centered at 660nm (PE-Cy5-A channel) to record the emission. P2 represents the population of PI$^+$ cells.
Direct fluorescence microscopic counting showed that in the sample containing live cells, most cells were detected as PI negative (PI−) indicating the presence of an intact plasma membrane (Figure 4.1A). On the contrary, in the sample containing heat shock-killed cells, PI was able to enter the cells and display an intense fluorescence (Figure 4.1A). Finally, in the mixture containing live and dead cells, PI− and PI+ cells were scored in a proportion close to 50% in agreement with the ratio used to prepare the sample (Figure 4.1A). The samples were subsequently analyzed to determine the flow cytometric measurement of PI uptake in 10,000 cells per assay. In samples containing untreated cells, in the conditions used, 75% of the cells were found to be PI− or live cells (Figure 4.1B). In heat treated controls, 99% of the cells were detected as PI+ and finally in samples containing a mixture of treated and untreated cells a perfect separation of live and dead cells was achieved (Figure 4.1B). These results are taken as a proof of principle to demonstrate that PI staining can be used to determine cell viability during stationary phase and therefore may be suitable to study chronological ageing.

4.4.2 Assessment of yeast chronological life span by propidium iodide staining and flow cytometry.

To evaluate the potential of propidium iodide staining and flow cytometry as an alternative or complementary method to determine yeast chronological life span, we performed a standard ageing experiment using PI and FCM to determine cell viability and construct a survival curve.
Figure 4.2. Chronological life span of W303-1A cells determined by propidium iodide (PI) staining and flow cytometry (FCM) analysis. (A) W303-1A cells were inoculated in synthetic complete media containing 2% glucose (SDC) and allowed to reach stationary phase (72 h). Subsequently, cell viability was determined at day 0, 2, 4, 7, 9 and 11 by PI staining and FCM analysis. (B) Chronological life span curve of W303-1A cells. Data represents percentage of live cells relative to day 0 as a function of time in stationary phase.
We run a typical chronological ageing experiment by inoculating W303-1A cells in synthetic complete media containing glucose (SDC) and allowing them to grow for 72 h to enter in stationary phase. This point was considered the beginning of the chronological ageing or day 0. Subsequently, we analyzed cellular viability by PI staining and FCM analysis at days 0, 2, 4, 7, 9 and 11. At day 0, 82.2% of the cells in the population were PI⁻ and thus alive and only 17.8% were PI⁺ and considered dead (Figure 4.2A). As expected, at consecutive days of chronological ageing, the percentage of PI⁺ (dead cells) progressively increased while the percentage of cells that retained membrane integrity and excluded PI became proportionally reduced (Figure 4.2A). The experiment was finished at day 11 when the number of PI⁻ cells was lower than 10%. The fraction of alive (PI⁻) cells at each time point was calculated as percentage of the cells alive at time 0. The data obtained was used to construct a survival curve by plotting the percentage of PI⁻ cells vs time (Figure 4.2B).

4.4.3 Validation of propidium iodide staining and flow cytometry as a method to determine yeast chronological life span.

Any new analytical method must pass the tests of reliability and reproducibility. To determine the reliability of the PI-FCM approach, we repeated the experiment and used the same yeast cell population to assess CLS using both PI-FCM and CFU methods. As shown in Figure 4.3A, the survival curves obtained in both cases are very similar and extensively superimpose, although consistently, in late stages of chronological age, the cells seem to lose the ability to reproduce more quickly than they lose the integrity of their membranes. This is probably due to the accumulation of alive pre-apoptotic cells that have already
lost their proliferative capacity as previously reported by other groups (Prudencio et al., 1998).

To assess the reproducibility of the PI-FCM method to study yeast chronological life span, we performed four independent in parallel (at the same time) repetitions (to analyze "intra-experimental" variability) and four independent repetitions performed at different weeks (to analyze “inter-experimental” variability) to determine the chronological life span of W303-1A. As shown in Figure 4.3B, repetitions started at the same time and carry out under the same exact conditions (i.e. same constant shaking speed, temperature, etc.) displayed a minimal “intra-experimental” variation and reported a median life span for W303-1A of 5.50 days (95% confidence interval 5.11 , 5.89) and a maximum life span of 10.7 days (95% confidence interval 10.5 , 10.9). On the other hand, repetitions executed during consecutive weeks, showed a slightly larger “inter-experimental” variation typical of ageing experiments performed at different times. Based on independent repetitions, the median life span of W303-1A was 4.98 days (95% confidence interval 4.24 , 5.73) and the maximum life span was 10.1 days (95% confidence interval 8.7 , 11.4) (Figure 4.3 C). In both case, the median and maximum life span calculated for W303-1A cells using the PI-FCM method were in agreement with previously reported average values for W303-1A life span in the growth conditions used here, determined by using the classical CFU method (Burtner et al., 2009; Magherini et al., 2009).
Figure 4.3. Validation of propidium iodide staining and flow cytometry as a method to determine yeast chronological life span. (A) Comparison of chronological life span curves of W303-1A cells determined by PI staining and FCM analysis or CFU obtained as in Figure 4.2B and explained in the Materials and Methods section. (B) Reproducibility of chronological life span determinations of W303-1A populations by PI-FCM tested in four independent experiments performed at the same time (intra-experimental variability). (C) Reproducibility of chronological life span determinations of W303-1A populations by PI-FCM tested in four independent experiments performed in successive weeks (inter-experimental variability). (D) Chronological life span of BY4741 and DBY2006 wild type strains determined by either PI-FCM analysis or CFU counting as explained in panel A. Data represents percentage of live cells relative to day 0 as a function of time in stationary phase. W303-1A wild type has been included for comparison purposes.

Together, these experiments demonstrate the high degree of reproducibility of CLS determination using this novel method. In all the experiments reported in Figures 4.3D and 4.4, triplicates were performed with independent cultures at the same time. A special mention should also be made about the extremely small standard deviation among the three samples of ten thousand cells used for each of the single data points when using PI-FCM analysis. Consistently, this standard deviation was lower than 1%, which
represents a great improvement in the accuracy of CLS analysis when compared
to methods such as CFU, characterized by a much larger experimental error.

The different *S. cerevisiae* strains commonly used in the laboratory are
known to have significant genetic variability (Mortimer and Johnston, 1986; van
Dijken et al., 2000). With the continuous expansion of yeast ageing research and
the involvement of a multiplicity of laboratories, researchers have found that
chronological life span extensively varies among different genetic background
(Fabrizio and Longo, 2003). To evaluate the potential use the PI-FCM method in
yeast ageing research, we decided to determine CLS of two different yeast
genetic backgrounds commonly used in this field, the short lived strains BY4741
(Burtner et al., 2009; Murakami et al., 2008; Powers et al., 2006) and DBY2006
(Bonawitz et al., 2007; Pan and Shadel, 2009). The long lived W303-1A strain
was used as a control. In agreement with previous results, W303-1A cells
displayed a CLS of around 10.5 days. The CLS determination of BY4741 and
DBY2006 was virtually identical when using the CFU or the PI-FCM methods to
discriminate dead and live cells. While BY4741 cells showed a maximum life
span of approximately 8 days, DBY2006, which are shorter lived, displayed a
maximum life span of around 3 days (Figure 4.3D). The survival integrals for
BY4741 and DBY2006 were compared to W303-1A by *t*-test and the
chronological life span of both stains was found to be significantly shorter than
that of W303-1A (W303-1A vs. BY4741 *p*-value = 0.00013, W303-1A vs.
DBY2006 *p*-value = 5.554E-09). These results are consistent with those reported
in the literature by several research groups (Burtner et al., 2009; Pan and Shadel, 2009).

4.4.4 Using propidium iodide staining and flow cytometry to assess the effect of genetic disturbances on yeast chronological life span.

Yeast has become a very valuable model system to identify genetic and environmental interventions that extend or reduce life span. The identification and study of these “modulators” of ageing is contributing towards understanding the regulation of the ageing process. We aimed to test the robustness of the PI-FCM method by using it to assess cellular viability of strains with expected reduced or extended CLS.

For this purpose, we have evaluated the life span of two respiratory deficient strains, W303-1A rho<sup>0</sup> and Δcyc3, expected to have a chronological life span shorter than wild type. It has been reported that intact mitochondrial function is required for maximum CLS of yeast populations (Aerts et al., 2009; Bonawitz et al., 2006). Rho<sup>0</sup> cells are cells devoid of mitochondrial DNA which encodes several essential components of the mitochondrial respiratory chain and therefore are unable to respire. The mutation in Δcyc3 cells affects the gene coding for the cytochrome c heme lyase that provides heme to apo-cytochrome c. Therefore, in the absence of CYC3 no functional cytochrome c is accumulated and the cell loses its ability to respire. We analyzed CLS in these strains by the PI-FCM method as well as CFU counting as described earlier. W303-1A wild type cells were included as a control. Both respiratory deficient strains, W303-1A rho<sup>0</sup> and Δcyc3, exhibited a significantly shorter life span when compared to wild type cells, with more than 90% of the cells no longer retaining viability after only 4
days (Figure 4.4A). The survival integrals for W303-1A $\rho^0$ and $\Delta$Cyc3 were compared to W303-1A by t-test and chronological life span for both stains was found to be significantly shorter than W303-1A CLS (W303-1A vs. W303-1 $\rho^0$ $p$-value = $8.896E^{-08}$, W303-1A vs. $\Delta$Cyc3 $p$-value = $2.542E^{-08}$). The results obtained by the PI-FCM and CFU protocols were similar but not identical. For both mutant strains, and particularly for the $\rho^0$ strain, statistical analysis comparing the survival integrals obtained by both methods showed we that the cells lose their capacity to divide and form a colony prior to losing the integrity of the membrane permeability barrier ($\rho^0$ PI vs. $\rho^0$ CFU $p$-value = 0.00016).

