IDENTIFICATION OF SELECTED AGEING-RELATED PROTEINS AND THE CHARACTERISATION OF PTC4 IN YEAST

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IDENTIFICATION OF SELECTED AGEING-RELATED PROTEINS AND THE CHARACTERISATION OF PTC4 IN YEAST

by

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LIST OF SYMBOLS

Amp	Ampere
A	Alanine
N	Asparagine
D	Aspartic acid
bp	Base pair
$CaCl_2$	Calcium chloride
$CuSO_4$	Copper (II) sulfate
cm	Centimeter
mm^3	Cubic millimeter
°C	Degree Celsius
Δ	Deletion
Na_2HPO_4	Disodium phosphate
δ	Doubling time
F	Farad
G_0	Gap phase
g	Gravity
G_1	Growth 1 phase
h	Hour
HCl	Hydrogen chloride
H_2O_2	Hydrogen peroxide
•OH	Hydroxyl radical
::	Insertion
kb	Kilobase
kDa	Kilodalton
K	Lysine
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar

mAmp	Milliampere
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
min	Minute
M	Molar
KH_2PO_4	Monopotassium phosphate
ng	Nanogram
N	Normality
Ω	Ohm
O_2	Oxygen
%	Percentage
KCl	Potassium chloride
s	Second
$^1O_2$	Singlet oxygen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$NaH_2PO_4.2H_2O$	Sodium phosphate monobasic dihydrate
$O_2^{\bullet -}$	Superoxide radical
U	Unit
V	Volts
H_2O	Water

LIST OF ABBREVIATIONS

A_{600}	Absorbance at a wavelength of 600 nm
Acetyl-CoA	Acetyl coenzyme A
APS	Ammonium persulfate
Amp^R	Ampicillin resistance
BrdU	5-bromo-2-deoxyuridine
CR	Calorie restriction
CCD	Chlorocatechol 1,2-dioxygenase
CLS	Chronological life span
CS	Cloning site
CFU	Colony forming unit
cAMP	Cyclic adenosine monophosphate
CDKs	Cyclin-dependent protein kinases
dNTP	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
D	Dilution factor
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
DSBs	Double-stranded DNA breaks
EMM_2	Edinburgh Minimal Medium
EDTA	Ethylenediaminetetraacetic acid
ERCs	Extrachromosomal rDNA circles
ETC	Electron transport chain
FoxOs	Forkhead transcription factors of the O class
HlyE	Haemolysin E
HSP	Heat shock protein
HOG	High-osmolarity glycerol
His	Histidine
HRP	Horseradish peroxidase
LiOAc	Lithium acetate
IgG	Immunoglobulin G

IDT	Integrated DNA Technologies
JTT	Jones-Taylor-Thomton
L	Length
Leu	Leucine
LB	Luria-Bertani
MIF	Migration inhibitory factor
MSc	Master of Science
mRNA	Messenger ribonucleic acid
PPM	Metal-dependent protein phosphatase
MEP	Mother enrichment program
MCS	Multiple cloning sites
Mb	Myoglobin
NADPH	Nicotinamide adenine dinucleotide phosphate
N	Number
OD	Optical density
Ori	Origin of replication
PBS	Phosphate-buffered saline
PPP	Phosphoprotein phosphatase
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PDS	Post-diauxic shift element
PKA	Protein kinase A
PTP	Protein tyrosine phosphatase
PDH	Pyruvate dehydrogenase
ROS	Reactive oxygen species
RLS	Replicative life span
rDNA	Ribosomal DNA
SC-Ura	Selective complete lacking uracil
SC-Ura-Leu	Selective complete lacking uracil and leucine
ss	Single-stranded
snRNAs	Small nuclear ribonucleic acids
snRNPs	Small nuclear ribonucleoproteins
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STREs	Stress responsive elements
SOC	Super optimal broth with catabolite repression

S	Survival
TOR	Target of rapamycin
TEMED	Tetramethylethylenediamine
T	Time
TORC1	TOR complex 1
TORC2	TOR complex 2
TSS	Transformation and storage solution
Tgf-β	Transforming growth factor-β
TBE	Tris-borate-EDTA
TE	Tris-EDTA
2μ	Two-micron
PP2C	Type 2C protein phosphatase
Ura	Uracil
V	Volume
YPD	Yeast extract peptone-dextrose

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IDENTIFIKASI PROTEIN BERKAIT PENUAAN TERPILIH DAN PENCIRIAN PTC4 DALAM YIS

ABSTRAK

Protein yang berkaitan dengan penuaan memainkan peranan dalam proses selular seperti tindak balas terhadap tekanan, apoptosis, sistem ubiquitin-proteasome dan laluan signal antara pelbagainya. Beratus-ratus protein yang berkaitan dengan penuaan telah dikesan, tetapi peranan mereka masih tidak diketahui. Dalam pengajian ini, ujian jangka hayat kronologi (CLS), ujian tekanan dan ujian pertumbuhan telah ditubuhkan. Kesan pelanjutan CLS protein berkaitan penuaan, Ptc4, Zwf1, Sme1 dan Sod1 yang dipilih daripada skrin CLS sebelum ini telah disahkan melalui ujian unit pembentuk koloni piawai (CFU) dan pemadaman gen berkaitan penuaan yang dipilih menyebabkan penurunan CLS dalam yis. Ptc4 menyumbangkan toleransi tekanan oksidatif dalam ujian tekanan oksidatif piawai dan memanjangkan CLS terbanyak antara protein berkaitan penuaan yang dipilih. Zwf1 dan Sod1 yang menyumbangkan toleransi tekanan oksidatif dalam sel muda, tidak menunjukkan toleransi tekanan oksidatif dalam sel selepas penuaan yang berpanjangan, mencadangkan kehilangan keupayaan toleransi tekanan oksidatif selepas penuaan yang berpanjangan. Selain itu, Ptc4 dan Zwf1 yang diekspres berlebihan menggalakkan percambahan sel semasa pertumbuhan sel dalam ujian pertumbuhan piawai, mencadangkan penglibatan mereka dalam sistem pembahagian atau pertumbuhan sel. Tambahan pula, kehilangan Ptc4 masih dapat menggalakkan percambahan sel tetapi kehilangan Zwf1 tidak menjejaskan kadar percambahan sel. Disebabkan Ptc4 dapat mengalakkan percambahan sel, kesan fosfatase protein jenis 2C yis lain (PP2Cs) terhadap kadar percambahan sel juga disiasat dalam ujian

pertumbuhan piawai. Berdasarkan keputusannya, ekspresi berlebihan atau kehilangan PP2C juga boleh menggalakkan percambahan sel, menunjukkan mekanisme pembahagian sel adaptif dikawal oleh PP2Cs di dalam yis. Secara keseluruhannya, kajian ini mengesahkan kepentingan protein berkaitan penuaan, Ptc4, Zwf1, Sme1 dan Sod1 dalam pengawalan jangka hayat sel dengan memainkan pelbagai peranan dalam laluan penentuan jangka hayat dan mengenal pasti penglibatan tujuh PP2Cs yis dan Zwf1 dalam pengawalan percambahan sel.

IDENTIFICATION OF SELECTED AGEING-RELATED PROTEINS AND THE CHARACTERISATION OF PTC4 IN YEAST

ABSTRACT

Ageing-related proteins play different roles in cellular processes such as regulating stress response, apoptosis, ubiquitin-proteasome system and signal transduction pathways amongst many others. Hundreds of ageing-related proteins have been revealed, but the roles of most of these ageing-related proteins are still unknown. In this study, the standardised chronological life span (CLS) assay, stress assay and growth assay were established. The CLS-extending effects of the ageing-related proteins, Ptc4, Zwf1, Sme1 and Sod1 selected from previous chronological life span (CLS) screen were validated through standardised colony forming unit (CFU) assay and the deletions of the selected ageing-related genes resulted in decreased CLS in yeast. Ptc4 contributes to oxidative stress tolerance in standardised oxidative stress assay and extends CLS the most among the selected ageing-related proteins. Zwf1 and Sod1 that contribute to oxidative stress tolerance in young cells, did not show oxidative stress tolerance in cells after prolonged ageing, suggesting the loss of oxidative stress tolerance capability after prolonged ageing. Besides, Ptc4 and Zwf1 promoted cell proliferation during cell growth upon protein overexpression in standardised growth assays, suggesting their involvement in cell division or growth pathways. Furthermore, loss of Ptc4 could still promote cell proliferation while loss of Zwf1 did not affect rate of cell proliferation. As Ptc4 could promote cell proliferation, the effects of other yeast type 2C protein phosphatases (PP2Cs) on rate of cell proliferation were also investigated in standardised growth assays. Based on the results, overexpression or loss of type 2C protein phosphatase (PP2C) could also

promote cell proliferation, suggesting an adaptive cell division mechanism is regulated by PP2Cs in yeast. Overall, this study confirms the importance of the ageing-related proteins, Ptc4, Zwf1, Sme1 and Sod1 in regulation of cell life span by playing various roles in longevity-defined pathways and identifies the involvement of seven yeast PP2Cs and Zwf1 in regulation of cell proliferation.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Ageing is a biological process that occurs with gradual structural and functional changes in living organisms at increasing lifetime. There are two types of ageing which are organismal ageing and cellular ageing. At organismal level, organisms like humans get older with clear appearance of wrinkles and grey hairs. At cellular level, cells like skin, neuron and muscle cells undergo ageing with the accumulation of cellular damage that causes loss of cellular function and eventually cell death. There are two types of cellular ageing which are replicative ageing and chronological ageing. Replicative ageing occurs in replicating cells such as skin cells that can replicate to produce new cells while chronological ageing occurs in non-replicating cells such as neuron and muscle cells that do not replicate (Longo *et al*., 2012).

With increasing population of elders, ageing studies become important to be focused on. Older people have high possibility to have chronic diseases that deteriorate their life qualities (Longo *et al*., 2012). In fact, ageing is the main risk factor of causing chronic human diseases such as type 2 diabetes mellitus, neurodegenerative disorders, hypertension, all types of arteriosclerosis, cancer and age-associated macular degeneration (Li *et al*., 2017; Martin, 2011). A good control of ageing is significantly demanded for preventing the disease attacks, and enabling older people to continue working and minimize health expenses (Longo *et al*., 2012).

Budding yeast, *Saccharomyces cerevisiae*, has been used as the model organism to study ageing (Longo *et al*., 2012), as well as the molecular mechanisms or cellular processes such as cell cycle progression (Wanke *et al*., 2005), proteostasis

(Kyryakov *et al*., 2012) and apoptosis (Owsianowski *et al*., 2008). Using yeast in ageing study is beneficial due to its short generation time, convenient and inexpensive experimental approaches as well as high-throughput methodologies available for genetic screen (Mirisola *et al*., 2014; Petranovic & Nielsen, 2008). Moreover, yeast and human have been evolved from a common ancestor around a billion years ago, yet yeast has 23 % of genes homologous and thousands of genes orthologous to human genes and gene functions of both yeast and human are strongly conserved that almost half (47 %) of the yeast genes can be humanised or substituted by human orthologs (Kachroo *et al*., 2015; Laurent *et al*., 2016; Liu *et al*., 2017) These enable yeast as a suitable model for studying human genetics and molecular biology of human ageing (Janssens & Veenhoff, 2016; Karathia *et al*., 2011; Mirisola *et al*., 2014).

In this ageing study, *S. cerevisiae* was used as the model organism. The ageing-related proteins (Ptc4, Zwf1, Sme1 and Sod1) in this study are involved in different cellular processes or pathways in yeast, which have been discussed in section 2.6 in this thesis. Initially, their effects on CLS in yeast were unknown based on database. The effects of these ageing-related proteins on CLS in yeast were then determined in previous CLS screen (Ong, 2018). These ageing-related proteins promoted CLS extension in the CLS screen. In this study, these ageing-related proteins were selected from the CLS screen to validate their CLS-extending effects in yeast. The type 2C protein phosphatase, Ptc4 was then characterised in the aspects of stress response and rate of cell proliferation and the effects of other type 2C protein phosphatases (PP2Cs) on rate of cell proliferation were also determined in yeast.

1.2 Hypothesis and objectives

A screening result of previous study performed by our group identified seven proteins, Ptc4, Zwf1, Sme1, Sod1, Cpr3, Kss1 and Ump1 that extend CLS upon protein overexpression in *S. cerevisiae* (Ong, 2018). The CLS-extending effects of top three best proteins, Ptc4, Zwf1 and Sme1 from the previous screen were validated by performing a standardized CLS assay in *S. cerevisiae* in this study. Some proteins extend CLS by enhancing stress tolerance (Fabrizio *et al*., 2004; Fabrizio *et al*., 2003; Sampaio-Marques *et al*., 2014). Therefore, the first hypothesis of this study is that the overexpressed proteins Ptc4, Zwf1, Sme1 and Sod1 that extend CLS in *S. cerevisiae*, elevate stress tolerance after prolonged chronological ageing. This hypothesis was tested by determining the stress responses of the overexpressed proteins during chronological ageing through standardised stress assays. As some of the overexpressed proteins promoted proliferation of aged cells during outgrowth in the CLS screen (Ong, 2018), thus the second hypothesis is that the ageing-related proteins can promote cell proliferation during cell growth. To investigate this hypothesis, the rate of proliferation of cells with the overexpressed proteins was determined by calculating doubling time of cells at log phase of normal growth through a standardised growth assay.

The main objective of this study is to determine the roles of the ageing-related proteins in regulation of cell life span and proliferation.

Specific objectives of this study are:

1. To investigate effects of selected ageing-related proteins on chronological life span in yeast.
2. To examine stress responses of selected ageing-related proteins during chronological ageing.
3. To determine effects of selected ageing-related proteins on rate of cell proliferation during cell growth.
4. To investigate effects of type 2C protein phosphatases including ageing-related protein phosphatase, Ptc4 on rate of cell proliferation during cell growth.