We subsequently assessed CLS in two mutant strains, $\Delta$tor1 and $\Delta$ras2, with deletions in nutrient sensor pathways known to extend chronological life span compared to wild type cells as mentioned in the Introduction (Longo and Finch, 2003; Wei et al., 2008). PI staining combined with FCM analysis and CFU assay were performed as previously described to analyze the chronological life span of both mutant strains using W303-1A wild type cells as control. The results obtained by both methods were equivalent. More than 50% of $\Delta$tor1 and $\Delta$ras2 cells remained alive after day 11 when most of wild type cells had lost viability (Figure 4.4B). After comparing the survival integrals for W303-1A $\Delta$tor1 and $\Delta$ras2 to W303-1A by t-test, the chronological life span for both stains was found to be significantly longer than W303-1A (W303-1A vs. W303-1 $tor1\Delta$ $p$-value = $6.223E^{-10}$, W303-1A vs. $ras2\Delta$ $p$-value = $5.777E^{-10}$).
Figure 4.4. Chronological life span of W303-1A strains with reduced or extended life span determined by propidium iodide (PI) staining and flow cytometry (FCM) analysis or colony formation units (CFU). (A) Chronological life span of respiratory deficient strains. W303-1A cells lacking mitochondrial DNA (rho<sup>0</sup>) and cyc3Δ cells were inoculated in synthetic complete media containing 2% glucose (SDC) and allowed to reach stationary phase (72 h). Subsequently, cell viability was determined by PI staining and FCM analysis or CFU. (B) Chronological life span of tor1Δ and ras2Δ strains. W303-1A tor1Δ and ras2Δ cells were grown as previously described. Subsequently, cell viability during stationary phase was determined by PI staining and FCM analysis or CFU. In both panels, data represents percentage of live cells relative to day 0 as a function of time in stationary phase. W303-1A rho<sup>+</sup> wild type has been included for comparison purposes.
These results are in agreement with previous reports showing that cell populations carrying the tor1Δ and ras2Δ mutations have significantly longer life spans than wild type cells (Wei et al., 2008). Particularly in the case of ras2Δ strain, the comparison of the survival integral using PI-FCM and CFU methods showed that the failure in the ability of cells to form colonies significantly preceded in time the loss membrane integrity (ras2Δ PI vs. ras2Δ CFU p-value = 0.0006). This observation highlights the importance of analyzing more than one parameter when studying yeast CLS and justifies the use of an approach, such as the PI-FCM method presented here, that does not rely on cellular reproduction.

Taken together, the results presented in this section demonstrate that PI staining combined with FCM analysis is a precise and robust method to study yeast CLS not only of wild type strains but also strains characterized by shorter or longer life span as a result of specific genetic disturbances.

4.5 DISCUSSION

4.5.1 What makes the propidium iodide staining and flow cytometric method valuable for yeast chronological ageing assessment.

The PI-FCM method described here is simple, rapid, reliable and reproducible. The simplicity of the method stands on the straightforward cell staining technique with PI and an automated FCM analysis. Once the cells are stained, a large amount of cells, usually 30,000 cells per population, can be accurately analyzed in a matter of seconds, certainly a quick turnaround. We have shown that the method is reliable, based on the broad agreement of the
data obtained with the traditional CFU assay and previously reported data. Also, the consistency of the results obtained from the independent experiments presented here indicates that the method is reproducible.

The PI-FCM method relies on the use of propidium iodide as a fluorescent dye that is excluded by an intact membrane permeability barrier in live cells but will stain dead cells with compromised membrane integrity. In contrast to classical CFU analysis, the method is not based on cell proliferation, which is an important distinction when estimating viability of non-dividing post-mitotic cells such as during yeast CLS.

Although the PI–FCM and CFU methods produce similar data, it is important to keep into consideration that they measure two different parameters. Some genetic or environmental perturbations could affect the ability to divide of cells in stationary phase populations while they could retain membrane integrity as a marker of life. Similarly, some stresses could induce transient breakage of the membrane permeability barrier while the cells retain their capacity to reproduce. Recently Davey et al., described that under certain levels of stress, a subpopulation of cells (approximately 7% of total cells) can transiently lose their membrane permeability and uptake propidium iodide, however short incubations after stress allow for the repair of the membrane damage (Davey and Hexley, 2011). As a consequence, this subpopulation of cells could be classified as dead cells during the analysis of chronological life span using PI–FCM. Despite the fact that this subpopulation of cells represents a small percentage, under the conditions used in this work and based on the comparison between PI–FCM and
CFU methods, we believe that if any, the population of cells that could have lost membrane permeability (PI+) but retained the ability to form a colony was insignificant.

In contrast, our studies have shown that in some instances, as exemplified in the cases of respiratory deficient strains or long-lived strains presented here, CLS determination by the PI-FCM method can provide a slightly different picture of cellular viability than CFU counting. In these cases, we speculate that the differences observed between the two methods could be theoretically explained by several reasons. First, cells undergoing cellular senescence and committed to programmed cell death or apoptosis could be in a pre-apoptotic state in which cellular division is already compromised while membrane integrity remains unaffected (Prudencio et al., 1998). Second, this population of cells could be in a non-reversible quiescent state in which cells have entered into a G0 phase of the cell cycle and are unable to re-enter the cell cycle after restoration of growing conditions because they have experienced some physiological change that make them unable to reproduce, as previously reported (Allen et al., 2006). In this line, particularly in the case of respiratory mutants, it could be plausible that cell membrane permeability might be maintained while intracellular energy levels could be too low for cells to be able to re-enter the cell cycle and form a colony.

An important advantage of the PI-FCM approach to study yeast CLS is the use of flow cytometry for the final analysis of the samples. This approach allows for the optional combination of cellular viability determination via PI staining with the assessment of additional cellular parameters, such as levels of reactive
oxygen species or mitochondrial membrane potential, simply by using specific fluorescence dyes. Additionally, expression of proteins with a potential impact on cellular ageing, such as those known to misfold and aggregate, could be easily studied by fusion to fluorescence tags. Finally, it is important to recognize that in contrast to the classical CFU technique, the PI-FCM method has the capacity to accommodate high-throughput studies, since PI staining and FCM analysis could be conveniently performed for a large number of strains or growth conditions using available modern automatic systems and multi-well plates. Nevertheless, after potential strains of interest or suitable growth/environmental conditions have been identified, confirmation analysis should be carried out in combination with other methods available to guarantee the avoidance potential artifacts.

4.5.2 Conclusions.

The simple technological conditions described here allowed the definition of a quick, precise, reproducible and robust propidium iodide staining based flow cytometric protocol (PI-FCM) for the quantitative assessment of cellular viability during yeast chronological life span. Although the method is straightforward and it should be possible to use it routinely, it should be emphasized that when the PI-FCM protocol is to be applied to yeast strains growing in conditions different than those reported here, preliminary experiments should be carried out to establish the optimal staining conditions and flow cytometer analysis settings. The method was validated by determining CLS of several yeast genetic backgrounds used in ageing research and by evaluating the effect of genetic interventions known to reduce or extend yeast life span. Thus, this method allows for the assessment of
post-mitotic cell viability and can be useful in all simple and high-throughput studies aiming to determine CLS in any yeast cell population, in screens for CLS modifiers as well as to determine CLS in yeast models of human age-related disorders affecting post-mitotic tissues.
5.1 SUMMARY

We have comprehensively explored the role of mitochondrial function on aging by genetically and pharmacologically modifying yeast cellular respiration and concomitant reactive oxygen species (ROS) production during the exponential and/or stationary growth phases, and determining their effects on chronological life span (CLS). Our results demonstrate that although respiration is essential during both phases for standard CLS, yeast cells have a large respiratory capacity and only deficiencies below a threshold (~40% of wild-type) significantly curtail CLS. Respiration controls CLS by altering stress resistance and particularly metabolism of storage nutrients, independently of ROS production during growth. Trehalose supplementation to media restores wild-type CLS to respiratory null cells. Furthermore, we show that caloric restriction (CR) extends CLS of cells respiring during growth above a threshold (at least ~45% of wild-type) and abolishes the requirement of respiration during the stationary phase. We conclude that mitochondrial respiratory thresholds regulate yeast CLS and its extension by caloric restriction.

5.2 INTRODUCTORY REMARKS

Biological aging can be defined as the progressive decline in the ability of a cell or an organism to resist stress, damage or disease. Eukaryotic biological
systems rely on oxidation and aerobic energy production for sustainability, and as a consequence are permanently exposed to the effects of reactive oxygen species (ROS) generated as respiratory byproducts. Among the multiple theories proposed to explain the aging process, the free radical/mitochondrial theory of aging (Harman, 1972; Miquel et al., 1980) is resisting the passage of time. According to this theory, production of ROS during respiration damages mitochondrial DNA (mtDNA) and disturbs aerobic energy production, setting up a "vicious cycle". Here a progressive energetic decline and ROS-induced cumulative oxidative damage to cellular macromolecules eventually overwhelms stress resistance and self-repair systems, thus resulting in further functional decline and aging. Although this is a longstanding theory, evidence supporting and contradicting its postulates, gathered from studies on diverse model organisms, continue to accumulate in the literature (Bonawitz and Shadel, 2007; Ristow and Schmeisser, 2011; Trifunovic and Larsson, 2008). Mice expressing an error-prone version of the catalytic subunit of mitochondrial DNA polymerase accumulate a substantial burden of somatic mtDNA mutations that limits respiration, and are associated with premature aging phenotypes and reduced life span although without affecting ROS production (Trifunovic et al., 2004). On the contrary, a mild increase in ROS has been reported in long-lived flies and worms carrying mutations that cause partial disruption of the mitochondrial respiratory chain (Miwa et al., 2004; Rea, 2005). Furthermore, overexpression of superoxide dismutases SOD1 and SOD2 moderately extends life span in yeast, flies and worms (Longo and Fabrizio, 2002). Overall, these studies suggest that
although oxidative damage is an important contributor, there is more to aging than ROS overproduction.

Over the last two decades, *Saccharomyces cerevisiae* models of aging have provided invaluable contributions toward the discovery of conserved longevity factors that modulate aging in mammals (Fontana et al., 2010; Kaeberlein, 2010). The facultative aerobe/anaerobe condition of *S. cerevisiae* provides an excellent opportunity to study how mitochondrial function and respiratory ROS production modulate aging. It has been established that lack of mitochondrial respiration severely affects the ability of yeast cells to achieve a standard wild-type chronological life span (CLS) (Aerts et al., 2009; Bonawitz et al., 2006; Ocampo and Barrientos, 2011b). CLS is a model for the aging process of post-mitotic cells, defined as the capacity of postdiauxic stationary (*G₀*) cultures to maintain viability over time. Mitochondrial function plays important roles in CLS extension mechanisms. This is the case of yeast strains with reduced “target of rapamycin” (TOR) nutrient sensor signaling, a longevity factor conserved from yeast to mammals. TOR-deficient yeast cells have a larger amount of mitochondrial respiratory chain enzymes, increased coupled respiration and ROS superoxide production which provide an adaptive signal during growth that extends CLS (Bonawitz et al., 2007; Pan et al., 2011; Pan and Shadel, 2009).

Caloric restriction (CR) is an intervention that extends the life spans of a variety of eukaryotic organisms from yeast to mammals. The mechanisms by which CR operates involve inhibition of nutrient-responsive kinases such as TOR
and Sch9 (Wei et al., 2008), a consequent general enhancement of stress resistance mechanisms, and metabolic remodeling involving a shift from fermentation to respiration which is particularly essential for CR extension of yeast CLS (Oliveira et al., 2008).

For CLS studies, yeast cells are usually aged in media containing 2% glucose. Under these conditions, cells rapidly divide while producing energy preferentially by fermenting pyruvate to ethanol, which is mostly secreted into the medium, while respiration is repressed in a glucose concentration-dependent manner. As glucose is being consumed, growth slows down and the diauxic shift occurs, involving up-regulation of nuclear genes important for mitochondrial biogenesis and use of ethanol for energy production through oxidative phosphorylation. Chronological life typically begins when cell division stops and cells rely on their own nutrient storages (such as glycogen and trehalose) for survival. It has recently been proposed that accumulation in the culture medium of toxic acetic acid produced during ethanol metabolism induces an apoptosis-like response involving mitochondrial ROS production that limits yeast CLS (Burtner et al., 2009; Kaeberlein, 2010) and that CR (usually 0.5% glucose) extends CLS by favoring respiratory metabolism and reducing the accumulation of acetic acid (Burtner et al., 2009). While acetic-acid induced cell death can mask the role of cell-intrinsic factors on modulating yeast longevity, we have recently shown that at least in the case of cells with reduced TOR signaling, increased mitochondrial respiration and ROS production are cell-intrinsic factors regulating CLS (Pan et al., 2011). However, how changes in mitochondrial
respiration and ROS generation interplay to regulate CLS remain largely unexplored.

For this study, we have comprehensively analyzed the role of mitochondrial function in yeast CLS by modulating respiratory function using genetic and pharmacological interventions. We report that yeast cells have a surprisingly large respiratory capacity to support standard wild-type CLS, which is curtailed only when respiration drops below a ~40% of wild-type threshold during exponential and stationary phases of growth. Cells respiring below the threshold display limited CLS owing to cell-intrinsic factors including rapid exhaustion of cellular energy storage, which can be reverted by trehalose supplementation of the growth medium. Our data suggest that respiration modulates CLS independently of ROS production during growth. Furthermore, we show that cells respiring above the threshold can benefit from CR-induced wild-type CLS extension, and that CR suppresses the requirement of respiration during stationary phase as a dominant trait of the program that extends CLS.

5.3 MATERIAL AND METHODS

5.3.1 Yeast strains, culture conditions.

The *S. cerevisiae* strains used included the wild-types W303-1A and BY4741. All strains carrying mutations affecting components of the mitochondrial respiratory chain were constructed in the W303 genetic background and are listed in Table 5.1.
Table 5.1. Genotype and source of *S. cerevisiae* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>aW303-1A</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1</td>
<td>Gift of Dr. Rothstein</td>
</tr>
<tr>
<td>aBY4741</td>
<td>MATa his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>Open Biosystems</td>
</tr>
<tr>
<td>aW303 rho&lt;sup&gt;0&lt;/sup&gt;</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1, rho&lt;sup&gt;0&lt;/sup&gt;</td>
<td>(Zambrano et al., 2007)</td>
</tr>
<tr>
<td>aW303 cyc3Δ</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcyc3::URA3</td>
<td>(Barrientos et al., 2003)</td>
</tr>
<tr>
<td>aW303 shy1Δ</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δshy1::URA3</td>
<td>(Barrientos et al., 2003)</td>
</tr>
<tr>
<td>aW303-C199</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δmss51::HIS3 + LEU2::Yip351-mss51&lt;sup&gt;C199&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>aW303 cox5aΔ</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox5a::HIS3</td>
<td>(Glerum and Tzagoloff, 1997)</td>
</tr>
<tr>
<td>aW303 cyc1Δ</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcyc1::URA3</td>
<td>(Barrientos et al., 2003)</td>
</tr>
<tr>
<td>aW303 + YEp352</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 + YEp352</td>
<td>(Ocampo et al., 2010)</td>
</tr>
<tr>
<td>aW303 + HAP4</td>
<td>MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 + YEp352-HAP4</td>
<td>(Ocampo et al., 2010)</td>
</tr>
</tbody>
</table>

5.3.2 Chronological life span determination.

All chronological life span determinations were performed in cells grown in liquid synthetic complete media containing 2% glucose (SDC) supplemented with standard amounts of amino acids and nucleotide bases as previously described (Sherman, 1991) including a four fold excess of the supplements tryptophan, leucine, histidine, methionine, adenine and uracil to avoid possible artifacts due to the auxotrophic deficiencies of the strains (Ocampo and Barrientos, 2011b; Sherman, 1991). For calorie restriction (CR) experiments, glucose concentration was reduced to 0.5%. For buffered media experiments, media was supplemented to final 80 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM citric acid (pH 4.7) prior to cell inoculation. Briefly, yeast strains from frozen stock (-80°C) were patched onto YPD agar
plates (2% glucose) and incubated at 30ºC. The following day, cells were inoculated into 10 ml of SDC media and grown overnight. After 24 hours, cells were inoculated into 50 ml of SDC media in 250-ml flasks to an optical density at 600 nm (OD<sub>600</sub>) of 0.250. Cultures were grown with shaking (250 rpm) at 30ºC. Maximum cell density is normally reached after 48 hours of growth in SDC, therefore 3 days after inoculation was considered as Day 0 of chronological life span. Subsequently, cellular viability was determined at the indicated days by colony formation unit (CFU) assay. Briefly, cell number was estimated by optical density (OD) for each population and serial dilutions of different cultures were plated onto 3 or 4 YPD plates at an approximate concentration of 100 cells per plate. Plates were incubated at 30ºC for 48 hours and CFU were counted.

5.3.3 Chemical treatments.

Cell cultures were supplemented with either 50μM antimycin A (Sigma) or 10μM oligomycin (Sigma). The treatments started either in the exponential phase when the cells were first inoculated or in the stationary phase 72h after inoculation. Fresh doses of the inhibitors were added every other day. In untreated cultures, an equal volume of drug vehicle (ethanol) was added as a control.

5.3.4 Endogenous cell respiration.

Endogenous cell respiration was assayed polarographically using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK) at 30ºC as described previously (Barrientos et al., 2002). For exponential phase respiration, cells were grown overnight in SDC media, re-inoculated in fresh SDC media at
the same confluence (OD$^{600} = 0.250$) and grown for 3-6 h, as indicated, to measure maximal cellular respiration. For stationary phase respiration, cell were treated as describe above and grown for 72 h (day 0) before measure respiration, to allow cell to reach stationary phase. The specific activities reported were corrected for KCN-insensitive respiration.

5.3.5 Media-swap experiments.

In the media-swap experiments, cultures were inoculated to OD$^{600}$ of 0.250 in 50 ml SD and grown for 72 h. Cells were pelleted and subsequently resuspended in the original medium (nonswap) or equivalently conditioned medium of the indicated strain (swap).

5.3.6 Quantification of reactive oxygen species (ROS).

Samples (equivalent of OD$^{600} = 2$) were taken from the indicated cultures, and the cells were pelleted. After removal of the media, the cell pellets were resuspended in phosphate-buffered saline (PBS) (500 µl containing 50 µM dihydroethidium (DHE; Molecular Probes) and incubated at 30°C for 10 min. Cells were then pelleted again, washed once with PBS and resuspended in PBS (500 µl) without dye. Flow cytometry was carried out on a Becton-Dickinson FACSCalibur flow cytometer. The DHE fluorescence indicated is the direct output of the FL3 (red fluorescence-detecting) channel without compensation. A total of 25,000 cells were analyzed for each curve.

5.3.7 Acetic acid detection assay.

Enzymatic detection of acetic acid was performed using Megazyme Acetic Acid Kit, following the manufacturer’s protocol. To measure the media acidic acid
concentration, 1 ml of stationary phase cells were pelleted and the supernatant were taken for the measurement media acidic acid concentration. The samples from the prior step were diluted 1:10 before the measurement. To calculate the sample acidic acid concentration, a standard curve with titration amount of acidic acid was generated for each experiment using the acidic acid standard provided by the Kit following manufacturer’s protocol. The amount of acidic acid per sample was calculated through the standard curve generated for each experiment.

5.3.8 Quantification of glycogen and trehalose.

Determination of glycogen and trehalose content was performed following the method previously described by Parrou et al (Parrou and Francois, 1997). Briefly, samples (equivalent of OD$_{600}$ = 2) were taken from the indicated cultures at the indicated times. First, cells were pelleted, resuspended in 0.25 ml of 0.25 M Na$_2$CO$_3$ and incubated for 4 h at 95ºC using screw-top Eppendorf tubes. Subsequently, samples were neutralized by addition of 0.15 ml of 1M acetic acid and 0.6 ml of 0.2 M Na-acetate. Next, for the trehalose content determination, 150 μl of the samples were digested overnight in the presence of trehalase (Sigma, St. Louis, MO, USA) (0.05 U/ml) at 37ºC under constant agitation. For the glycogen content determination, for 150 μl of the samples were digested overnight in the presence of amyloglucosidase (Sigma, St. Louis, MO, USA) (1.2 U/ml) at 57ºC under constant agitation. Finally, glucose release was quantified using the Glucose (GO) Assay Kit (Sigma, St. Louis, MO, USA) following the manufacturer’s protocol.
5.3.9 Statistical analysis

All experiments were done at least in triplicate. The number of CFU at day 0 (72 hours after inoculation) was considered to be the initial survival baseline (100%) and was used to calculate the percentage of survival at the different later time points. All data is presented as means ± SD of absolute values or percent of control. Values were compared by Student’s t-test. \( P < 0.05 \) was considered significant.