CHAPTER 2

LITERATURE REVIEW

2.1 Ageing and its causal factors

Ageing occurs at organismal and cellular levels. Organismal ageing is resulted by the progressive accumulation of molecular damage, cellular senescence, tissue degeneration and organ dysfunction. It can become the risk driver for development of various diseases such as cancer, cardiovascular and neurodegenerative disorders as well as premature mortality (Chen *et al*., 2007; Sławińska & Krupa, 2021). Cellular ageing is driven by the progressive accumulation of biomolecular damage in cells, contributing to cellular dysfunction and ultimately cell death (Viña *et al*., 2007). Cellular senescence is one such pathway that is activated upon permanent cell cycle arrest and can lead to cellular ageing. The causal factors such as oxidative stress due to accumulation of reactive oxygen species (ROS) in cells, genomic instability resulted by telomere shortening or ribosomal deoxyribonucleic acid (rDNA) copy number loss, and molecular damage in DNA, protein and lipid can induce cellular senescence and thus organismal and cellular ageing (Lee & Ong, 2021; Richardson & Schadt, 2014; Rodríguez-Rodero *et al*., 2011; Von Zglinicki, 2002).

Previous studies reported that protein function can be affected by oxidative damage induced by ROS to specific amino acid sequences *in vitro*, especially the proteins that carry metal ions such as iron and copper (Richardson & Schadt, 2014; Stadtman, 2006). Apart from affecting protein function, oxidative damage can also change protein structure, resulting in protein oligomerization and aggregation (Pierce *et al*., 2008; Richardson & Schadt, 2014). Protein damage and accumulation of protein oligomers or aggregates lead to cellular ageing (Richardson & Schadt, 2014;

Salmon *et al*., 2009). In addition, DNA damage can cause cellular senescence and apoptosis. The increase in DNA damage due to DNA repair machinery dysfunction can lead to increase in irreversible DNA mutations, thus accelerating ageing (Garinis *et al*., 2008; Richardson & Schadt, 2014). ROS can also oxidise polyunsaturated lipid chains, resulting in production of aldehydes that can react with DNA bases and amine functional group ($-NH_2$) of proteins to cause DNA and protein mutations (Juan *et al*., 2021).

Another causal factor of ageing is genomic instability due to telomere shortening or rDNA copy number loss (Lee & Ong, 2021). Telomere situated at chromosomal end, which contains 3' repetitive guanine-rich sequence of DNA in its single-stranded (ss) DNA overhang (Eugène *et al*., 2017; Giraud-Panis *et al*., 2010), protects chromosomal end from end-to-end fusion, degradation, and misrecognition of the ends as double-stranded DNA breaks (DSBs) (Dieckmann *et al*., 2016; Tham & Zakian, 2002). Telomeres shorten with each cell division until they lose the ability to protect the ends of chromosomes, leading to cellular replicative senescence and ageing (Mc Auley *et al*., 2017; Richardson & Schadt, 2014). In addition, DNA break repair by intrachromosomal recombination leads to formation of extrachromosomal rDNA circles (ERCs), resulting in rDNA copy loss (Kobayashi & Sasaki, 2017) that causes rDNA instability and subsequently cellular ageing (Hein *et al*., 2012; Saka *et al*., 2016).

2.2 *Saccharomyces cerevisiae* as a model organism for human ageing study

Saccharomyces cerevisiae is generally known as baker's yeast or brewer's yeast. *Saccharomyces* is a genus under kingdom of fungi. It is a unicellular eukaryote that consists of membrane-bound organelles, such as a nucleus and mitochondria,

and has similar cell structure of higher eukaryotic cells such as mammalian cells (Duina *et al*., 2014).

S. cerevisiae cells divide or proliferate as quickly as once every 90 min, through budding. Unbudded yeast cells have diameter of approximately 5 μm which is between the size of bacteria and human cells. Haploid **a** and haploid α are the types of yeast cell mating. Mating between haploid **a** and haploid α produces diploid. Diploids undergo meiosis and sporulation, and produce four haploid spores with two haploids of each mating type when growing in nutrient-poor condition (Duina *et al*., 2014).

In 1978, transformation of *S. cerevisiae* with a plasmid was successfully performed (Hinnen *et al*., 1978). Hence, geneticists were able to mutate genes and control gene expression in yeast. Yeast replication origins allow plasmids to replicate in yeast cells. A combination of approaches such as polymerase chain reaction (PCR) and homologous recombination enable integration of foreign gene into yeast genome and disruption of targeted gene (Duina *et al*., 2014). With the available molecular techniques, a library of various yeast strains with deleted genes was established (Winzeler *et al*., 1999) and enabled the study of longevity-associated genes.

In 1996, the genome of *S. cerevisiae* was the first completely sequenced eukaryotic genome. Its genomic DNA of 12,068 kilobases (kb) is further subdivided into 16 chromosomes (Goffeau *et al*., 1996). Saccharomyces Genome Database (http://www.yeastgenome.org) is available to public and can be accessed to know the information of every single yeast gene.

Amino acid sequences and functions of many ageing-related proteins are highly conserved in yeast and human (Aggarwal & Brosh, 2012; Cazzanelli *et al*., 2018). For instance, loss of yeast slow growth suppressor one (Sgs1) and human

Werner syndrome ATP-dependent helicase (WRN) homolog cause premature ageing (Kawabe *et al*., 2001). Moreover, yeast silent information regulator two (Sir2) and human sirtuin one (*SIRT1*) homolog promote life span extension (Figarska *et al*., 2013; Kaeberlein *et al*., 1999). Furthermore, cellular processes that regulate ageing such as mitochondrial metabolism (Baccolo *et al*., 2018; Barros *et al*., 2010), proteostasis (Ruan *et al*., 2017), stress response regulatory pathways (de la Torre-Ruiz *et al*., 2015; Eleutherio *et al*., 2018), genomic stability maintenance (Lee & Ong, 2021) and nutrient signalling pathways (Kapahi *et al*., 2010; Rahimi *et al*., 2013) are also well conserved in yeast and human. These allow understanding of human ageing mechanism through studying of ageing-related proteins and pathways in yeast using yeast genetics. In addition, ageing studies on mammals such as mice and rats require long time duration as the mammals have longer life span (3-5 years) (Flurkey *et al*., 2007; Gorbunova *et al*., 2008) while yeast has shorter life span of 1-3 weeks. Therefore, yeast is preferable to be a model organism for human ageing study (Zimmermann *et al*., 2018).

2.3 Growth phases of Saccharomyces cerevisiae

Budding yeast, *Saccharomyces cerevisiae* cells undergo growth with four phases which are lag phase, exponential or log phase, post-diauxic phase and stationary phase in life-time (Figure 2.1). During lag phase, yeast cells adapt to the environment and do not proliferate and grow. Nutrients in growth medium are excessive for later growth (Alsuhaim *et al*., 2013). The subsequent phase is log phase in which the cells grow and proliferate rapidly with average doubling time of 90 min at growth temperature of 30°C. The proliferating cells undergo fermentation which is

the glycolysis process of breaking down glucose to produce nonfermentable ethanol and energy (Gray *et al*., 2004).

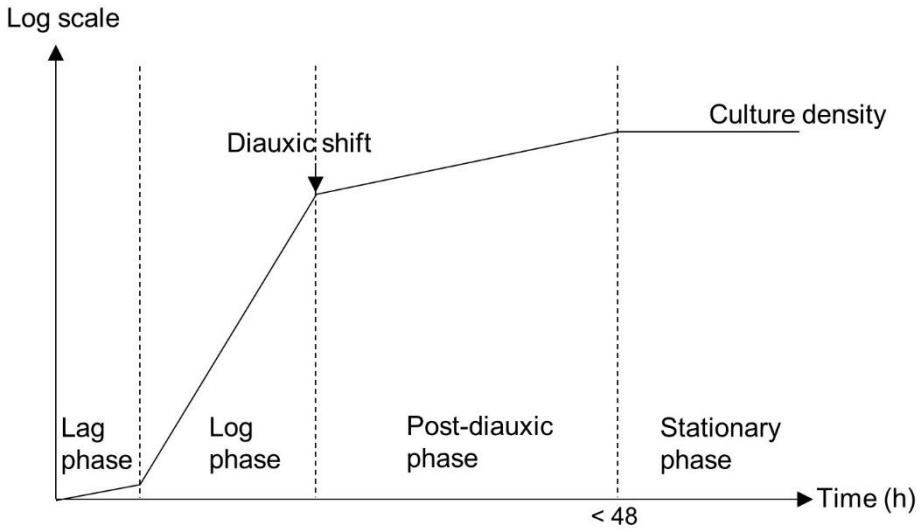

Figure 2.1 Growth phases of *Saccharomyces cerevisiae* (adapted from Busti *et al*., 2010)

When glucose is consumed thoroughly by cells in the medium, diauxic shift occurs. At diauxic shift, the yeast cells stop proliferation and convert fermentation to respiration. After converting to respiration, the cells enter post-diauxic phase in which they resume their proliferation and growth at slow mode and consume ethanol accumulated in medium. When the ethanol and other nonfermentable carbon sources are depleted, the cells stop proliferation and have undergone replicative ageing. The cells then enter stationary phase in which most of the cells are quiescent and undergo chronological ageing. These quiescent cells are capable to re-enter cell cycle and resume replication to produce daughter cells when the old growth medium is replaced with fresh medium (Busti *et al*., 2010; Werner-Washburne *et al*., 1993) (Figure 2.1).

Yeast cells in stationary phase have condensed chromosomes (Yang *et al*., 2006) and thickened cell wall (Smith *et al*., 2000). They exhibit significantly decreased gene transcription and translation rate compared to cells at log phase (Choder, 1991), strongly repressed expression of genes which encode ribosomal proteins (Gray *et al*., 2004), activated transcription of stress responsive genes, accumulation of storage carbohydrates such as glycogen and trehalose, elevated stress tolerance (Gray *et al*., 2004; Swinnen *et al*., 2006) and induced autophagy (Noda & Ohsumi, 1998).

2.3.1 Regulation of cell growth and proliferation in *Saccharomyces cerevisiae*

Mammalian cells were shown to have finite proliferative capacity known as "Hayflick limit" (Hayflick & Moorhead, 1961) which subsequently triggers replicative senescence. Cell proliferation, a process that leads to an increase in cell number via cell division (Kaldis, 2016), is essential for tissue renewal and maintaining cell homeostasis for sustaining survival of living organisms (Campisi & d'Adda di Fagagna, 2007). However, nonstop proliferation can be detrimental, leading to development of cancer. Therefore, cellular senescence is a beneficial protective process that suppresses cancer development because it suspends the proliferation of damaged cells and mitotic progression of malignant cells (Muñoz-Espín & Serrano, 2014). A regulation of cell proliferation is thereby important to ensure optimal conservation of healthy and functional cells.

Two nutrient signalling pathways, target of rapamycin (TOR) pathway and cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway that regulate cell growth and proliferation in budding yeast are well defined. The downstream target of both pathways, serine/threonine protein kinase (Rim15), is

a key regulator protein of cell cycle progression. Rim15 is essential for cell entry into G_0 or stationary phase (Pedruzzi *et al*., 2003) and is inactivated via phosphorylation in TOR pathway and PKA pathway (Reinders *et al*., 1998). When both pathways are inhibited, Rim15 becomes active and drives cell entry into G_0, leading to cell cycle arrest. The downstream transcription factors of Rim15 such as multicopy suppressor of *SNF1* mutation proteins two and four (Msn2 and Msn4) subsequently bind to stress responsive elements (STREs) present in the promoter of stress-responsive genes to regulate stress responsive gene expression to support survival of non-dividing cells (Boy-Marcotte *et al*., 1998; Martínez-Pastor *et al*., 1996). The details about both the pathways in regulation of cell growth and proliferation are mentioned in section 2.3.1(a) and 2.3.1(b).

2.3.1(a) Target of rapamycin (TOR) pathway regulates cell growth and proliferation

TOR proteins, Tor1 and Tor2 in the TOR pathway were originally recognized in *S. cerevisiae* by mutations of TOR proteins which provide resistance to rapamycin (Helliwell *et al*., 1994). TOR proteins present in all eukaryotes. Tor1 and Tor2 are structurally and functionally similar. Both TOR proteins regulate cell growth (Loewith *et al*., 2002). Tor2 has an additional function of cell cycle-dependent polarization of actin cytoskeleton (Schmidt *et al*., 1996). Both TOR proteins form two complexes in the signalling pathway, which are TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 comprises of Tor1 or Tor2, kontroller of growth one (Kog1) and lethal with sec13 protein eight (Lst8) while TORC2 consists of Tor2, adheres-voraciously-to-target-of-rapamycin-2 protein one, two or three (Avo1, Avo2, Avo3) and Lst8 (Loewith *et al*., 2002; Wedaman *et al*., 2002).

TOR pathway is activated in the presence of nitrogen and carbon sources (Crespo *et al*., 2002; Shamji *et al*., 2000). TOR pathway promotes cell growth and proliferation by regulating several cell activities such as inducing ribosome biogenesis, protein translation, early G_1 progression and actin organization as well as downregulating stress response and inhibiting autophagy (Barbet *et al*., 1996; Inoue & Nomura, 2017; Noda & Ohsumi, 1998; Powers & Walter, 1999; Zurita-Martinez & Cardenas, 2005). TORC1 phosphorylates type 2A phosphatase-associated protein 42 (Tap42) in the presence of nutrients such as nitrogen and carbon sources, thus promoting interaction of Tap42 with NAD-dependent protein lipoamidase sirtuin-4 (Sit4) and type 2A protein phosphatase (PP2A) catalytic subunits (Pph21/22) (Como & Arndt, 1996; Jiang & Broach, 1999) while autophagy-related one (Atg1) and autophagy-related 13 (Atg13) which are important regulators of autophagy are phosphorylated and inactivated by protein kinase A and TORC1 kinase, thus inhibiting the association of Atg1 and Atg13 to prevent the induction of autophagy (Stephan *et al*., 2009; Stephan *et al*., 2010) (Figure 2.2).