5.4 RESULTS

5.4.1 Respiratory rate of yeast strains broadly correlates with their chronological life span.

To analyze how mitochondrial respiratory function regulates yeast CLS, it was important to choose the most appropriate respiratory competent \( S. \) \( \text{cerevisiae} \) strain for our studies. The several laboratory strains commonly used in aging research are genetically and physiologically heterogeneous, which is somehow reflected in their CLS (Fabrizio and Longo, 2007; Ocampo and Barrientos, 2011b). Some of these strains, including BY4741, are S288c derivatives which carry a mutation affecting the \( HAP1 \) gene (Gaisne et al., 1999). Hap1 is a heme-dependent activator of a number of genes involved in electron-transfer reactions, which also acts as a repressor of some genes in the absence of heme or oxygen. Therefore, S288c-derived strains are modified in many aspects of their respiratory and oxygen metabolism, as reflected by their poor ability to respire when compared with strains carrying a wild-type \( HAP1 \) gene such as W303 (Supplemental Figure S5.1A-B). However, all the strains reduce
their metabolic rate when they reach the stationary phase and their respiratory rate at day 0 in the stationary phase (72h after inoculation) is 20% of their exponential phase respiratory rate (Supplemental Figure S5.1C). It is tempting though to correlate respiratory rate with CLS. In fact, the W303 strain is long-lived with a maximum CLS of ~11 days, while the BY4741 strain is shorter-lived with a maximum CLS of 8 days (Burtner et al., 2009; Ocampo and Barrientos, 2011b). Importantly, this difference in CLS does not correlate with the amount of toxic metabolites, mainly acetic acid, in the culture media, which is lower in the short-lived genetic background (Supplemental Figure S5.1D). Neither has it been correlated with ROS generation, since BY4741 produces less ROS than W303 cells during the exponential phase and similar amounts during the stationary phase (Supplemental Figure S5.1E). While the plausible influence of additional physiological factors prevents us from extracting conclusions, these preliminary experiments allowed us to decide to use W303 cells with robust respiration for further studies on respiration and CLS.

5.4.2 A mitochondrial respiratory threshold regulates yeast chronological life span.

To comprehensively analyze the role of mitochondrial function during yeast CLS, we have used three groups of W303 respiratory-deficient mutants. The first group included cells unable to respire because they either are devoid of mitochondrial DNA (rho0 cells) or carry a null cyc3 allele (cyc3Δ) and thus lack cytochrome c heme lyase and functional cytochrome c (Figure 5.1A and B). As reported for several respiratory mutants (Aerts et al., 2009; Bonawitz et al., 2006; Ocampo and Barrientos, 2011b), the absence of respiration in rho0 and cyc3Δ
strains severely limits their CLS from a wild-type maximum of 11 days to a maximum of 3 days (Figure 5.1C). The second group included two mutant strains which respire poorly: shy1Δ and C199. Shy1 is a cytochrome c oxidase (COX) assembly chaperone (Barrientos et al., 2002). The C199 strain carries a point mutation in Mss51, another COX assembly factor (our unpublished results). Both shy1Δ and C199 cells retain an endogenous respiration of ~15% of wild-type during the exponential phase but respiration during the stationary phase was undetectable (Figure 5.1D and E). The residual 15% respiration in these strains was not able to support standard wild-type CLS, which was reduced to a maximum of 4 days (Figure 5.1F). The third group of strains analyzed included cox5aΔ and cyc1Δ. Cox5 and cytochrome c exist in two isoforms differentially regulated by oxygen levels. The normoxic genes are COX5a and CYC1 while the hypoxic genes are COX5b and CYC7. In the absence of the normoxic genes, the hypoxic genes have some leaky expression, which allow for residual respiration (Barrientos et al., 2003). In our growth conditions, during the exponential phase both mutant strains retained a respiratory rate of 40-50% of wild-type (Figure 5.1G). During the stationary phase instead, both mutant strains had a respiratory rate similar to that of wild-type cells (Figure 5.1H). Interestingly, cox5aΔ and cyc1Δ strains follow a survival curve and have a CLS indistinguishable from that of wild-type cells (Figure 5.1I). These results indicate that in yeast, cellular respiration is essential to maintain a standard wild-type CLS. However, yeast cells have a large respiratory capacity to sustain CLS, which is significantly
affected only when the respiratory rate in exponential phase drops below a 40-50% of wild-type respiratory threshold.

Figure 5.1. Mitochondrial respiratory thresholds regulate yeast CLS. (A and B) Endogenous respiration of W303, rho0 and cyc3Δ cells during exponential (A) and stationary (B) phases of growth. (C) CLS of W303, rho0 and cyc3Δ cells. (D and E) Endogenous respiration of W303, shy1Δ and C199 cells during exponential (D) and stationary (E) phases of growth. (F) CLS of W303, shy1Δ and C199 cells. (G and H) Endogenous respiration of W303, cox5aΔ and cyc1Δ cells during exponential (G) and stationary (H) phases of growth. (I) CLS of W303, cox5aΔ and cyc1Δ cells. Error bars represent the mean ± SD with $P$ values denoted by $^* = P < 0.05$ and $^{**} = P < 0.01$. 
5.4.3 Stationary phase respiration is essential for standard yeast CLS.

To further evaluate the role of respiration in modulating yeast CLS and its relative relevance during the different phases of growth, we have taken a pharmacological approach, supplementing the culture media with either 50μM antimycin A (AA), a respiratory chain complex III inhibitor, or 10μM oligomycin, a specific mitochondrial ATP synthase inhibitor. Under these conditions, AA completely inhibited cell respiration when supplemented at the exponential or stationary phase (at 72 h) (Figure 5.2A and D). In the presence of 10μM oligomycin instead, the cells respired at a rate 40% that of untreated cells when added at the exponential phase and 10-20% when added at the stationary phase (Figure 5.2A and D). The effects of these inhibitors on respiration resembled those induced by mutations that totally (rho0 or cyc3Δ) or partially (cox5aΔ or cyc1Δ) limit cell respiration.

Addition of AA starting at the exponential phase severely limited CLS as reported (Fabrizio et al., 2003), similar to the rho0 mutation (Figure 5.2C), while supplementation of oligomycin had no effect on CLS, similar to the results obtained with cox5aΔ and cyc1Δ. On the contrary, when the respiratory inhibitors were added only during the stationary phase, the results obtained were slightly different. Complete respiratory inhibition with AA reduced CLS from 14 days in non-treated cells to 8 days, and the 25% of residual cell respiration in the stationary phase in oligomycin-treated cells supported a CLS slightly reduced to a maximum of 12 days (Figure 5.2E).
Figure 5.2. Effect of mitochondrial respiratory inhibitors on yeast CLS. (A and B) Endogenous respiration during exponential (A) and stationary (B) phases of growth of W303 cells treated with 50μM antimycin A or 10μM oligomycin through the exponential and stationary phases. (C) CLS of W303 cells treated with 50μM antimycin A or 10μM oligomycin through the exponential and stationary phases. (D) Endogenous respiration during the stationary phase of growth of W303 cells treated with 50μM antimycin A or 10μM oligomycin, through only the stationary phase. (E) CLS of W303 cells treated with 50μM antimycin A or 10μM oligomycin through only the stationary phase (72h). Error bars represent the mean ± SD with p values denoted by * = P < 0.05 and ** = P < 0.01.
These results show that although respiration during the stationary phase is essential for standard wild-type CLS, both the absence of or limitation on respiration exclusively during this phase have a milder effect on CLS than that occurring through the exponential and stationary phases. The different effects of oligomycin on CLS when supplemented in the two growth phases could suggest some kind of pre-conditioning when the drug is added during the exponential phase that pre-adapts the cells to minimize respiratory inhibition during the stationary phase and remain able to achieve a standard wild-type CLS. In support of this view, cells treated with oligomycin starting during the exponential phase respire in the stationary phase at a rate 80% of wild-type, while treatment exclusively during the stationary phase reduces respiration to 20% of wild-type (Figure 5.2B and 2D). Although it is difficult to titrate respiration exclusively during the stationary phase, our results further suggest the existence also in this phase of a respiratory capacity threshold above which the cells maintain standard wild-type CLS.

5.4.4 Cell-extrinsic factors are not responsible for the shorter CLS of respiratory mutant stains.

To gain insight into the mechanisms responsible for the shorter life span of cells with a respiratory rate below the capacity threshold, we have evaluated the contribution of cell-extrinsic and intrinsic factors.

Because acetic acid produced during ethanol metabolism creates a toxic environment for yeast cells that limits yeast CLS (Burtner et al., 2009; Kaeberlein, 2010), we sought to determine whether in respiratory mutants CLS inversely correlates with acetic acid levels produced during growth. As a first
observation, the pH of the culture media after 72 h of growth of \( \text{rho}^0 \), \( \text{cyc3} \Delta \), \( \text{shy1} \Delta \) and C199 respiratory-mutant strains was significantly less acidic (3.30) than for wild-type cells (2.75). The amount of acetic acid produced by these respiratory-deficient strains is actually ten-fold lower than by wild-type cells (Figure 5.3A) thus discarding this molecule as the factor responsible for the shorter life span of these strains. To further eliminate a role of cell-extrinsic factors on limiting CLS in cells respiring below the threshold, we performed a media swap experiment between W303 wild-type (\( \text{rho}^+ \)) and \( \text{rho}^0 \) cells. Cells were grown to stationary phase and at this point, cultures were centrifuged; in one batch the culture media were exchanged and CLS analyzed. Control wild-type and \( \text{rho}^0 \) cells in their own media reproduced their typical survival curve and CLS (Figure 5.3B). On the contrary, \( \text{rho}^0 \) cells transferred to wild-type media displayed a yet shorter life span while the CLS of \( \text{rho}^+ \) cells switched to \( \text{rho}^0 \) media was significantly extended (Figure 5.3B), probably as a consequence of the acetic acid concentrations present in each culture medium. Thus, we conclude that the shorter life span of cells with respiratory capacity below the threshold is the result of cell intrinsic factors.

On another approach to eliminate the influence of acetic acid accumulation, we chronologically aged respiratory-competent and -deficient strains in buffered media (pH 4.7). In these conditions, CLS of wild-type cells is known to be significantly extended although the cells continue to lose viability over time (Burtner et al., 2009). Here, we wanted not only to confirm that the rapid decline in survival of strains with respiratory rate below the capacity
threshold is acetic acid-independent. We also sought to test whether we had failed to observe a difference between the survival of wild-type cells and cells that retain 40-50% respiratory capacity ($\text{cox5a}\Delta$, $\text{cyc1}\Delta$ and oligomycin-treated wild-type cells) because the presence of acetic acid in the media could mask the differences.