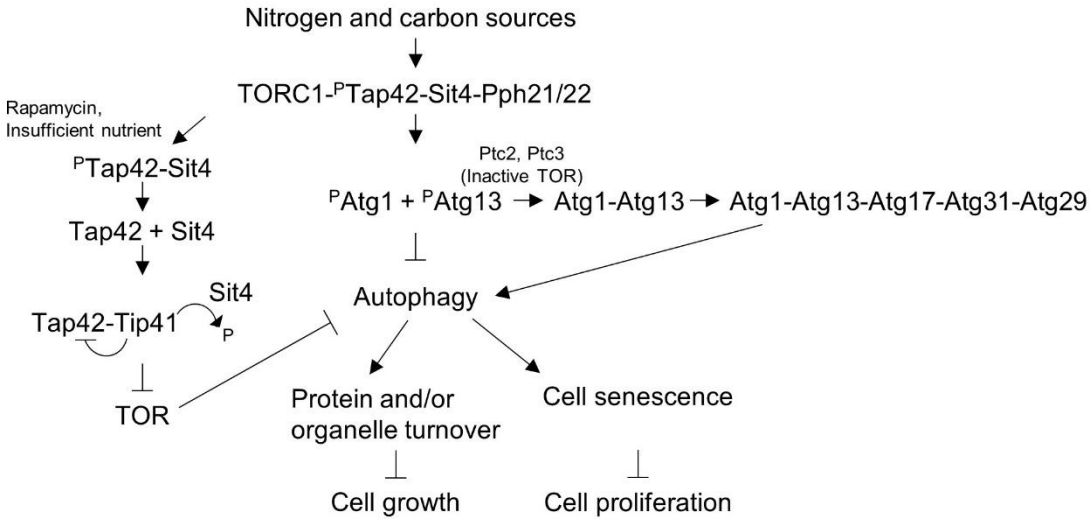

Figure 2.2 TORC1 regulates autophagy that inhibits cell growth and proliferation

Rapamycin treatment or loss of TOR function by nutrient starvation, stress condition or during diauxic shift, results in blocking translation initiation and transcription of ribosomal proteins, stimulating cell growth arrest early in G_1, inducing cell entry into stationary phase (G_0), producing storage carbohydrates (glycogen and trehalose) and inducing autophagy (Barbet *et al*., 1996; Noda & Ohsumi, 1998; Schmelzle & Hall, 2000). In rapamycin-treated cells, rapamycin binds to FKBP12 prolyl isomerase. This rapamycin-FKBP12 complex targets on Tor1 and Tor2 in TORC1 and inhibit the function of TORC1 while the complex cannot bind to TORC2, thus the unique function of actin polarization of TORC2 is not inhibited by the complex (Loewith *et al*., 2002; Sabers *et al*., 1995). Dissociation of Tap42 from Sit4 occurs under nutrient insufficiency or treatment with rapamycin. Enhanced association of Tap42 interacting protein 41 (Tip41) with Tap42 instead occurs with the help of Sit4 to dephosphorylate Tip41 while type 2C protein phosphatase (Ptc1) is essential for maintaining normal level of Tip41 (González *et al*., 2009). Tip41 interacts with and inhibits Tap42, thus TOR pathway is inactivated (Jacinto *et al*., 2001). Type 2C protein phosphatases (Ptc2 and Ptc3) stimulate autophagy by dephosphorylating Atg1 and Atg13 when TOR is inactive (Memisoglu *et al*., 2019).

The dephosphorylated Atg1 and Atg13 interacts with each other and also associates with autophagy-related Atg17-Atg31-Atg29 to form Atg1 complex (Fujioka *et al*., 2014; Kabeya *et al*., 2009). The Atg1 complex then localises at phagophore assembly site (PAS) and stimulates the downstream proteins to induce generation of autophagosomes to engulf and transport intracellular substrates to vacuoles in yeast for degradation and nutrient recycle (Kamada *et al*., 2010; Kawamata *et al*., 2008; Suzuki *et al*., 2007; Yamamoto & Noda, 2020). Autophagy

that occurs can lead to inhibition of cell growth by inducing protein and/or organelle turnover and inhibition of cell proliferation by inducing cellular senescence (Wang & Levine, 2010) (Figure 2.2).

2.3.1(b) Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway regulates cell growth and proliferation

cAMP-dependent PKA pathway is conserved in all eukaryotic organisms, except in plant phyla (Kim *et al*., 2007). PKA is made of a regulatory subunit encoded by bypass of cyclic-AMP requirement one (*BCY1*) and three catalytic subunits encoded by Takashi's protein kinase one, two or three (*TPK1, TPK2*, and *TPK3*) (Cameron *et al*., 1988; Toda *et al*., 1987). cAMP-PKA pathway stimulates cell growth and proliferation by inducing ribosome biogenesis, downregulating the expression of STREs-controlled genes and inhibiting autophagy in response to nutrients (Baroni *et al*., 1989; Besozzi *et al*., 2012; Budovskaya *et al*., 2004; Neuman-Silberberg *et al*., 1995; Smith *et al*., 1998; Zurita-Martinez & Cardenas, 2005). In addition, PKA pathway also downregulates the expression of genes such as glycogen accumulation one (*GAC1*) and glycogen synthase two (*GSY2*) required for glycerol synthesis (Francois *et al*., 1992; Hardy *et al*., 1994).

In the presence of glucose, adenylate cyclase encoded by cyclic AMP requirement 1/cell division cycle 35 (*CYR1*/*CDC35*) is activated by GTP-bound G proteins (Ras and G protein-coupled receptor one, Gpr1) (Cannon *et al*., 1986; Rolland *et al*., 2000; Suzuki *et al*., 1990). Ras proteins, homologous to RAS proto-oncogene proteins (Ras1 and Ras2), are activated by cell division cycle 25 (Cdc25), which induces the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP), and inhibited by inhibitory regulator proteins (Ira1 and Ira2), which induces the GTP hydrolase (GTPase) activity of Ras proteins. Gpr1 interacts

with G protein α subunit (Gpa2) for the activation of adenylate cyclase. The activated adenylate cyclase stimulates the synthesis of cyclic adenosine monophosphate (cAMP). cAMP binds to the inhibitory regulatory subunit of PKA, thus releasing the catalytic subunits of PKA to activate PKA. In feedback inhibition on cAMP synthesis, cAMP can be degraded by low- and high-affinity phosphodiesterases, Pde1 and Pde2 respectively, thus downregulating the level of cAMP and PKA activity (Kataoka *et al*., 1985; Kraakman *et al*., 1999; Ma *et al*., 1999; Mbonyi *et al*., 1988; Nakafuku *et al*., 1988; Nikawa *et al*., 1987; Paiardi *et al*., 2007; Robinson *et al*., 1987; Sun *et al*., 1994; Tanaka *et al*., 1991; Temeles *et al*., 1985; Van Aelst *et al*., 1991; Xue & Hirsch, 1996; Yun *et al*., 1997). Other than glucose, intracellular acidification also stimulates cAMP synthesis (Colombo *et al*., 1998). Active PKA, similarly to TORC1, phosphorylates and inactivates Atg1 and Atg13 and thus inhibits the association of Atg1 and Atg13 to prevent the stimulation of autophagy (Stephan *et al*., 2009; Stephan *et al*., 2010) (Figure 2.3).