**Figure 5.3. Regulation of CLS by mitochondrial function is acetic-acid independent.** (A) Determination of acetic acid concentration in the growth media of the indicated strains. (B) CLS of W303 ($\rho^+$) or $\rho^0$ cells in their original media or subjected to media swap in the stationary phase. (C) CLS of W303, $\text{cox5a}\Delta$ and $\text{cyc1}\Delta$ cells in the presence of buffered SDC media. (D) CLS of W303 cells grown in buffered SDC medium, treated with 50μM antimycin A (AA) or 10μM oligomycin (OLI) from inoculation (Exp) or stationary phase (Sta) until the termination of the experiment. Error bars represent the mean ± SD.

As expected, when grown in buffered media, the CLS of all the strains was significantly longer than in non-buffered conditions (Figure 5.3C). Noticeably, although $\rho^0$ cells had a CLS double than in non-buffered conditions, they still had a CLS significantly shorter than wild-type cells which was extended ~2.5-fold
(Figure 5.3C and 1C). For the cox5aΔ strain we measured a CLS comparable or even slightly longer than wild-type cells (Figure 5.3C), thus supporting the view that 40-50% of cell respiration is enough to sustain wild-type CLS. Pharmacological treatments of wild-type cells during the exponential phase in buffered media confirmed the results obtained with the mutant strains (Figure 5.3D). CLS extension by buffering the medium is probably accounted for by acetic acid quenching as mentioned earlier. However another important contributing factor, probably most significant for respiratory null mutant strains, is the decrease in energy expenditure required to maintain the internal cellular pH during the stationary phase, which has been estimated to account for 40-60% of all energy spent by the cells.

We used the same media-buffered conditions to test the effect of complete or partial pharmacological respiratory inhibition exclusively during the stationary phase. Partial respiratory inhibition with oligomycin was enough to shorten maximum CLS from 28 to 23 days, while complete inhibition with AA further reduced it to 16 days (Figure 5.3D).

These results confirmed that respiration during the exponential and stationary phases is essential for standard wild-type life-span.

5.4.5 Modulation of CLS by respiration is independent of ROS accumulation during growth.

To determine whether ROS could be responsible for the shorter CLS of mutant strains with respiratory rate below the capacity threshold, we estimated the amount of ROS in these strains using the fluorescent probe dihydroethidium (DHE) (Pan et al., 2011). As expected, in rho0 and cyc3Δ cells unable to respire,
ROS levels were significantly lower than in wild-type cells during both phases of growth (Figure 5.4A), and thus increased ROS accumulation in these strains is not responsible for their limited CLS. Similar low ROS levels were measured for \textit{cox5aΔ} and \textit{cyc1Δ} cells (Figure 5.4B) which have wild-type CLS.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5_4.png}
\caption{ROS production during growth does not affect yeast CLS. (A and B) ROS production determined as DHE fluorescence in the indicated strains during exponential or stationary phases of growth. (C and D) ROS production determined as DHE fluorescence during exponential and stationary phase in W303 cells treated with 50μM antimycin A or 10μM oligomycin (C) through the exponential and stationary phases or (D) through only the stationary phase. Error bars represent the mean ± SD with p values denoted by * = p <0.05 and ** = p <0.01.}
\end{figure}

Antimycin A and oligomycin are known to promote ROS generation. Cells treated with either drug during the exponential phase generate higher levels of ROS than do untreated cells (Figure 5.4C). For oligomycin-treated cells, which respire above the capacity threshold that supports standard wild-type CLS, increased ROS levels did not induce a detrimental effect on CLS either in buffered or non-buffered media (Figures 5.2C and 5.3D). ROS production has
been recently shown to provide an adaptive signal during growth of TOR1 mutants that extends CLS (Pan et al., 2011). However, this does not seem to be the case for oligomycin-treated cells, since their survival curve and maximum CLS were the same as for the cox5aΔ and cyc1Δ strains (Figures 5.1I and 5.2C), which generate lower ROS levels than wild-type cells (Figure 5.4B). And AA treated cells, despite generating higher ROS levels, have a survival curve and maximum CLS comparable to the rho0 and cyc3Δ strains (Figures 5.1C and 5.2C) where ROS levels are significantly lowered (Figure 5.4A). However, when the cells were exposed to the two drugs exclusively during the stationary phase, their respiration was below the threshold required for standard CLS (Figure 5.2D) and their ROS production during this phase was significantly reduced (Figure 5.4D). In conclusion, in the conditions used here, the cellular respiratory rate regulates CLS in a manner largely independent of ROS production during growth.

5.4.6 Caloric restriction alters the requirement of stationary phase respiration.

Caloric restriction (CR) is the best known non-genetic intervention capable of extending life span in a wide variety of organisms from yeast to primates (Fontana et al., 2010). In yeast, CR is modeled by growing the cells in 0.5% glucose instead of the non-restricted 2% concentration. Because glucose represses the expression of genes required for respiration in yeast, CR attenuates the repression and facilitates a switch from fermentative to respiratory metabolism. To disclose whether respiratory thresholds regulate CLS extension by CR we started by examining cellular respiration of caloric-restricted wild-type
cells during the exponential and stationary phases. Caloric-restricted cells respire during exponential growth at a rate 30% higher than non-restricted cells (Oliveira et al., 2008), Figure 5.5A) and have a more than two-fold extension of their maximum CLS up to 27 days (Figure 5.5C). Caloric-restricted cells reach the stationary phase at a time similar to non-restricted cells (not shown). Unexpectedly however, during the stationary phase at 72h of growth (day 0 of chronological life), caloric-restricted cells barely respire (Figure 5.5B). This observation suggests that the metabolic remodeling program that goes along with the entry of caloric-restricted cells into the stationary phase alters the requirement of respiration during chronological aging. We subsequently used our models of respiratory deficiency to further explore this possibility. Respiratory null rho0 cells as well as wild-type cells treated with AA during the exponential phase had a similar CLS in non-restricted and caloric-restricted media (3.5 and 5.5 days for rho0 cells and 4 and 3.5 days for AA-treated cells, respectively; Figure 5.5C and 5.5D), indicating that cells need to respire during growth to significantly benefit from the CR-induced CLS extension. However, when CR was performed in cox5aΔ cells, their respiration also increased by 15-20% in the exponential phase but was barely detectable during the stationary phase (Figure 5.5B) and CLS extension by CR was similar to wild-type cells (Figure 5.5C). Additionally, caloric-restricted cells treated with oligomycin during the exponential phase had a residual 45% of wild-type respiration during growth and very poor respiration during the stationary phase (Figure 5.5B), but were still able to achieve a wild-type CLS extension (Fig 5.5D). Thus, caloric restricted cells with respiratory
capacity above at least a 45% threshold during growth have wild-type CLS extension. The CR effect on longevity involves shutting down respiration during the stationary phase. Supplementation of the cultures with AA or oligomycin after 72h of growth at day 0 of chronological age did not affect CLS (Figure 5.5D), thus further indicating that respiration during the stationary phase is not essential for yeast CLS extension by CR.

Figure 5.5. Mitochondrial respiratory thresholds regulate CLS under caloric restriction. (A and B) Endogenous respiration during exponential (A) and stationary (B) phases of growth of W303, cox5αΔ and W303 cells treated with 10μM oligomycin through the exponential and stationary phases. Cells were grown in regular (Non-CR) or caloric restriction (CR) SDC media. (C) CLS of W303, rho0 and cox5αΔ cells grown under caloric restriction (CR). (D) CLS of W303 cells treated with 50μM antimycin A (AA) or 10μM oligomycin (OLI) from inoculation (Exp) or stationary phases (Sta) until the termination of the experiment under caloric restriction (CR). Error bars represent the mean ± SD.
5.4.7 Increasing mitochondrial respiration during growth is not enough to extend CLS.

To obtain a CR-independent view of how increased mitochondrial respiration during exponential growth could modulate CLS, we used a wild-type strain overexpressing *HAP4* (Ocampo et al., 2010). Hap4 is the catalytic subunit of the Hap2,3,4,5 transcriptional complex known to play an important role in globally activating transcription of nuclear genes involved in mitochondrial respiration during transition from fermentation to respiration (Buschlen et al., 2003). The *HAP4* over-expressor strain respires at a rate 150% of wild-type during growth and 50% during the stationary phase (Supplemental Figure S5.2A). *HAP4* overexpression has been reported to slightly extend CLS when the cells were transferred to water upon reaching stationary phase (Piper et al., 2006). However, in our experimental conditions, we did not observe any positive effect on CLS (Supplemental Figure S5.2B). It must be noted that contrary to caloric restriction, *HAP4* overexpression induces an increase in mitochondrial biogenesis that is not accompanied by an increase in expression of either stress response genes, with the exception of Sod1, or in genes involved in metabolism of energy reserves (Lascaris et al., 2003). These results indicate that enhanced mitochondrial respiration during growth is not enough to extend CLS, when not accompanied by a CR-like metabolic remodeling that slows down energy consumption and enhances cell protection systems during the stationary phase.
5.4.8 Do respiratory thresholds modulate CLS by regulating stress resistance and the metabolism of energy reserves?

Subsequently, we wanted to explore whether mitochondrial function regulates stress resistance mechanisms and the accumulation and consumption of reserve energy sources that sustain life in the stationary phase (Longo and Fabrizio, 2002). The results presented in Figure 5.6A show that when rho<sup>0</sup> cells reach the stationary phase they are less resistant to oxidative (1h in 100mM H<sub>2</sub>O<sub>2</sub>) and heat-shock (10 min at 55°C) stresses than wild-type cells or cells respiring above the 40% threshold, such as cox5aΔ.