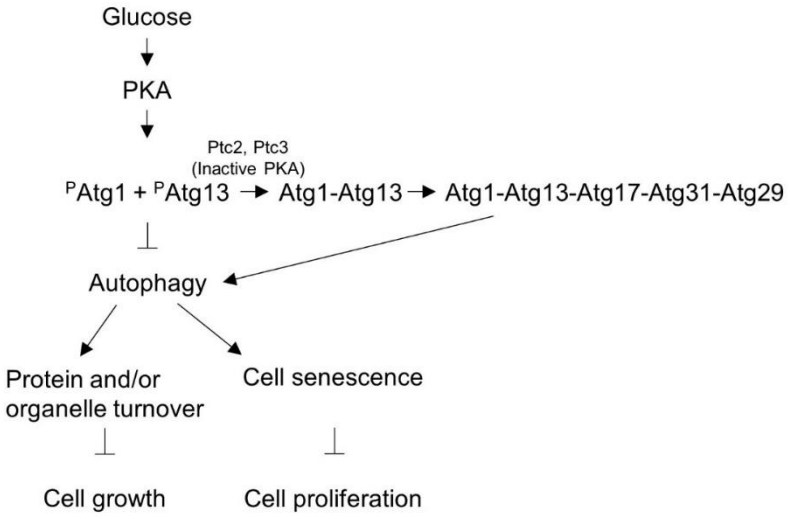

Figure 2.3 PKA regulates autophagy that inhibits cell growth and proliferation

Loss of PKA function due to nutrient starvation leads to cell growth arrest early in G_1 and cell entry into G_0 followed by accumulation of storage carbohydrates (glycogen and trehalose), elevated stress tolerance and induction of autophagy, similarly shown by TOR-deficient cells (Engelberg *et al*., 1994; Matsumoto *et al*., 1983; Pedruzzi *et al*., 2003; Smith *et al*., 1998). Ptc2 and Ptc3 dephosphorylate Atg1 and Atg13 to stimulate autophagy which can in turn inhibit cell growth and proliferation when PKA is inactive (Memisoglu *et al*., 2019) (Figure 2.3).

2.4 Ageing paradigms of yeast

Replicative ageing and chronological ageing are two primary paradigms in study of yeast ageing (Longo *et al*., 2012). Replicative life span (RLS) is defined as the total number of times a mother cell proliferates through asymmetric budding during replicative ageing before cellular senescence (Mortimer & Johnston, 1959) and enables the ageing study of dividing cells such as stem cells, fibroblasts and lymphocytes. Chronological life span (CLS) is defined as the length of time that a nondividing cell is viable at stationary phase during chronological ageing and enables the ageing study of postmitotic cells such as neuron and muscle cells (Longo *et al*., 2012; Sampaio-Marques *et al*., 2014) (Figure 2.4).

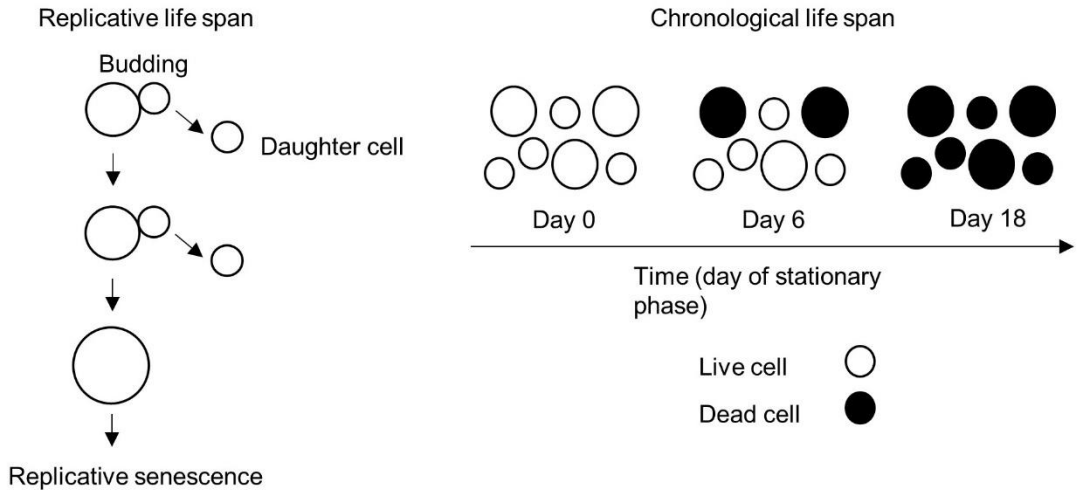

Figure 2.4 Replicative and chronological life span of budding yeast (adapted from Chadwick *et al*., 2016)

2.4.1 Replicative life span

Replicatively aged yeast cells stop proliferation after completing around 20-25 numbers of divisions and are in a short post-replicative state followed by cell lysis (Longo *et al*., 2012). Accumulation of ERCs in yeast mother cells (Janssens & Veenhoff, 2016; Sinclair & Guarente, 1997), loss of proteostasis, deregulated nutrient sensing pathways, and mitochondrial dysfunction are the hallmarks of replicative ageing in yeast (Janssens & Veenhoff, 2016; López-Otín *et al*., 2013).

RLS is determined by counting the number of daughter cells produced by an individual mother cell through asymmetric budding before cellular senescence (Sampaio-Marques *et al*., 2014) (Figure 2.4). RLS analysis is primarily conducted by microdissection. This method involves using a thin needle to separate daughter cells from mother cells manually under a microscope (Longo *et al*., 2012). However, microdissection is time-consuming and is not suitable for high-throughput studies. Moreover, it is difficult to observe cell or organelle morphology and track molecular markers in individual cells during RLS (Xie *et al*., 2012). Hence, there are alternative

methods developed to measure RLS such as biotinylation (Denoth Lippuner *et al*., 2014; Xie *et al*., 2012), mechanical trapping (Denoth Lippuner *et al*., 2014; Lee *et al*., 2012), elutriation (Denoth Lippuner *et al*., 2014; Woldringh *et al*., 1995), affinity purification (Denoth Lippuner *et al*., 2014) and mother enrichment program (MEP) (Denoth Lippuner *et al*., 2014; Lindstrom & Gottschling, 2009).