Storage carbohydrates, mainly glycogen and trehalose, are used as energy sources during stationary phase. As a consequence, mutants unable to accumulate or utilize them have significantly shorter CLS (Favre et al., 2008; Samokhvalov et al., 2004). Respiratory null strains have defective trehalose synthesis and although they accumulate high levels of glycogen during exponential growth, they readily mobilize as soon as glucose is exhausted in the medium (Enjalbert et al., 2000). In agreement with these observations, at -14h (58h after inoculation) we measured high levels of glycogen (70% of wild-type) and a significantly reduced amount of trehalose (50% of wild-type) in cells respiring below the 40% threshold (Supplemental Figure S5.3). A time-course analysis of glycogen and trehalose content during the stationary phase in wild-type and cox5aΔ cells showed comparable amounts of these storage carbohydrates at days 0, 4 and 9 of chronological life, with a tendency to be larger in cox5aΔ cells. Glycogen and trehalose content progressively decreased following similar trends in both strains as they were used during the stationary
phase (Figure 5.6B). However, in rho\(^0\) cells, storage carbohydrate content was already low at day 0 as reported (Enjalbert et al., 2000) and basically exhausted after day 4 (Figure 5.6B). A similar tendency was measured in our pharmacological models (Figure 5.6C and D). Cells treated with AA during the exponential phase exhausted their storage carbohydrates soon after reaching the stationary phase, while oligomycin-treated cells metabolized them as did wild-type cells (Figure 5.6C). In cells treated with AA or oligomycin during the stationary phase, the rate of storage mobilization inversely correlated with the amount of residual respiration (Figure 5.6B). Our results show a striking correlation between stored carbohydrate content and cell survival. Wild-type and cox5a\(\Delta\) strains that have a maximum CLS of ~11 days already consumed approximately 80% of their stored carbohydrates by day 9 (Figure 5.6B). In respiratory-deficient strains, which rapidly mobilize their stored carbohydrates during stationary phase, exhaustion of these stores may be responsible for their shorter CLS. To further test this hypothesis, we measured CLS of respiratory-deficient and competent strains cultured in media supplemented with 1% trehalose. Trehalose supplementation did not significantly extend the CLS of wild-type cells (probably limited by acetic acid accumulation; Figure 5.6E) independently of the growth phase in which was added. Instead, trehalose supplementation only during the stationary phase, when trehalose assimilation is known to be poor (Jules et al., 2004), slightly extended CLS of rho\(^0\) cells. Significantly, the CLS of rho\(^0\) cells was extended even beyond wild-type length
when trehalose was added during the growth phase (Figure 5.6F) and could be efficiently assimilated (Jules et al., 2004).

**Figure 5.6. Mitochondrial dysfunction curtails yeast CLS by decreasing stress resistance and altering the reserve nutrients metabolism.** (A) Hydrogen peroxide and heat stress resistance of W303, rho⁰ and cox5Δ cells at day 0 during the stationary phase. (B) Time-course of glycogen and trehalose content during the stationary phase in W303, rho⁰ and cox5Δ cells. (C and D) Time-course of glycogen and trehalose content during the stationary phase of W303 cells treated with 50μM antimycin A (AA) or with 10μM oligomycin (OLI). (C) from inoculation (Exp) or (D) stationary phases (Sta) until the termination of the experiment. (E and F) CLS of (E) W303 and (F) rho⁰ cells grown in SDC medium supplemented with trehalose at the moment of inoculation (Exp) or at the stationary phase (Sta). Error bars represent the mean ± SD.
5.5 DISCUSSION

Despite some controversies surrounding the mitochondrial theory of aging (Harman, 1956, 1972; Miquel et al., 1980), it is widely accepted that mitochondria play fundamental roles in the mechanisms of aging and in life span extension strategies. How changes in mitochondrial respiration and ROS generation interplay to regulate aging, however, remain to be fully understood. Here we have used genetic and pharmacological yeast models of respiratory deficiency to determine the minimum respiratory capacity required to sustain standard wild-type CLS. In agreement with previous reports (Aerts et al., 2009; Bonawitz et al., 2006; Ocampo and Barrientos, 2011b) we show that mitochondrial function is essential for CLS. However, our results indicate that yeast cells have an excess respiratory capacity to sustain CLS, which only when depleted below a ~40% of wild-type threshold acts as a factor limiting CLS (Figure 5.7). Cells respiring below this threshold cells have a CLS 73% shorter than wild-type cells. A fundamental aspect of yeast CLS is the ability of cells to remodel their metabolism from fermentation to respiration when glucose levels decline and are exhausted during the diauxic shift. This remodeling is also accompanied by a significant reduction in metabolic rate, which is particularly profound when cells are grown in complete medium (Fabrizio and Longo, 2007), but which we appreciated also in cells growing in synthetic medium. Cells that respire above the 40% threshold during growth are somehow able to properly adjust their metabolic rate after the diauxic shift in a manner similar to that of wild-type cells. In contrast, cells respiring below the 40% of wild-type threshold during growth
have extremely poor respiratory capacity in the stationary phase. We have
explored the relevance of the stationary phase respiration for CLS by allowing
wild-type cells to reach the stationary phase – the moment at which respiration
was fully or partially inhibited. Full inhibition of stationary phase respiration with
AA reduced CLS by 40% of wild-type and inhibition down to residual 20% of
stationary phase wild-type respiration with oligomycin only reduces CLS by 20%
of wild-type. These results indicate that while respiration in the stationary phase
is essential for standard wild-type CLS, respiratory defects seem less detrimental
once the cells have accumulated nutrient stores during growth and undergone
their metabolic remodeling during the diauxic shift.

Figure 5.7. Model of CLS regulation by mitochondrial respiration. Mitochondrial respiratory
thresholds regulate yeast CLS and its extension by caloric restriction (see explanation in the text).
For simplicity, the effect of cell extrinsic factors is not included.
Acetic acid-induced apoptosis has been recently proposed as the major factor responsible for yeast CLS. While there is no doubt that acetic acid toxicity can mask the effect of some longevity factors, so far mutations in several nutrient sensor conserved pathways, including TORC1 (Pan et al., 2011) and RAS2 (Burtner et al., 2009) have been shown to extend CLS through acetic acid-independent mechanisms. Similarly, three pieces of evidence allow us to conclude that mitochondrial respiratory thresholds regulate CLS in an acetic acid-independent manner. Short-lived respiratory mutants accumulate very low amounts of acetic acid. As a consequence, our media swap experiments showed that wild-type CLS is extended when transferred at the beginning of their chronological age to media where respiratory-deficient strains were grown. Finally, when the cells were grown in buffered media to eliminate the acetic acid factor, the differences remained between strains with respiratory capacity below and above the threshold required to sustain wild-type CLS. In buffered media, however, CLS of all strains was extended as reported for wild-type cells (Burtner et al., 2009), thus offering an opportunity to identify new factors that modulate survival of non-dividing yeast cells as proposed (Kaeberlein, 2010).

The concept of mitochondrial ROS exclusively as the toxic molecular inducers of cellular damage and responsible for disease and aging has been challenged over the last few years. While ROS are known to limit the long-term survival of yeast cells during CLS and have been shown to have a detrimental effect during aging of species of fly, worm and mouse, a growing amount of evidence supports a role for mitochondrial ROS as signaling molecules (Pan et
al., 2011; Ristow and Schmeisser, 2011). The results presented here further indicate that the relationship between respiration, ROS and life span is complex. All respiratory-deficient strains below the 40% of wild-type threshold have low ROS production (at least as measured by DHE) during growth and stationary phase as expected, thus confirming high ROS is not the cause of their limited survival. The two strains that respire at the 40% threshold also generate significantly lower amounts of ROS during both growth phases, but no CLS extension was observed. Finally, AA- and oligomycin-treated cells produced a larger amount of ROS during growth. It is particularly remarkable that oligomycin-treated cells with a residual 40% respiration and 1.5-fold increase in ROS production maintain a standard wild-type CLS. Taken together our results suggest that the effect of respiratory threshold regulating CLS is largely independent of mitochondrial ROS.

What could be the key factor responsible for the shorter CLS of mutant strains with respiratory rates below the capacity threshold? During CLS, yeast cells entering the stationary phase have already sensed the shortage of glucose and have responded by expressing cell protection systems that allow them to survive. In dividing yeast cells, mitochondrial function has been shown to be required for resistance to oxidative stress even if they are able to normally express the required anti-oxidant enzymes (Grant et al., 1997). Here, we have shown that respiratory-deficient cells are very sensitive to oxidative and thermal stresses when they reach the stationary phase. The stress sensitivity could be related to a defect in an energy-requiring process needed for either ROS
detoxification or oxidative damage repair that cannot be fulfilled in the absence of respiration as previously proposed (Grant et al., 1997). Furthermore, the metabolism of stored nutrients in cells respiring below the 40% threshold is altered. Respiratory-deficient cells accumulate high amounts of glycogen during growth. The synthesis of trehalose instead, which occurs when glucose is exhausted and cells start utilizing ethanol, is known to be limited in respiratory-deficient strains (Enjalbert et al., 2000). Trehalose plays additionally roles as a stress protectant in biological systems (Francois and Parrou, 2001). Therefore, its poor synthesis and rapid depletion in respiratory-deficient cells probably contributes to their stress sensitivity. Trehalose is also essential to enable yeast cells to survive starvation conditions and then rapidly proliferate upon return to favorable growth conditions by fueling cell cycle progression (Shi et al., 2010). However, respiratory-deficient cells are not just impaired in cell cycle re-entry, as shown by the fact that their survival curve is similar when constructed by counting colony formation units or by counting propidium iodide positive cells by flow cytometry (Ocampo and Barrientos, 2011b). Glycogen is mobilized faster than the more stable trehalose. Both of these reserve carbohydrates are quickly consumed when the cells reach the stationary phase because in the absence of respiration the cells must rely on fermentation, which generates a 14-fold lower energetic yield. Glycogen and trehalose are virtually exhausted in respiratory-deficient cells by the day of their maximal CLS, thus suggesting that starvation is an important factor limiting their survival. In support of this possibility, trehalose supplementation of growth media significantly extends CLS of respiratory null
cells. The beneficial effect of trehalose on longevity is not restricted to yeast. Trehalose treatment has been shown to extend life span of *C. elegans* even when administered from the old-adult stage (Honda et al., 2010). Also, by acting as a chemical chaperone, trehalose has been shown to alleviate polyglutamine-induced pathology in mice (Tanaka et al., 2004) and thus it could be a promising health-promoting therapeutic compound.