Previous findings found some genes that regulate RLS in yeast. For instance, *RAS1* and *RAS2* impose opposite effects on RLS. Deletion of *RAS1* prolongs RLS while deletion of *RAS2* shortens RLS (Sun *et al*., 1994). Besides, deletion of either longevity assurance gene one (*LAG1*) or longevity assurance gene two (*LAG2*) shortens RLS (D'Mello *et al*., 1994). Deletion of superoxide dismutase one (*SOD1*) or double deletions of *SOD1* and superoxide dismutase two (*SOD2*) dramatically decreases RLS (Longo *et al*., 1996). Moreover, deletion of serine/threonine protein kinase gene (*SCH9*) extends RLS in yeast (Fabrizio *et al*., 2004). Furthermore, overexpressed silent information regulator two (*SIR2*) extends RLS (Kaeberlein *et al*., 1999).

Other than the RLS-associated genes, calorie restriction and several nutrient signalling pathways were reported to be involved in regulation of RLS. Calorie restriction (CR) extends RLS in yeast and the activation of Sir2 was suggested to be required for the RLS extension. In addition, downregulation of TOR pathway or PKA pathway extends RLS (Kaeberlein *et al*., 2005; Lin *et al*., 2000).

2.4.2 Chronological life span

Chronologically aged yeast cells are in nondividing state at stationary phase. CLS of the yeast cells is generally determined by a traditional method, colony forming unit (CFU) count. This is performed by growing the cells until stationary phase, followed by doing serial dilution and plating the diluted nondividing cell

cultures on nutrient-rich agar. Number of colonies formed on the agar is then counted to calculate cell survival. This method consumes much time and resources and is not suitable for high-throughput assay (Jett *et al*., 1997; Powers III *et al*., 2006). Therefore, several CLS methods such as outgrowth kinetics assay (Murakami *et al*., 2008), barcoded competition-based assay (Matecic *et al*., 2010), fluorescence labelling (Chadwick *et al*., 2016) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)-based assay (Belak *et al*., 2018) were developed to minimize time and resource consumption. These methods are also applicable in high-throughput manner.

Several RLS-associated genes are involved in regulation of CLS as well in yeast. For instance, deletion of *SOD1* or double deletion of *SOD1* and *SOD2* can cause dramatic reduction of RLS and CLS in yeast (Longo *et al*., 1996). Overexpression of both Sod1 and Sod2 increased yeast CLS by 30 %, while the overexpression of either Sod1 or Sod2 alone could result in minor yeast CLS extension (Fabrizio *et al*., 2003). Moreover, overexpression of Sir2 prolongs yeast CLS (Orozco *et al*., 2013). Besides extending RLS, deletion of *SCH9* also prolongs CLS in yeast. CR extends RLS and CLS in yeast (Wei *et al*., 2008). In addition, downregulation of TOR pathway or PKA pathway extends RLS and CLS by enhancing stress tolerance (Fabrizio *et al*., 2001; Fabrizio & Longo, 2003; Powers III *et al*., 2006).

2.5 Life span regulators in *Saccharomyces cerevisiae*

The budding yeast, *Saccharomyces cerevisiae* is useful for studying mechanism of regulating life span. Ageing studies in budding yeast discovered nutrient signalling pathways, TOR pathway and PKA pathway that regulate cell life

span. Other than the nutrient sensing pathways, CR was also demonstrated to regulate life span of budding yeast and higher eukaryotes including mammals. The lessons of life span regulation learnt from budding yeast thus make possible the pharmacological modulation of longevity mechanisms to extend human life span.

2.5.1 Nutrient signalling pathways regulate cell life span

The two major nutrient signalling pathways, TOR pathway and PKA pathway which are essential for cell growth, can result in organismal ageing (Longo, 2003; Wei *et al*., 2008). Several studies reported that downregulation of the nutrient signalling pathways extends cell life span by activating stress response pathway. The Rim15 in stress response pathway, which is converged by both major pathways, activates the stress-resistance transcription factors, Glg1-2 suppressor (Gis1) and Msn2/4 which induce the transcriptional activation of stress-associated genes to promote cell protection against stresses, finally leading to cell life span extension (Carmona-Gutierrez & Büttner, 2014; Pedruzzi *et al*., 2003; Swinnen *et al*., 2006). In addition to the activation of Rim15, downregulated TOR pathway increases mitochondrial respiration and membrane potential that leads to induction of mitochondrial ROS signalling which reduces ROS synthesis. Reduced level of intracellular ROS can lead to cell life span extension due to reduced ROS damage to cellular biomolecules (Longo *et al*., 2012) (Figure 2.5). The mechanisms of both nutrient signalling pathways are reviewed in section 2.5.1(a) and 2.5.1(b).

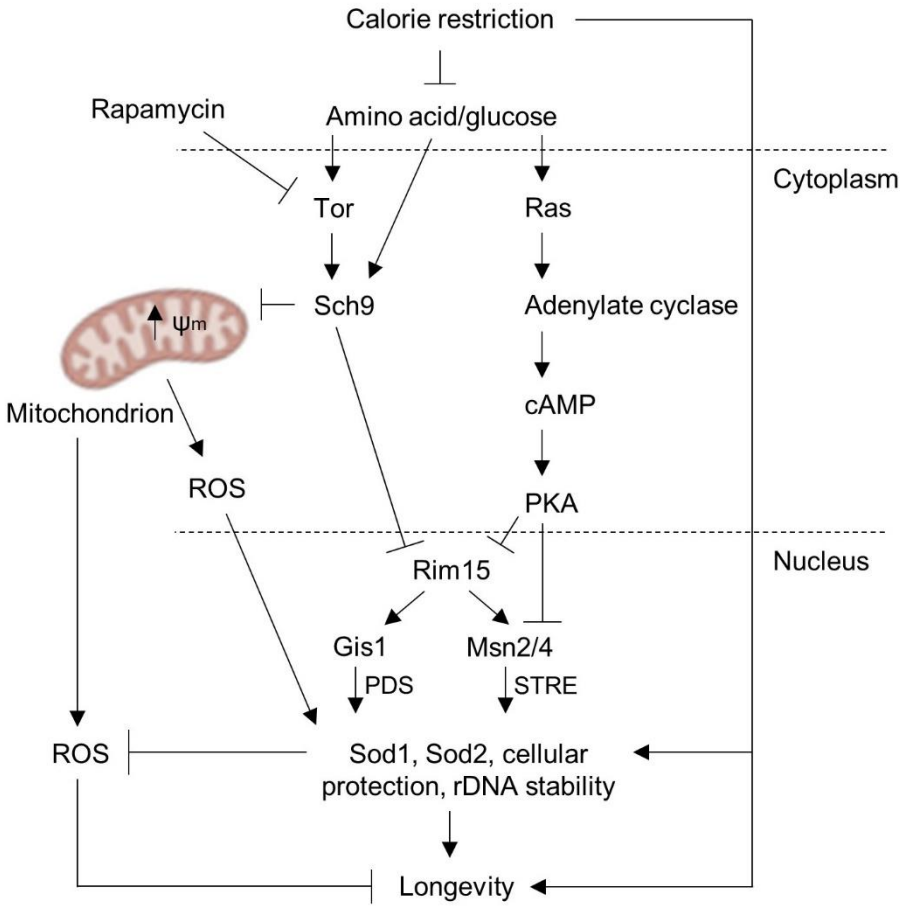

Figure 2.5 Nutrient signalling pathways, TOR pathway and PKA pathway in regulation of yeast longevity

Abbreviation: cyclic adenosine monophosphate, cAMP; reactive oxygen species, ROS; post-diauxic shift element, PDS; stress responsive element, STRE (adapted from Longo *et al*., 2012) (image created with BioRender.com).