Caloric restriction (CR) is the most robust environmental intervention known to retard aging and extend life span across eukaryotic life. In yeast, a significant portion of the CR effects on longevity are mediated through the down-regulation of signaling pathways involving nutrient responsive kinases such as Ras/cAMP/PKA, TOR and Sch9, which converge on the activation of stress resistance transcription factors that will induce the expression of anti stress defenses and accumulation of storage carbohydrates (Wei et al., 2008). CR also involves a metabolic remodeling including a shift from fermentation to respiration which is essential for CLS extension (Oliveira et al., 2008). Growing evidence indicates that the longevity pathways play an important role in the regulation of mitochondrial biogenesis. Deletion of *TOR1* extends yeast CLS primarily by enhancing mitochondrial translation and respiration (Bonawitz et al., 2007) and by ROS adaptive signaling (Pan et al., 2011). The Ras/cAMP/PKA pathway senses excessive ROS to signal to the Hap2,3,4,5 transcriptional system and regulates mitochondrial biogenesis (Chevtzoff et al., 2009). In yeast, CR is mimicked by growing the cells in 0.5% glucose which minimizes the glucose-induced repression of oxidative phosphorylation genes, thus allowing the cells to
increase their respiratory capacity during growth. However, the metabolic remodeling occurring during the diauxic shift allows caloric-restricted cells to dramatically reduce their metabolic rate in the stationary phase and consume their stored nutrients at a rate slower than cells grown in 2% glucose (Goldberg et al., 2009). Our results show that these cells retain only 5-10% of the respiratory capacity of cells grown in non-restricted conditions and that respiration during the stationary phase is not essential to have maximal CLS extension. However, cells must respire during growth to benefit from CR-induced CLS extension, although respiration above at least ~45% threshold supports wild-type CLS extension (Figure 5.7). On the other hand, increased respiration during growth is not sufficient per se to extend CLS. This is indicated by the fact that HAP4 overexpression has no effect on CLS. HAP4 overexpression basically affects a set of genes involved in mitochondrial biogenesis with little crosstalk with other regulatory networks (Lascaris et al., 2003). Significantly, although HAP4-overexpressing cells resemble cells that undergo a physiological diauxic shift, there are important differences. Notably it has been reported that no increase occurs in several categories of genes including the category of “metabolism of energy reserves” and most antioxidant defenses with the exception of SOD1 (Lascaris et al., 2003).

In conclusion, respiratory thresholds regulate yeast CLS and its extension by caloric restriction. In humans, threshold effects have been described in mitochondrial diseases as related to mtDNA heteroplasmy and energy thresholds, defined as the amount of respiratory enzyme that can be inhibited
before observing a respiratory phenotype have also been identified in many human tissues (Rossignol et al., 2003). Respiratory thresholds to support cellular life probably vary for every tissue. Respiratory deficiencies underlying the pathogenic mechanisms of classical mitochondrial disorders are also frequently associated with age-related neurodegenerative diseases. In both instances, interventions mimicking CR might lower the energetic thresholds of a set of tissues which if accompanied by an enhancement of cellular protection systems, might contribute to extending health span.

5.6 SUPPLEMENTAL DATA

**Figure S5.1. Comparison of different yeast genetic backgrounds.** (A and B) Endogenous respiration of W303 and BY4741 cells during exponential (A) and stationary (B) phases of growth. (C) Endogenous respiration of W303 during exponential and stationary phase of growth. (D) Determination of acetic acid concentration in the growth media of the indicated strains. (E) ROS production determined as DHE fluorescence in the indicated strains during exponential or stationary phases of growth. Error bars represent the mean ± SD with p values denoted by * = P <0.05 and ** = P <0.01.
Figure S5.2, related to Figure 5. Increasing mitochondrial respiration during growth is not sufficient to extend CLS. (A) Endogenous respiration of W303 carrying an empty plasmid (YEp352) or a plasmid for the overexpression of HAP4 (HAP4). (B) CLS of W303 carrying an empty plasmid (YEp352) or a plasmid for the overexpression of HAP4 (HAP4). Error bars represent the mean ± SD with p values denoted by * = P < 0.05 and ** = P < 0.01.

Figure S5.3, related to Figure 6. Accumulation of glycogen and trehalose during exponential phase of growth in respiratory competent and deficient strains. Glycogen and trehalose content in W303, rho° and cox5aΔ cells during exponential phase of growth (14 h after inoculation). Error bars represent the mean ± SD.
CHAPTER 6
DISCUSSION

Biological aging can be defined as the progressive decline in the ability of a cell or an organism to resist stress, damage or disease and it is considered a major risk factor for many of the diseases affecting modern societies including cardiovascular conditions, cancer and neurodegenerative diseases. Among the different age-related diseases, neurodegenerative proteinopathies, including polyglutamine (polyQ) diseases such as Huntington’s disease, are a group of disorders in which a single protein or a set of proteins misfold and aggregate resulting in a progressive and selective loss of anatomically or physiologically related neuronal systems.

Mitochondria lay at the center of cellular life and death. Mitochondria, frequently referred as the “powerhouses of the cell”, are attractive candidates to play fundamental roles in aging and age-related diseases due to their functions in energy and reactive oxygen species (ROS) generation in the cells as well as their involvement in cell death. Data supporting the relevance of mitochondrial dysfunction on aging and age-related disease such as neurodegenerative proteinopathies has accumulated over the years generated from studies of post-mortem tissues as well as from the research performed using multiple model organisms. However, nowadays, it is still not completely clear if mitochondrial dysfunction and ROS accumulation are the cause or the consequence of the process of aging and neurodegeneration, and what their contribution to these processes is. We have taken advantage of the suitable properties of
S. cerevisiae as a model organism, and have used yeast models of neurodegenerative proteinopathies and aging to investigate some of the open questions regarding mitochondrial function, aging and age-related diseases. We have assessed the extent and relevance of mitochondrial dysfunction on polyQ-induced cytotoxicity and chronological life span, have disclosed the mechanism by which mutant polyQ domains and aging affect normal mitochondrial function, and have evaluated whether the enhancement of mitochondrial biogenesis and respiratory function could serve as potential therapeutic strategies to ameliorate the symptoms of polyQ diseases and delay the process of aging.

Using yeast models of polyQ disorders, we have observed that expression of mutant polyQ domains induces cellular and mitochondrial toxicities that are proportional to the length of the polyQ tract. Increasing toxicity with increased polyQ expansion is a hallmark of neuronal polyQ pathology. In our models, mutant polyQ domains induce a significant growth defect that is accompanied by a decrease in cell endogenous respiration and alterations in several mitochondrial biophysical properties including membrane potential, ability of mitochondria to regulate its volume and mitochondrial protein synthesis.

Concerning the mechanism by which polyQ domains affect mitochondrial function, several hypotheses have been proposed, including direct interaction of polyQ domains with mitochondrial membranes (Choo et al., 2004; Panov et al., 2002), transcriptional dysregulation affecting the expression of genes that control mitochondrial biogenesis (Cui et al., 2006; McCampbell et al., 2000), expression and import defects of nuclear-encoded mitochondrial proteins (Benchoua et al.,...
and cytoskeletal disruption affecting vesicular and mitochondrial trafficking (Trushina et al., 2004). Similarly, in yeast models of polyQ disease, previous studies have already allowed us to propose that also a combination of direct and indirect mechanisms could account for polyQ-induced mitochondrial toxicities (Solans et al., 2006). In this line, we have now found that wild type and mutant polyQ domains directly interact with the mitochondrial outer membrane very early after expression is induced. Since mutant polyQ domains are known to misfold and serve as a seed for oligomerization, we have hypothesized that mutant polyQ oligomers interacting with the outer mitochondrial membrane could be one of the causes of mitochondrial dysfunction.

To evaluate the role of mitochondrial dysfunction on polyQ diseases, we have tested the effect of enhancing mitochondrial biogenesis and respiratory function on polyQ-induced cytotoxicity. We found that overexpression of HAP4, the catalytic subunit of the transcriptional activator Hap2,3,4,5p complex, known to increase mitochondrial biogenesis and respiratory function (Fontanesi et al., 2008; Lascaris et al., 2003), ameliorates 103Q toxicity and prevents the severe decline of mitochondrial respiration and other mitochondrial physiological parameters. This suppression mechanism highlights the importance of mitochondrial dysfunction as a contributor to polyQ-induced toxicity. During the course of my thesis, a link between transcriptional dysregulation and mitochondrial function impairment was proposed by the discovery that in mouse models, mutant huntingtin causes disruption of mitochondrial function by inhibiting expression of PGC-1α, a transcriptional coactivator functional homolog
of the yeast *HAP4*, that regulates several metabolic processes including mitochondrial biogenesis and respiration (Cui et al., 2006). In these models, *PGC-1α* overexpression significantly ameliorated mutant htt-induced toxicity in a similar way that *HAP4* in our yeast models (Cui et al., 2006).

We are aware that the suppression by Hap4 overexpression is only partial which clearly indicates, as previously described by us and others, that pathways other than mitochondrial function are targets of mutant polyQ domains (e.g. cytoskeletal disturbances (Solans et al., 2006), ER stress (Duennwald and Lindquist, 2008) and decreased cytosolic protein synthesis). However, we can conclude that in our yeast models, mitochondrial dysfunction is one of the important contributors to polyQ-induced cytotoxicity and that strategies for increasing mitochondrial biogenesis, either alone or as part of multitarget therapeutic interventions, might prove valuable to combat polyQ expansion diseases by delaying the development of clinical symptoms in patients.

Despite some controversies surrounding the mitochondrial theory of aging (Harman, 1956, 1972; Miquel et al., 1980), it is widely accepted that mitochondria play fundamental roles in the mechanisms of aging and in life span extension strategies. However, how changes in mitochondrial respiration and ROS generation interplay to regulate aging remains to be fully understood. We have take advantage of the yeast model of aging, chronological life span (CLS), that has been suggested may mimic the aging process of postmitotic cells, to analyze fundamental questions about the role of mitochondrial dysfunction and ROS production in the aging process. By using genetic and pharmacological
interventions, we have been able to unveil the existence of a mitochondrial respiratory threshold under which mitochondrial dysfunction affects the chronological life span of yeast. Similarly, in humans, threshold effects have been described in mitochondrial diseases as related to mtDNA heteroplasmy and energy thresholds, defined as the amount of respiratory enzyme that can be inhibited before observing a respiratory phenotype have also been identified in many human tissues (Rossignol et al., 2003). In addition, contrary to what expected, we have observed that the effect of mitochondrial dysfunction on CLS is independent of ROS generation during growth, questioning once again the free radical theory of aging and the role that ROS may play in the aging process. In this line, while ROS are known to limit the long-term survival of yeast cells during CLS and have been shown to have a detrimental effect during aging of species of fly, worm and mouse, a growing amount of evidence supports a role for mitochondrial ROS as beneficial signaling molecules (Pan et al., 2011; Ristow and Schmeisser, 2011).

We proposed alterations on stress resistance and accumulation and consumption of energy stores (glycogen and trehalose) that maintain viability during the yeast stationary phase of growth, as the mechanisms through which severe mitochondrial dysfunction may limit standard aging. In addition, we have found that mitochondrial function is an essential component of interventions that extend life span. Caloric restriction (CR) is the most robust environmental intervention known to retard aging and extend life span across eukaryotic life. We have observed that CR modifies respiratory function during the different phases
of yeast growth and eliminates the requirement of respiration during stationary phase. Nevertheless, we have also found that mitochondrial dysfunction affects CLS extension by CR indicating that a minimum mitochondrial function during growth is required to benefit from life extension interventions.

Aging represents the major risk factor for neurodegenerative proteinopathies. For this reason, to increase the relevance and significance of studies using yeast models of polyQ disease and aging, we have developed novel yeast models of neurodegenerative proteinopathies where the expression of mutant proteins can be exclusively induced in non-dividing cells during yeast stationary phase, therefore resulting in a combination of yeast polyQ disease and chronological life span models. To achieve this objective, we have engineered innovative metabolism-independent inducible expression systems that allow for protein expression at different stages of growth and under different metabolic conditions. In addition, a novel method to measure yeast chronological life span was also established by the use of viability dyes and flow cytometry that allows for quick and reliable determination of yeast CLS independently of the capacity of the cells to divide and form a colony, a confounding variable when working with cells expressing polyQ domains. By studying these novel models of polyQ-toxicity during yeast CLS, we have found that mutant polyQ domains significantly shorten yeast CLS and that enhancement of mitochondrial biogenesis and respiratory function by different mechanisms during exponential growth ameliorates mutant polyQ toxicities and restores standard wild-type chronological life span in yeast cells (Appendix A1).
In conclusion, using yeast models of cellular aging and polyQ disorders we have showed that mitochondrial dysfunction is an important contributor to the process of aging and age-related neurodegenerative diseases. Preserving mitochondrial function is essential for standard wild-type aging. Enhancement of mitochondrial biogenesis ameliorates polyQ cytotoxicity and is a required component of interventions that retard the aging process.

Figure 6.1. Model for the suppression of age-related neurodegenerative proteinopathies and biological aging by enhancement of mitochondrial biogenesis.
APPENDIX A1

NOVEL MODELS OF POLYQ TOXICITY DURING YEAST CLS

To increase the relevance and significance of our studies, novel yeast models of polyQ toxicity were generated to study the effect of mutant polyQ on yeast chronological life span. In these models, polyQ domains fused to GFP were placed under the control of a β-estradiol inducible GAL1 promoter that can activate protein expression in non-dividing cells during stationary phase. To evaluate the effect of polyQ expression on yeast CLS, cells transformed with constructs for the expression of wild type (25Q) and mutant (103Q) huntingtin fused to GFP were inoculated in minimum media containing glucose (WOGLU) and allowed to reach stationary phase (48 h). Protein expression was subsequently activated by addition of β-estradiol and the percentage of live cells was evaluated every 2 days by propidium iodide staining combined with FACS analysis as previously described (Ocampo and Barrientos, 2011b). Protein expression and aggregation were confirmed by fluorescence microscopy (Figure A1.1A) and FACS (data not shown). Expression of mutant polyQ domains significantly reduced chronological life span of the cells compared to wild type or non-induced controls resulting in more that 90% of the cells dead after day 6 (Figure A1.1B). The reduction in the life span was proportional to the amount of protein induced when different concentrations of the inducer β-estradiol were added to the media (Figure A1.1C) although even in the presence of only 50nM β-estradiol a significant reduction in the life span of the cells was observed.
Figure A1.1. Mutant polyQ domains reduce yeast chronological life span. (A) Visualization of wild type (25Q) and mutant (103Q) polyQ expression during stationary phase of growth under the control of a β-estradiol inducible GAL1 promoter. (B) Chronological life span of cells expressing wild type and mutant polyQ domains during stationary phase. (C) Chronological life span of cells expressing different levels of mutant polyQ by addition of different concentrations of β-estradiol.
At this point, we have been able to show that expression of mutant polyQ domains significantly reduces yeast CLS. After our previous results analyzing the role of respiration during yeast CLS, we can hypothesize that mutant polyQ domains which are known to induce mitochondrial dysfunction in rapidly dividing cells may reduce yeast CLS by reducing the respiratory capacity of the cells during stationary phase below the proposed minimum threshold. At present, due to the low respiratory rate during stationary phase and the presence of a mixture of populations of live and dead cells after mutant polyQ domains have been induced, we do not have data to demonstrate mitochondrial dysfunction induced by mutant polyQ domains in stationary phase during CLS, however we are currently working to obtain some results in this area. On another aspect of the project, we have previously showed and published that increase in mitochondrial biogenesis by $HAP4$ overexpression was able to partially suppress mutant polyQ toxicity in rapidly dividing cells expression polyQ domains (Ocampo et al., 2010). For this reason, we wanted to test if environmental or genetic manipulations aiming to increase mitochondrial biogenesis could partially suppress the effect of mutant polyQ expression also on yeast CLS. First, we decided to test the effect of caloric restriction (CR) on mutant polyQ toxicity, an intervention that we have showed induces a higher rate of cellular respiration during exponential phase of growth (Figure 5.5). For this purpose, cells were grown for 48 hours under CR, allowed to reach stationary phase and subsequently protein expression was activated and cell death was monitored. As expected, cells grown under caloric restriction had a longer life span that cells grown under normal conditions in
agreement with previous data (Wei et al., 2008) (Figure A1.2.A). In addition, caloric restriction was able to partially suppress the toxic effect of mutant polyQ domains during stationary phase significantly extending the life span of the cells compared to cells grown in regular media (Figure A1.2A). Protein expression and aggregation was not affected by caloric restriction (Figure A1.2B).

In yeast, glucose is one of the strongest repressor of respiratory function. Therefore, under caloric restriction, respiratory rate is increased due to the lower glucose concentration present in the media (0.5%). Taken this into account, we decided to test if growing cells in media containing only respiratory substrates such as ethanol and glycerol instead of glucose and therefore achieving a maximum respiratory function had an effect on the toxicity of mutant polyQ domains. Cells were grown in minimum media containing ethanol and glycerol (WOEG) or control media containing glucose (WOGLU). After cells have reached stationary phase (48 h), protein expression was activated by addition of 200nM β-estradiol and cell viability was monitored. We observed that cells grown in respiratory media presented a significantly longer life span compare to cells grown in fermentative media containing glucose (Figure A1.2C) as has been previously described (Piper et al., 2006). In cells grown in respiratory media containing ethanol and glycerol, 103Q toxicity was found to be partially but significantly suppressed indicating that an increase in mitochondrial function and respiratory metabolism is able to suppress mutant polyQ life span reduction (Figure A1.2C). Protein expression and aggregation was not affected by growing the cells in media containing ethanol and glycerol (Figure A1.2D). Finally, we
wanted to test if increasing mitochondrial biogenesis in cells grown in the presence of glucose could also suppress mutant polyQ toxicity. In order to do that, we overexpressed HAP4, the catalytic subunit of the HAP complex, a transcriptional activator of nuclear encoded mitochondrial genes. We have described before that in rapidly dividing cells, HAP4 overexpression promotes an increase in mitochondrial biogenesis and respiratory function that partially suppresses mutant polyQ toxicity. Mutant polyQ (103Q) cells carrying an empty plasmid or a plasmid overexpressing HAP4 were grown in media containing glucose (WOGLU) with the appropriate selection for plasmid maintenance during 48 hours until cells reached stationary phase. At this point, protein expression was induced by addition of 200nM β-estradiol and cell viability was monitored. We found that HAP4 overexpression was not able to extend yeast CLS as it has been previously reported (Piper et al., 2006), in fact, a slightly reduction in the life span was observed (Figure A1.2E). Chronological life span extension by HAP4 overexpression has been only reported once and the discrepancy with our data maybe due to the different yeast genetic background used or different culture conditions since in this specific article, cells were transferred to water after stationary phase is reached. Surprisingly, despite no observing any life span extension by HAP4, we found that HAP4 overexpression was able to suppress the toxicity of mutant 103Q almost completely (Figure A1.2E). As previously shown, protein expression and aggregation was not affected the cells in media containing ethanol and glycerol (Figure A1.2F)
Figure A1.2. Increase in mitochondrial biogenesis and respiratory function suppresses the toxicity of mutant polyQ domains during stationary phase. Suppression of mutant polyQ toxicity domains by growing cells: (A) Under caloric restriction (CR) (C) In respiratory media containing ethanol and glycerol (WOEG) (E) Overexpressing HAP4 to increase mitochondrial biogenesis. Mutant polyQ expression levels of cell growing (B) Under caloric restriction (CR) (D) In respiratory media containing ethanol and glycerol (WOEG) (F) Overexpressing HAP4 to increase mitochondrial biogenesis
We have found that increasing mitochondrial biogenesis and respiratory function by three different means was able to suppress the reduction of yeast chronological life span induced by mutant polyQ domains. These data agrees with our published work where working with rapidly dividing cells, we also demonstrated that HAP4 was able to partially suppress mutant polyQ toxicity. It is important to note that in this case, we are working with non-dividing cells and that the effect of the different suppression mechanisms that we tested on the toxicity of polyQ domains is probably due to a preconditioning of the cells before protein expression is activated. Caloric restriction, media containing respiratory substrates or HAP4 overexpression are altering the metabolism of the cells and increasing mitochondrial biogenesis already during exponential phase of growth in the 48 hours period before protein expression is activated. We believe that what happens during this time frame before the cells reach stationary phase is crucial for the fate of the cells and that it will not only influence the effect of mutant polyQ domains or other stresses in the life span of the cells but it will also affect how cells will be able to manage these stresses and ultimately survive.
REFERENCES