2.5.1(a) Target of rapamycin (TOR) pathway regulates cell life span

Downregulation of TOR pathway by deletion of the pathway gene *TOR1* or *SCH9*, lack of amino acid, or treatment with rapamycin, results in extension of RLS and CLS and enhanced stress tolerance in yeast (Kaeberlein *et al*., 2005; Longo, 2003; Powers III *et al*., 2006; Sampaio-Marques *et al*., 2014). Enhanced stress tolerance by activating the stress-resistance transcription factors, Msn2/4 and Gis1, and mitochondrial superoxide dismutases (Sod1 and Sod2), is essential for CLS

extension through cellular protection against oxidative damage (Fabrizio *et al*., 2001, 2003, 2004; Sampaio-Marques *et al*., 2014; Wei *et al*., 2008). However, another study claimed that expression of the stress-resistance transcription factors and Sod2 confers stress tolerance but does not lead to CLS extension. The study shows that downregulated TOR pathway extends CLS in *S. cerevisiae* substantially by enhancing mitochondrial respiration through promoting mitochondrial gene expression (Bonawitz *et al*., 2007). In addition, inhibition of TOR pathway maintains rDNA stability by stimulating the translocation of transcription factors Msn2 and Msn4 from cytoplasm to nucleus to induce pyrazinamidase/nicotinamidase one (*PNC1*) expression. *PNC1* expression activates Sir2 that suppresses E-pro transcription through removal of nicotinamide to enable rDNA break repair via nonhomologous recombination pathway. This nonhomologous recombination-mediated DNA break repair maintains rDNA stability and promotes RLS extension in budding yeast (Ha & Huh, 2011; Lee & Ong, 2021; Medvedik *et al*., 2007) (Figure 2.5).

2.5.1(b) Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway regulates cell life span

Downregulation of cAMP-PKA pathway by decreasing glucose concentration or mutating component of cAMP-PKA pathway such as *CYR1*, extends RLS and CLS and enhances stress tolerance in yeast (Lin *et al*., 2000; Longo, 2003; Sampaio-Marques *et al*., 2014). Deletion of *RAS1* or *RAS2* (gene activator of PKA pathway), has opposite effects on RLS and CLS. Deletion of *RAS1* extends RLS but slightly reduces CLS, while deletion of *RAS2* reduces RLS but extends CLS (Fabrizio *et al*., 2003; Kaeberlein *et al*., 2007; Sun *et al*., 1994).

Stress-resistance transcription factors (Msn2/4, Gis1), Sod1 and Sod2 are essential to promote cellular protection against stress damage for CLS extension by downregulated PKA pathway (Fabrizio *et al*., 2003, 2004; Sampaio-Marques *et al*., 2014). Several stress resistance genes that contain STREs in their promoters such as *SOD1*, *SOD2*, catalase T (*CTT1*), discoidin domain receptor tyrosine kinase two (*DDR2*), heat shock protein 12 (*HSP12*) and trehalose-6-phophate synthase/phosphatase two (*TPS2*) are activated by Msn2/4 in response to stresses such as nutrient starvation, temperature shift, osmotic shock and oxidative damage (Boy-Marcotte *et al*., 1998; Fabrizio & Longo, 2003; Martínez-Pastor *et al*., 1996; Schmitt & Mcentee, 1996; Smith *et al*., 1998). Superoxide dismutases, Sod1 and Sod2 catalyse the free radical superoxide anion dismutation into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) while *CTT1* encoded catalase T reduces H_2O_2 into two water (H_2O) molecules and O_2 (Smith *et al*., 2010; Wang *et al*., 2018). Regulatory reaction of Ddr2 in response to stress remains unknown. Hsp12 stabilises cell membrane in response to stress (Welker *et al*., 2010) while Tps2 dephosphorylates trehalose-6-phosphate to produce trehalose (De Virgilio *et al*., 1993) that provides protection of cells and cellular proteins from oxidative damage (Benaroudj *et al*., 2001; Mizunoe *et al*., 2018) and induces autophagy (Kim *et al*., 2021).

Gis1 induces transcriptional activation of PDS-controlled genes such as *SSA3* and can also induce partial transcriptional activation of the STREs-controlled genes such as heat shock protein 12 (*HSP12*) and heat shock protein 26 (*HSP26*) (Pedruzzi, 2000). Stress seventy subfamily A (*SSA3*) encoded heat shock protein 70 (Hsp70) isoform stimulates degradation of α-synuclein via autophagy to decrease α-synuclein toxicity (Gupta *et al*., 2018) and enhances thermotolerance (Hasin *et al*., 2014).

Hsp26 stabilises proteins, prevents protein aggregation and decelerates the thermal inactivation of proteins upon thermal stress (Haslbeck *et al*., 1999, 2004).

Intriguingly, triple deletions of *MSN2/4* and *RIM15* cause a major reduction in CLS but extension in RLS. Moreover, overexpressions of superoxide dismutases (Sod1 and Sod2) that extend CLS, lead to reduction in RLS and avoid budding by 30-40 %. The negative regulation of RLS in *S. cerevisiae* by Msn2/4, Rim15 or Sod2, was suggested to be unrelated to ageing (Fabrizio *et al*., 2004). However, a later study reported that Msn2/4 is important for regulation of yeast RLS. Activated Msn2/4 upon downregulation of nutrient signalling pathways induces the expression of nicotinamidase gene *PNC1* for stabilization of rDNA, thus extending yeast RLS (Medvedik *et al*., 2007) (Figure 2.5).

2.5.2 Calorie restriction (CR) regulates cell life span

CR is a decrease of calorie content via limitation of nutrients without malnutrition (*Jiang et al*., 2000). CR is the only non-genetic intervention that extends life span in wide range of living organisms from yeast to mammals (Fontana *et al*., 2010). CR is imposed in yeast by lowering the concentration of glucose from 2 % to utmost 0.01 % or reducing available amino acids in medium (Jiang *et al*., 2000). CR in yeast revealed the essential activation of Sir2 deacetylase (Lin *et al*., 2000) to inhibit the formation of extrachromosomal rDNA circle and maintain rDNA stability, thus delaying ageing (Kobayashi *et al*., 2004; Lee & Ong, 2021). In fact, activation of mammalian Sir2 (SIRT1) in mammalian cells could also extend CR-associated life span (Cohen *et al*., 2004). A study reported that CR enhanced mitochondrial respiration and increased ROS level that stimulated antioxidant defensive system to protect CR cells from oxidative stress-induced damage. This stimulation is known as CR mitohormetic effect (Ristow & Zarse, 2010) which results in yeast RLS